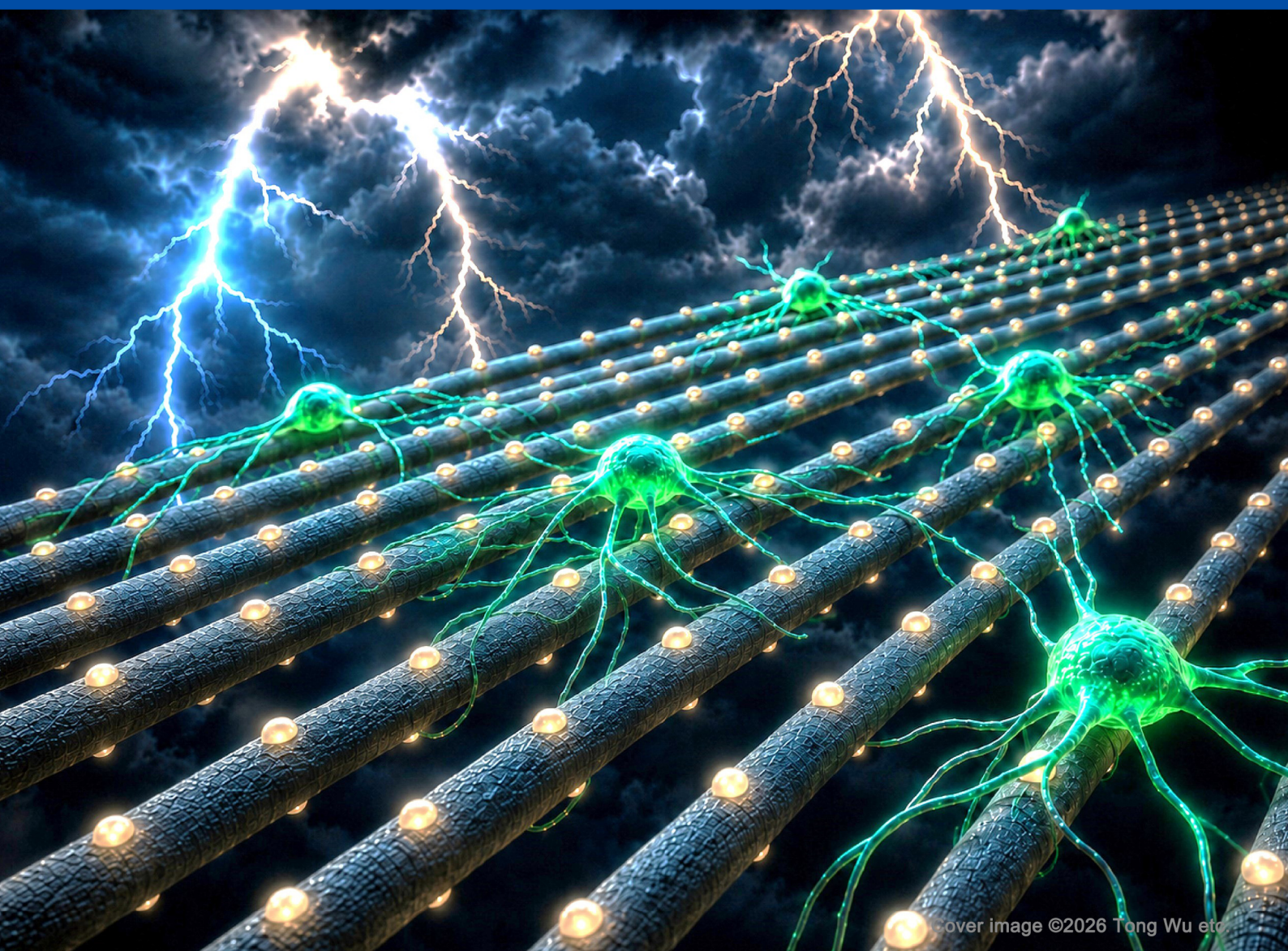


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Synergistic Effects of Topographical Guidance and Electrical Stimulation on Modulation of BMSC Behaviors Using Electrospun Nanofibers Decorated with Nanoscale Protrusions

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In the originally published article [1], one of the references cited in Section 1 has been retracted. This is the retracted reference:

Zhang, J.; Pritchard, E.; Hu, X.; Valentin, T.; Panilaitis, B.; Omenetto, F.G.; Kaplan, D.L. Stabilization of vaccines and antibiotics in silk and eliminating the cold chain. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 11981–11986. <https://doi.org/10.1073/pnas.1206210109>.

The authors would therefore like to update the original article by removing the retracted citation, and adjust the other reference numbers accordingly. The retracted reference was cited only to provide general background context and was not used to support the experimental design, data analysis, interpretation of results, or conclusions of the study.

In addition, due to a file-versioning error, two concentration values, “100 mM” and “ICA, 25 mM” were added to the sentence “We synthesized biocomposite samples by varying the concentration of BSA with fixed concentrations of zinc nitrate (100 mM) and 2-imidazolecarboxaldehyde (ICA, 25 mM)” in page 49, Section 3. To avoid any misunderstanding, the authors would like to revise the sentence by removing these values.

This update does not affect the scientific validity and conclusions of the article.

Reference

1. Wang, Y.; Tadepalli, S.; Baldi, H.; Morrissey, J.J.; Singamaneni, S. Effect of Protein Loading Density on the Structure and Biopreservation Efficacy of Metal-Organic Frameworks. *Mater. Interfaces* **2024**, *1*, 47–57. <https://doi.org/10.53941/mi.2024.100003>.



Polymer Microfibers: Fabrication, Structural Design, Functionalization, and Biomedical Applications

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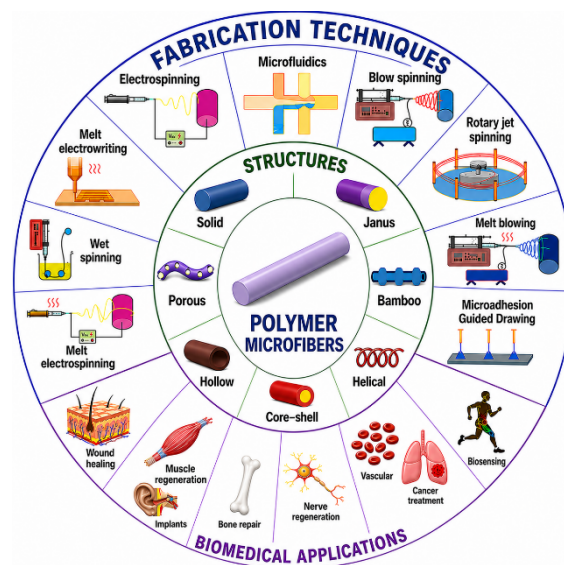
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Abstract: Microfibers are rapidly emerging as versatile building blocks for next-generation biomedical devices due to their precisely tunable morphology, chemistry, and functionality. However, most existing reviews primarily focus on electrospun nanofibers or broadly discuss nanofiber systems, where the focus on microfiber areas is limited. Herein, this review presents a comprehensive and systematic roadmap for the design and fabrication of microfibers tailored for healthcare applications. We first survey state-of-the-art fabrication technologies emphasizing their respective capabilities in controlling fiber diameter, alignment, internal architecture, and production throughput. We then discuss material selection strategies encompassing natural, synthetic, and hybrid composite systems. Subsequently, we examine key structural motifs, and elucidate how architectural design governs mass transport, cell-fiber interactions, and spatiotemporally controlled therapeutic release. We further outline functionalization strategies that transform passive microfibers into smart platforms. Finally, we highlight representative biomedical applications with high translational potential and commercial microfiber-based products. We conclude by discussing current translational challenges and future perspectives. Thus, this review provides practical design principles and strategic insights to accelerate the development and clinical translation of microfiber-based biomedical technologies.



Keywords: microfiber; fabrication; material selection; structural design; functionalization; biomedical applications

1. Introduction

The convergence of materials science and biomedical engineering has opened new frontiers in developing advanced materials for healthcare, among which microfibers have emerged as one of the critical component [1–4]. Microfibers, fibers with diameters typically ranging from 1 to few hundred micrometers, possess tunable structural, mechanical, and functional properties that make them ideal candidates for a wide range of biomedical applications [5–7]. Their unique ability to mimic the architecture of native extracellular matrices (ECM), combined with high surface-area-to-volume ratios, provides a favourable environment for cell adhesion, proliferation, and differentiation, making them particularly attractive in regenerative medicine and tissue engineering [8–11]. Over the past two decades, significant progress has been made in fabricating microfibers using a variety of top-down and bottom-up approaches [12]. Techniques such as electrospinning, melt blowing, rotary jet spinning, wet spinning, microfluidic spinning [13], and melt electrowriting [14] have enabled the production of microfibers with diverse morphologies, including solid, porous, core-shell, and hollow [15] structures [16–21]. Each fabrication technique offers distinct advantages and limitations, influencing the fiber properties and subsequent application



performance [22]. The evolution of these fabrication strategies has also allowed for precise control over fiber alignment, diameter, mechanical strength, and hierarchical organization, which are essential for designing application-specific microfiber scaffolds [23].

In addition to structural control, the integration of functional materials into microfibers has transformed them from passive matrices into dynamic and responsive platforms [24–26]. The incorporation of bioactive molecules, drugs, nanoparticles, and conductive or stimuli-responsive polymers has led to the development of smart microfibers capable of performing therapeutic or diagnostic tasks in response to biological cues [27–29]. These functionalized fibers are being actively explored in wound healing, controlled drug delivery, biosensing, and the tissue regenerations such as bone, nerve, and blood vessels [30,31]. Furthermore, the choice of base materials, ranging from biocompatible natural polymers like collagen, chitosan, and silk fibroin to synthetic biodegradable polymers such as polylactic acid (PLA), polycaprolactone (PCL), polyglycolide (PGA) and polyurethane (PU) plays a vital role in determining the degradation rate, mechanical behaviour, and biological response of microfibers [32,33]. Material selection, combined with smart design and surface engineering, enables the development of multifunctional systems tailored to specific biomedical needs [34].

This review aims to provide a comprehensive overview of the strategies employed to develop microfibers for biomedical applications. We first explore various fabrication techniques and their principles, followed by a discussion on the selection of materials and control over microfiber structure. Next, we focus on functionalization strategies that impart stimuli-responsiveness, therapeutic activity, or sensing capabilities to microfibers. Finally, we highlight major biomedical applications where these fibers are making a transformative impact, such as in wound healing, bone and muscle regeneration, nerve and vascular repair, biosensing, and cancer treatment.

2. Fabrication Techniques

Microfibers are prepared in a number of processes, starting with the selection of appropriate precursor materials and fabrication techniques. The choice of preparation techniques is determined by the desired characteristics of microfibers, including structure design, porosity, surface area, and mechanical strength. Figure 1 shows an overview of different techniques.

2.1. Solution Electrospinning

Electrospinning is a versatile and extensively used technique for fabricating microfibers, particularly in biomedical applications [35–37]. The method employs electrostatic forces to stretch a charged polymer solution into ultrafine fibers that are collected on a conductive substrate [38]. This approach allows precise control over fiber diameter, morphology, and alignment, enabling the design of scaffolds that closely mimic the fibrous architecture of native ECM [11,39].

The electrospinning process involves applying a high voltage (typically 5–30 kV) to a polymer solution dispensed through a needle connected to a syringe pump [40]. Once the electrostatic forces exceed the surface tension of the solution, a charged jet is ejected from the tip of the needle, forming what is known as a Taylor cone [41–43]. This jet undergoes elongation and thinning due to electrostatic repulsion and bending instabilities, and as the solvent evaporates in flight, solid fibers are deposited on the collector. The final fiber morphology can be finely tuned by adjusting key parameters such as solution viscosity, conductivity, and concentration, applied voltage, tip-to-collector distance, and environmental conditions like temperature and humidity [44,45]. Advanced forms of electrospinning have been developed to extend its functionality and address specific biomedical needs. Coaxial electrospinning enables the formation of core-shell fibers, useful for drug encapsulation and sustained release [46]. Needleless electrospinning enhances production throughput by generating multiple jets from a free liquid surface [47].

In electrospinning, different types of natural and synthetic polymers are explored [48]. Moreover, by integrating conductive or electroactive materials, the morphology and properties of fibers are tuned for different applications [49]. For example, Schönlein et al. fabricated nanocomposite microfiber scaffolds with PLA and polydopamine-coated barium titanate (BTO) for enhanced piezoelectric properties of 120% in comparison to pristine PLA microfiber scaffolds [50]. One key trend in utilizing electrospinning is the creation of highly specialized fiber architectures and composites. Mares-Bou et al., for instance, employed both uniaxial and coaxial electrospinning to fabricate polyvinyl alcohol (PVA)-based microfibers for controlled drug release [51]. Similarly, Li et al. explored the complexity of fiber morphology by generating ultraporous interweaving microfibers from immiscible PCL- polyethylene oxide (PEO) binary blends [52]. Beyond morphology, the electrospinning technique is instrumental in integrating bioactivity directly into the fiber structure. Roman et al. electrospun and incorporated the therapeutic agent paclitaxel into aligned PLA microfibers to treat spinal cord injuries [24]. Furthermore, for bone tissue engineering, Steffi et al. developed electrospun PCL/silk fibroin (SF) microfibers

loaded with 17-B estradiol (E2) [53]. The capacity of electrospinning technique for cell encapsulation is also critical for creating complex tissue constructs. Guo et al. modified an aqueous solution-electrospinning method (cell-electrospinning) to directly encapsulate C2C12 myogenic precursors within aligned fibrin/PEO microfiber bundles, overcoming the common limitation of cells only adhering to the exterior of conventional scaffolds [54]. Expanding on structural control, Fattahi et al. introduced 3D near-field electrospinning (3D-NFES), a low-cost, high-resolution method utilizing hobbyist 3D printers to fabricate repeatable 3D polymeric fiber patterns [55]. Neuhäusler et al. specifically highlighted the integration of electrospun microfibers into bioinks and 3D-bioprinted constructs to address the diffusion limit inherent in hydrogels, demonstrating the crucial role of electrospinning in the future of scalable biofabrication [56].

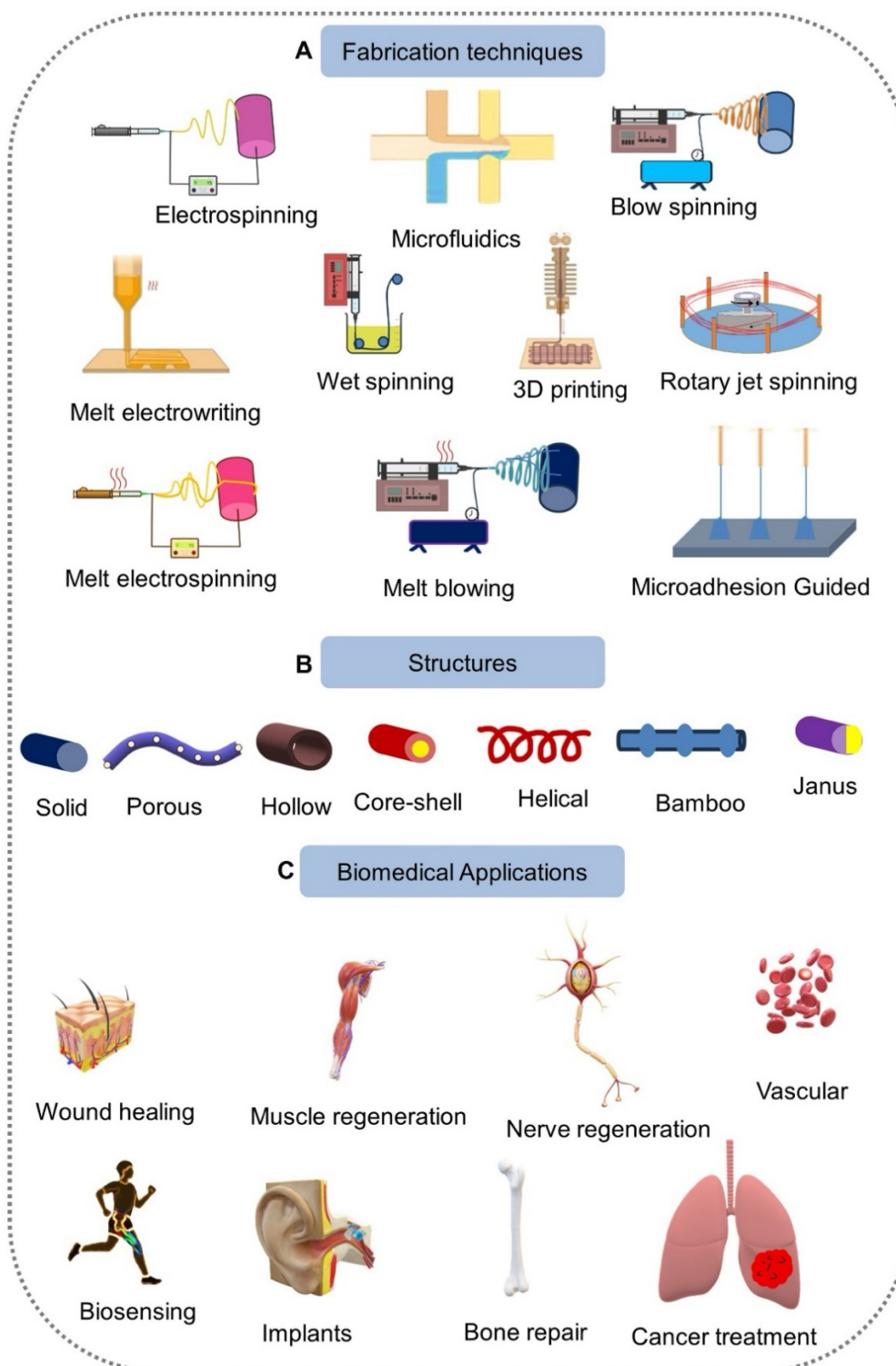


Figure 1. A brief outline of the contents discussed in this review illustrating (A) microfiber fabrication techniques, (B) secondary structures, and (C) their biomedical applications.

2.2. Melt Electrospinning

Melt electrospinning is a fiber fabrication technique that combines the principles of electrospinning and melt processing, using a polymer melt instead of a solvent-based solution to produce continuous microfibers [57–59]. In melt electrospinning, a thermoplastic polymer such as PCL, PLA, or PEO is heated above its melting point and extruded through a nozzle under the influence of a high-voltage electric field [60,61]. The molten polymer is drawn into thin fibers as it travels from the nozzle to the collector, where it solidifies upon cooling. The applied voltage, melt temperature, flow rate, and collector distance play critical roles in determining the fiber diameter and morphology [62–64]. Unlike solution electrospinning, where solvent evaporation contributes to jet thinning, melt electrospinning relies purely on electrostatic stretching and thermal behaviour, often leading to fibers in the range of 1–50 μm [65–67]. Melt electrospun fibers exhibit good mechanical strength, slow degradation rates, and excellent biocompatibility, making them ideal for long-term implantation and load-bearing tissue scaffolds [68,69]. Additionally, unlike solution-based methods, melt electrospinning eliminates toxic solvents, making it highly desirable for direct clinical applications and pharmaceutical manufacturing [70].

In melt electrospinning, the fine control over fiber architecture is invaluable for mimicking the native ECM. Castilho et al. employed melt electrospinning to fabricate highly organized scaffolds from a hydroxyl-functionalized polyester, poly(hydroxymethylglycolide-co- ϵ -caprolactone) (PHMGCL), for cardiac tissue engineering [71]. They showed that the prepared scaffolds significantly improved the alignment and differentiation of cardiac progenitor cells compared to traditional electrospun counterparts. Beyond single polymers, melt electrospinning is a powerful platform for engineering composite and blend microfibers with complex internal morphologies. Talebpour et al. conducted the comprehensive study on morphology development in melt-electrospun fibers from immiscible binary polymer systems, specifically polystyrene (PS) and PCL [72]. They demonstrated that by tuning parameters such as composition ratio, viscosity, and the use of a PS-*b*-PCL block copolymer as a compatibilizer, they could control the internal morphology, producing highly aligned microfibrils within the fibers. This level of internal structure control is crucial for designing smart microfibers with advanced functions, such as anisotropic mechanical response or directional drug release. Moreover, addressing the challenge of industrial scalability, Koenig et al. investigated the effect of additives and process parameters on the pilot-scale manufacturing of PLA sub-microfibers using melt electrospinning [73]. By combining a 600-nozzle pilot-scale device with conductive and viscosity-reducing additives like sodium stearate (NaSt) and sodium chloride (NaCl), they optimized the process to achieve sub-micron fiber diameters. This research is vital for translating melt electrospinning from a laboratory technique into a high-throughput, environmentally friendly manufacturing platform for polymer microfibers, making the technology viable for mass production in the biomedical and textile industries.

Despite its advantages, melt electrospinning faces challenges such as high processing temperatures, limited material options (thermoplastics only), and relatively low resolution and throughput [74–76]. Nevertheless, its solvent-free nature and compatibility with biodegradable polymers used in FDA-approved medical devices have positioned it as a promising technique for fabricating microfibers for tissue regeneration and other biomedical applications.

2.3. Rotary Jet Spinning (RJS)

RJS, also known as forcespinning, is a centrifugal fiber fabrication technique that produces microfibers by harnessing high-speed rotational forces instead of relying on electrostatic fields, as in electrospinning [77–79]. RJS has gained increasing attention in the biomedical field due to its ability to process a wide variety of polymer solutions or melts without the need for high voltage or electrical conductivity, and it offers significantly higher fiber production rates [80]. The technique is simple, cost-effective, and particularly well-suited for large-scale manufacturing of fibrous scaffolds for wound healing, tissue engineering, and drug delivery [81–83].

In a typical RJS setup, a polymer solution or melt is loaded into a rotating reservoir or cylindrical spinneret equipped with one or more orifices. When the spinneret is rotated at high speeds (typically thousands of revolutions per min), centrifugal forces eject the polymer through the orifices as fine jets [84]. As these jets travel outward, solvent evaporation or melt cooling causes the polymer to solidify into continuous microfibers, which are collected on a surrounding surface or drum collector. The morphology and diameter of RJS fibers can be tuned by adjusting key parameters such as polymer concentration and viscosity, spinneret rotation speed, nozzle diameter, ambient temperature and humidity, and solvent volatility [85]. With optimized conditions, RJS can produce fibers ranging from tens of nanometers to several micrometers in diameter [17]. The remarkable versatility and scalability of RJS have established it as a pivotal technology for fabricating advanced polymer microfibers in regenerative medicine, by offering high throughput and control over fiber morphology and alignment, enabling the precise recapitulation of native ECM architecture [86]. For example, Machado-Paula et al. explicitly leveraged RJS to create PCL

scaffolds embedded with hydroxyapatite (nHap) and carbon nanotube (CNT) for bone repair [87]. Further, Zamproni et al. fabricated porous PLA microfibers for mesenchymal stem cell (MSC) delivery to treat central nervous system injuries [85]. Beyond simple nonwoven meshes, the RJS platform has been ingeniously modified to fabricate complex three-dimensional devices and hierarchically structured materials. Motta et al. introduced focused rotary jet spinning (FRJS), a system combining RJS with a focused air stream, to achieve the on-demand manufacturing of heart valves (FibraValves) in a matter of minutes [88]. Similarly, Yang et al. employed wet rotary jet spinning (WRJS) combined with a salting-out process to produce PVA hydrogels with biomimetic hierarchical structures [77].

The adaptation of RJS into the immersion approach further expands its utility in producing hydrogel-based and tissue-specific constructs. MacQueen et al. employed the immersion rotary jet spinning (IRJS) process to produce gelatin microfibers at high production rates (~ 100 g/h) [89]. By tuning process conditions, they achieved fiber diameters comparable to natural collagen fibers, fabricating aligned scaffolds that successfully guided the formation of aligned muscle tissue from smooth muscle and myoblast cells. This versatile IRJS technique was also critical for developing smart therapeutic wound dressings. Ahn et al. engineered biomimetic and estrogenic fiber dressings composed of soy protein isolate (SPI) and hyaluronic acid (HA) [90]. For rapid hemostasis using RJS microfibers, Fu et al. developed a highly porous cellulose acetate/thermoplastic polyurethane aerogel and demonstrated a substantial reduction in blood loss and clotting time in trauma models [91].

Additionally, the ability to use polymer melts instead of solutions makes RJS an environmentally friendly option by eliminating organic solvents and their associated toxicity [80]. This melt-based variant is particularly useful for producing implantable devices and scaffolds where solvent residues are undesirable. Despite its many benefits, RJS does face some limitations [92]. The resulting fiber mats are typically non-aligned unless specific collection strategies or modifications are used. The resolution and spatial patterning of fibers are also less precise compared to melt electrowriting or microfluidic spinning [93–95]. Nevertheless, ongoing developments in collector design, multi-nozzle configurations, and hybrid processes are improving the versatility of RJS.

2.4. Melt Blowing

Melt blowing is a solvent-free, high-throughput fiber fabrication technique that utilizes the combination of polymer extrusion and high-velocity hot air to produce micro- and submicron-scale fibers [96,97]. Unlike electrospinning, which relies on electrostatic forces, melt blowing uses mechanical and aerodynamic forces to attenuate polymer melts into fine filaments [98]. The process is industrially scalable and widely used for applications requiring nonwoven fibrous mats with high porosity and surface area, such as filtration, wound care, and barrier materials.

In a typical melt blowing setup, a thermoplastic polymer, commonly polypropylene, PCL, PLA etc. is melted in an extruder and pushed through a die consisting of narrow capillaries [99]. Simultaneously, streams of hot, high-pressure air are directed through orifices adjacent to the polymer nozzles [100]. These air jets exert drag forces on the extruded polymer, causing rapid fiber stretching and thinning. The solidified fibers are collected on a rotating or stationary collector as a random, nonwoven web. Fiber diameters typically range from 0.5 to 20 μm , though submicron fibers can also be achieved with process optimization. The key parameters influencing the melt blowing process include polymer melt viscosity, air pressure and temperature, die-to-collector distance, throughput rate, and ambient conditions [101,102]. These variables control fiber diameter, web thickness, pore size, and fiber orientation. Compared to electrospinning, melt blowing generally results in thicker, randomly oriented fibers but offers significantly higher production rates and eliminates the use of potentially toxic solvents.

Melt-blown microfibers are particularly attractive due to their breathability, softness, and adaptability to large-area manufacturing. Nonwoven mats fabricated by melt blowing can be engineered to have antibacterial, absorbent, or barrier properties, making them suitable for wound dressings, surgical masks, and protective medical garments. Additionally, biodegradable polymers like PLA and PCL have expanded the use of melt-blown fibers in temporary implants and resorbable scaffolds. Although melt blowing offers less control over fiber alignment and architecture compared to techniques like melt electrowriting or electrospinning, its speed, scalability, and simplicity make it a valuable method for producing microfiber-based biomaterials, especially where large-area coverage and economic feasibility are essential.

To this end, Wang et al. successfully enhanced the performance of PLA-based fabrics by preparing PLA/polyethylene glycol@sodium dodecyl sulfate (PLA/PEG@SDS) microfibers using a modified melt-blown process [103]. Their work highlighted the ability to tune the average fiber diameter between 1 and 13.9 μm by regulating key process parameters. These modified nonwovens demonstrated remarkably efficient wetting performance, paving the way for their industrial application as wound dressings. In the domain of high-efficiency

air filtration for filtering respiratory protective devices (FRPDs), Brochocka et al. developed a multifunctional composite from polypropylene (PP) and poly(ethylene terephthalate) (PET) using a melt-blowing technique [104]. This method involved the simultaneous application of powdered functional modifiers, a biocidal agent (biohalosite) and a superabsorbent polymer via independent injection systems directly into the elementary fiber stream. This innovation yielded a nonwoven with very good antimicrobial activity, high filtration efficiency, and excellent water absorption capacity, which is critical for improving wearer comfort by mitigating the adverse effects of moisture accumulation in the breathing zone. Similarly, González-Sánchez et al. employed melt-blowing to incorporate copper nanoparticles (CuNP) into polyester to create antimicrobial non-woven fabrics, demonstrating activity against pathogens like *E. coli* for use in hospital environments [105]. For composite structures, Kang et al. utilized melt-blown thermoplastic polyurethane (TPU) nonwovens as a mechanically robust substrate layer onto which an electrospun nanofiber layer containing AgCl nanoparticles was deposited [106]. This double-layered approach provided a composite with superior mechanical properties, improved filtration efficiency, and potent antimicrobial activity of 99.9%.

2.5. Wet Spinning

Wet spinning is one of the oldest and most established fiber fabrication techniques, traditionally used in the textile and biomedical industries to produce continuous fibers from polymer solutions [107–110]. Unlike electrospinning methods, wet spinning is a solvent–non-solvent exchange process in which a polymer solution is extruded through a spinneret into a coagulation bath containing a non-solvent that induces phase separation and fiber solidification. This technique is particularly valuable for processing natural or bio-derived polymers that are difficult to electrospin due to poor solubility, low viscosity, or sensitivity to electric fields [111,112].

The process begins by preparing a homogenous polymer solution, which is then pumped through a fine nozzle into a coagulation bath, typically water, ethanol, or a salt solution, depending on the solvent system used [113–115]. Upon contact with the non-solvent, the polymer undergoes rapid precipitation or gelation, forming solid or semi-solid fibers. These fibers are then collected, washed to remove residual solvent or coagulant, and often subjected to post-processing steps such as stretching, crosslinking, or drying to enhance mechanical properties and dimensional stability. Key process parameters include polymer concentration, flow rate, nozzle diameter, bath composition, temperature, and the draw ratio applied to the fibers during collection [116]. One significant application of wet spinning lies in engineering gradient scaffolds for the complex interfaces found in the body. Calejo et al. pioneered the use of wet spinning to create a 3D fibrous scaffold with a continuous transition from mineralized to non-mineralized tissue [25]. They produced continuous, aligned composite microfibers from polycaprolactone (PCL)/gelatin and PCL/gelatin/hydroxyapatite (HAp).

Wet spinning is also crucial for synthesizing scaffolds with tailored mechanical and morphological features. Han et al. introduced a method to create microribbon-like elastomers by wet-spinning gelatin microfibers and subsequently drying them in acetone [117]. This drying process caused an asymmetrical collapse of the microfibers, yielding microribbons that could then be photocrosslinked into macroporous and highly flexible 3D scaffolds. This architecture supported robust proliferation of hASCs, illustrating how solvent choice and post-processing steps can be exploited in wet spinning to transition from simple fibers to complex, macro-porous elastic structures. In the realm of tissue engineering, Zhang et al. developed a new wet-spinning system using a blended solution of edible oil and hexane as the coagulation bath to fabricate PCL microfiber scaffolds [118]. They achieved control over fiber diameter (7–27 μm) and porosity (68–82%), demonstrating that the resultant oriented microfibers guided the oriented growth and infiltration of smooth muscle cells (SMCs).

Furthermore, the technique is adept at incorporating functional materials for advanced electronic and smart applications. Zheng et al. successfully fabricated biocompatible CNT-based hybrid microfibers via wet spinning for use as implantable electrochemical actuators and flexible electronics [119]. Similarly, Wang et al. achieved continuous meter-scale wet-spinning of cornlike composite fibers by anchoring polyaniline (PANI) onto regenerated cellulose (RC) from recycled waste cotton fabrics [109]. This green approach allowed for the mass production of multifunctional electronic fibers, with the corn-like morphology of the PANI layer, controlled by the coagulation bath's ammonium persulfate (APS) content, enhancing the fiber's performance as an eco-friendly flexible strain sensor.

The inherent structure of wet-spun fibers, characterized by a dense outer skin and a porous inner core, offers unique advantages. Puppi et al. applied an additive manufacturing protocol involving the patterned deposition of a polysulfone (PSU)/N-methyl-2-pyrrolidone (NMP) solution into a coagulation bath [120]. The resulting PSU medical implants possessed a transversal cross-section with a dense external skin and a macroporous/microporous inner structure due to the non-solvent induced phase separation (NIPS), highlighting the intrinsic architectural

control offered by the technique for medical implants. In a similar vein, Yang et al. fabricated shear-patterned alginate hydrogel microfibers using wet spinning [121]. They demonstrated that the shear force during spinning introduced microgroove patterns on the fiber surface.

However, wet spinning also presents certain limitations. The fiber diameters are generally larger than those obtained via electrospinning (typically in the range of tens to hundreds of micrometers), and the process is less amenable to producing ultra-fine structures [122–124]. In addition, precise control over fiber alignment and deposition requires specialized collection systems. Despite these challenges, wet spinning remains a valuable technique in the fabrication of biomedical microfibers due to its versatility in material compatibility, ability to form continuous and robust fibers, and potential for biofunctionalization.

2.6. Microfluidic Spinning

Microfluidic spinning is an innovative and highly controlled fiber fabrication technique that utilizes microfluidic devices to generate continuous microfibers through the manipulation of fluid flows within microscale channels [125–127]. This method offers exceptional control over fiber diameter, composition, morphology, and structure, making it especially valuable for biomedical applications that demand uniformity, reproducibility, and multifunctionality [128,129]. Unlike traditional spinning techniques that rely on bulk processes or high mechanical forces, microfluidic spinning enables gentle processing conditions, making it suitable for incorporating sensitive biological components [130].

The basic setup for microfluidic spinning involves a microfluidic chip with one or more inlet channels where polymer solutions and crosslinking agents flow in a co-axial or side-by-side configuration [131]. The spinning fluid (typically a hydrogel precursor or polymer solution) is sheathed or surrounded by a coagulating or crosslinking stream. As the fluids interact within the microchannels, solidification occurs through ionic crosslinking, solvent exchange, or photopolymerization, depending on the system. The resulting fibers are continuously extruded from the outlet and collected in a bath or on a moving stage. Parameters such as flow rate ratio, channel geometry, fluid viscosity, and external stimuli (e.g., UV light or temperature) govern the final fiber properties [132,133]. Microfluidic spinning excels in fabricating microfibers with advanced architectures such as core-shell, hollow, multilayered, and anisotropic structures. Chen et al., for instance, utilized a co-axial microfluidic spinning strategy to continuously produce all-polymeric, core-shell stretchable conductive fibers [134]. These fibers featured a polyurethane (PU) elastic shell and a conductive core of poly(3,4-ethylene dioxathiophene) polystyrene sulfonate (PEDOT:PSS). This design imparted a high conductivity (over $220 \text{ S} \cdot \text{m}^{-1}$) alongside an impressive stretchability (up to 400% strain). Similarly, Geiger et al. highlighted the use of microfluidic spinning to create microfibers entirely from the conductive polymer PEDOT:PSS for neural applications [135]. By precisely adjusting the flow rates of the spinning dope and shear fluid within a PDMS microfluidic chip, they were able to precisely control the fiber diameter between 1.2 to 3 μm . These conductive microfibers possessed mechanical properties comparable to natural fibrin fibers.

Beyond basic structural and conductive properties, microfluidic spinning allows for the intricate design of microfibers with complex, bio-inspired topographies and therapeutic capabilities. Shang et al. developed bioinspired multifunctional microfibers featuring spindle-knots and joints using a microfluidic spinning [136]. This integrated technique, which involves fluid coating and knot emulsification precisely tuned by adjusting flow rates, grants high controllability over the size and spacing of the knots. For post-operative care, Peng et al. demonstrated the fabrication of multifunctional analgesic sutures [137]. By using microfluidic spinning coupled with a solvent extraction process, PCL microfibers were continuously produced with encapsulated ropivacaine (ROP), a potent analgesic. Furthermore, microfluidic spinning is instrumental in constructing sophisticated *in vitro* tissue models and engineered scaffolds. Masuda et al. focused on the maturation of human induced pluripotent stem cell (iPSC)-derived cardiac microfibers (CMFs), fabricated by using a double-coaxial laminar flow microfluidic device to achieve the desired shape [138]. The architectural control is further emphasized by Liu et al., who proposed a phase inversion-based microfluidic spinning (PIMS) method to produce strong, stretchable, and biocompatible helical microfibers [139]. Thus, the meticulous control offered by microfluidic spinning thus makes it a pivotal technique for creating the smart, functional, and geometrically complex polymer microfibers.

Despite its advantages, microfluidic spinning is currently limited by low throughput and complexity in device fabrication [140]. Producing large quantities of fibers requires parallelization or scale-up of microfluidic channels, which adds to fabrication challenges. Moreover, the process often requires careful optimization of fluid compatibility and surface treatments to prevent clogging and ensure reproducible fiber formation [141]. Nonetheless, the precision and adaptability of microfluidic spinning make it a powerful tool for producing next-generation microfibers for biomedical applications [142,143]. Ongoing developments in soft lithography, 3D

printing of microfluidic chips, and integration with bioprinting platforms are expected to enhance the scalability and functionality of this technique.

2.7. 3D Printing

3D printing, also known as additive manufacturing, is a transformative fabrication technique that enables the layer-by-layer construction of three-dimensional structures from digital models [144–146]. In the context of microfiber fabrications, 3D printing offers precise control over scaffold geometry, pore size, and internal architecture, factors that are critical for mimicking the structural and functional characteristics of native tissues [147–149].

In 3D printing of microfibers, polymers are usually extruded through a nozzle and deposited layer by layer in a predefined pattern to form a solid structure [150]. Depending on the technique, the printing material may be a thermoplastic polymer, hydrogel, or composite bioink. Thermoplastic-based printing methods like fused deposition modeling (FDM) use heat to melt and deposit the polymer, which solidifies upon cooling [151]. Some systems also incorporate ultraviolet (UV) or visible light sources to induce rapid crosslinking of photocurable bioinks during or after deposition [152]. Moreover, by adjusting printing parameters such as nozzle speed, pressure, and deposition path, it is possible to control fiber orientation, gradient properties, and multiscale architecture, thereby influencing cell behavior and tissue integration [153–155].

The integration of smart functionalities into 3D-printed microfibers is rapidly advancing, with a focus on remote stimulation and targeted regeneration. Cedillo-Servin et al. reported the rational design and fabrication of magneto-active microfiber meshes using 3D printing of a magnetized PCL-based composite [156]. By incorporating uniformly dispersed iron oxide nanoparticles, they created hexagonal scaffolds with tunable fiber diameter and a zonal distribution of magnetic material. Beyond structural and magnetic control, 3D printing enables the precise immobilization of growth factors to drive functional tissue outcomes. Ainsworth et al. combined 3D printing with atmospheric-pressure plasma jet (APPJ) treatment to create PCL microfiber meshes with covalently immobilized transforming growth factor beta-1 (TGFB1) for guided cartilage regeneration [157]. 3D printing is also addressing the challenge of fabricating vascular grafts that are both mechanically stable and anti-thrombogenic. Shen et al. developed a 3D-printed electrospun graft loaded with tetramethylpyrazine [158].

Despite its immense potential, 3D printing faces challenges in microfiber fabrication, primarily related to resolution, throughput, and material limitations [151,159]. Achieving fiber diameters in the micron or submicron range requires precise control over printing parameters and specialized equipment, which can increase cost and complexity [160]. Additionally, ensuring long-term mechanical stability, biodegradation kinetics, and regulatory approval for printed constructs remains an active area of research. Nevertheless, 3D printing continues to evolve rapidly, driven by advances in bioprinting, multi-material integration, and in situ fabrication techniques [161].

2.8. Melt Electrowriting (MEW)

MEW is an emerging fiber fabrication technique that merges principles from electrospinning and additive manufacturing to produce highly controlled, micron-scale fiber architectures [162–164]. Unlike conventional electrospinning, which typically results in randomly deposited fibers due to jet instability, MEW enables direct, layer-by-layer (LbL) desposition of molten polymer filaments with minimal jet whipping, allowing precise control over fiber placement and scaffold geometry [165–167]. This deterministic fiber deposition makes MEW particularly attractive for biomedical applications where structural fidelity and pore architecture are critical [168].

In MEW, a thermoplastic polymer such as PCL is heated above its melting point and extruded through a fine nozzle under low pressure [169,170]. A moderate voltage (typically 2–10 kV) is applied between the nozzle and a conductive collector, which guides the fiber's trajectory and enhances stretching [171]. The collector, often mounted on a programmable stage, moves in predefined patterns to direct fiber deposition and build up 3D scaffolds with high spatial resolution [172]. Unlike traditional solution-based electrospinning, MEW operates at much lower flow rates and avoids solvent-related issues, making it more suitable for biomedical applications where solvent toxicity and residuals are a concern. Several parameters govern the MEW process, including melt temperature, applied voltage, nozzle-to-collector distance, polymer viscosity, and collector speed [68,173,174]. These variables influence fiber diameter, deposition accuracy, inter-fiber spacing, and scaffold porosity. The resulting microfibers can be arranged in well-defined grid-like or biomimetic architectures. A major advantage of MEW is its ability to produce mechanically robust scaffolds with highly organized, reproducible structures [175]. These scaffolds exhibit superior load-bearing capacity and support cell alignment and tissue organization, making them ideal for regenerating mechanically active tissues such as cartilage, intervertebral discs, and tendons. Furthermore, MEW scaffolds can be functionalized with bioactive agents, coated with ECM proteins, or combined with hydrogels to create hybrid systems with improved biological performance [176–178].

A key strength of MEW lies in its ability to rationally design and fabricate scaffolds with specific mechanical properties tailored to target tissues. For instance, Mueller et al. established a design strategy to create small-diameter vascular grafts with programmable compliance [179]. By meticulously controlling the helical microfiber winding angle in PCL scaffolds, they exploited the structure-function relationship of the MEW architecture, achieving tailored compliance that spanned the physiological range of both arteries and veins. This precise mechanical matching is critical for minimizing the compliance mismatch that often leads to high failure rates in synthetic grafts. Similarly, Castilho et al. leveraged MEW to engineer ultrastretchable scaffolds for cardiac tissue engineering [173]. Their work demonstrated that controlled hexagonal microstructures could exhibit large biaxial deformations, delivering up to 40 times more elastic energy than rudimentary MEW fiber scaffolds.

Beyond mechanical stability, MEW is increasingly utilized in advanced composite scaffold design and microphysiological systems. Lai et al. combined MEW with solution electrospinning to produce antibacterial and osteogenic dual-functional micro-nano composite PCL scaffolds for calvarial defect repair [180]. The precision of MEW also extends to constructing sophisticated *in vitro* models, as shown by Janssen et al., who engineered an immunocompetent Intestine-on-a-Chip (IoC) [181]. This model integrated half-pipe-shaped MEW scaffolds with extrusion-based bioprinted hydrogels, allowing for spatial epithelial-immune cell interactions. Moreover, MEW demonstrated its potential to control over polymer processing and architecture to produce smart microfibers, where high porosity is crucial for rapid dissolution kinetics. Keßler et al. utilized MEW to create highly porous, rapid-dissolving scaffolds loaded with an amorphous solid dispersion of Indomethacin [177].

While MEW offers customized design control, and reproducibility, it also has some limitations [182–184]. The range of usable polymers is restricted to thermoplastics with appropriate melt rheology, and the process throughput is relatively low due to its single-nozzle, LbL nature [185,186]. Nonetheless, ongoing developments such as multi-nozzle arrays, temperature-gradient control, and integration with biological printing are expanding its capabilities.

2.9. Micro-Adhesion Based Drawing

Micro-adhesion based drawing technique mimics spider silk drawing by utilizing adhesion between a polymer droplet and a collector surface to generate fibers during mechanical withdrawal [187,188]. Fiber diameter and alignment can be finely controlled by adjusting pulling speed, contact time, and surface tension [189]. This technique allows direct deposition of microfibers onto complex geometries or moving surfaces, making it ideal for site-specific fabrication on biomedical devices, implants, or skin-interfacing electronics. The simplicity and precision of micro-adhesion based drawing hold promise for personalized therapeutic textiles and soft robotics [190].

In summary, microfiber fabrication techniques have evolved significantly, enabling precise control over fiber diameter, architecture, alignment, porosity, and multifunctionality for diverse biomedical applications. As summarized in Table 1, each technique possesses distinct advantages and limitations in terms of resolution, material compatibility, throughput, cost, and clinical applicability, making the selection of fabrication strategy highly dependent on the intended biomedical application. Continued advances in fabrication technologies, process integration, and scalable manufacturing are expected to further accelerate the clinical translation of microfiber-based biomedical systems.

Table 1. Comparative overview of key microfiber fabrication techniques for biomedical applications.

Technique	Scalability	Resolution	Material Compatibility	Cost	Ref.
Electro-spinning	Moderate to high; scalable using multi-nozzle, or roll-to-roll systems	High control; typically fiber diameter in nano–micro range)	Broad	low to moderate cost; but scale-up requires advanced collectors	[56,191]
Melt electro-writing	Low to moderate; slower fabrication rate than electrospinning	high spatial precision; fibers in microscale range	Limited	High equipment complexity; requires controlled heating, pressure, voltage, and motion stage	[172]
Rotary jet spinning	High; can produce large-area fibrous mats rapidly	Moderate diameter control; fibers often in nano- to microscale range	Broad	Moderate cost; simpler than electrospinning for large-scale production	[77,81]
Melt electro-spinning	Moderate; but process control is challenging	Moderate control; usually produces microfibers larger than solution electrospun fibers	Limited	Moderate to high; requires heating and high-voltage control	[75,76]
Wet spinning	High; industrially scalable and continuous fiber production is possible	Moderate; fiber diameter controlled by system parameters	Broad	Moderate; relatively simple setup but requires coagulation chemistry optimization	[77,109]

Table 1. *Cont.*

Technique	Scalability	Resolution	Material Compatibility	Cost	Ref.
Microfluidic spinning	Low to moderate; excellent control but limited throughput	Very high control over cross-section, diameter, core-shell, hollow, Janus, and multicompartiment structures	Broad	High device-design complexity; requires microfluidic chips and precise pumps	[140,192]
Melt blowing	Very high; industrially scalable and used for nonwoven production	Low to moderate; produces mostly random fibers with broad diameter distribution	limited	High industrial equipment cost but low cost per unit at scale	[101,106]
3D printing	Moderate; scalable for customized constructs	Moderate to high; depends on nozzle size, ink rheology, and printing system	Broad	Moderate to high; requires printer, optimized bioink, and post-crosslinking	[193,194]
Micro-adhesion-guided drawing	Low; currently mainly laboratory scale	High control over fiber placement and alignment at small scale	Limited	Moderate; requires patterned substrates and controlled drawing/spinning	[187,188]

3. Choice of Materials

The selection of appropriate materials is a critical step in the design and fabrication of microfibers for biomedical applications [171,195]. The material not only determines the mechanical and degradation properties of the fiber but also influences its biocompatibility, drug-loading capacity, and interaction with cells and tissues [196]. Depending on the intended function, whether for tissue regeneration, wound healing, drug delivery, or biosensing, materials must be carefully chosen to ensure structural integrity, biological performance, and compatibility with the selected fabrication technique [197,198]. Table 2 provides a comprehensive summary of the polymers, including fabrication process, microfiber diameter, final structure, and their biomedical applications or significant outcomes.

Table 2. List of different polymers and fabrication techniques of microfibers and their biomedical applications.

Materials	Fabrication Technique	Fiber Diameter (μm)	Secondary Structure	Applications	Ref.
PCL	Wet spinning	15	Solid	Tissue regeneration	[199]
PLLA/cBTO	Electrospinning	1.8	Solid	Piezoelectric scaffold	[50]
Alg-GelMA	Microfluidic spinning	380–510	Hollow	Peripheral nerve regeneration	[200]
PEDOT: PSS	Microfluidic wet-spinning	3	Solid	Nerve guidance conduits	[135]
PLA	FDM 3D printing	45	Solid	Piezoelectric biosensor	[201]
GelMA	Bioprinting	20–120	Solid Janus	Tumor angiogenesis	[202]
PHB/ε-PLL	Solution blowing	1	Solid	Antimicrobial activity	[203]
Gelatin	Microfluidic spinning	685–715	Hollow	Application in blood capillary	[193]
poly(styrene- <i>b</i> -isobutylene- <i>b</i> -styrene)	solution blow spinning	1–20	Solid	Antibacterial and antithrombosis coating	[204]
Na-alginate	Microfluidic spinning	140–200	Janus-hollow	Tissue construction	[142]
PDO	Centrifugal spinning	15–19	Solid	Skin tissue engineering	[205]
PEDOT/PDA/Silk	extraction-protection	10	Core-sheath	Wound healing	[206]
Polyacrylamide/Mxene	Microfluidic spinning	233	Porous	Joint monitoring	[207]
hyaluronic acid/SWCNT	Wet Spinning	50	Porous	Implantable electrochemical actuator	[119]
PCL	Electrospinning	2–10	Solid	Biomedical application	[19]
PLGA	Microfluidic spinning	20–230	Porous	Tissue engineering	[127]
PCL	3D printing	11	Solid	Bone regeneration	[208]
PEG/PVA/Alginic acid	Microfluidic spinning	300–500	Cavity knot	Tissue engineering	[209]
PCL	Melt electrowriting	10–15	Solid	Tympanic membrane replacement	[162]

Natural polymers are widely used due to their inherent biocompatibility and resemblance to the native ECM in both composition and function [210]. Materials such as collagen and gelatin are frequently employed in skin and cartilage regeneration owing to their cell-adhesive properties [211]. Silk fibroin, another natural protein polymer, offers superior mechanical strength and is often used in bone, nerve and ligament scaffolds [212]. Polysaccharides like chitosan provide antimicrobial activity and are suitable for wound healing, while alginate and hyaluronic acid are commonly used in hydrogel-based spinning systems that encapsulate cells or bioactive agents [213]. Despite these advantages, natural polymers often require blending or crosslinking to improve mechanical stability and consistency.

Synthetic polymers offer greater control over fiber properties, including degradation rate, mechanical strength, and processability [214]. PCL, PLA, PGA, and poly(lactic-co-glycolic acid) (PLGA) are among the most commonly used biodegradable polyesters, well-suited for a wide range of tissue engineering and drug delivery applications [169,213,215]. Polyurethane (PU) is valued for its elasticity and durability in skin and cardiovascular devices, while polyvinylidene fluoride (PVDF) is often selected for its piezoelectric properties in sensing applications [84,216]. Water-soluble polymers like PEO and PVA are used in systems requiring rapid dissolution or transient performance [217]. Synthetic polymers are generally easier to process and offer more predictable performance, but often lack intrinsic bioactivity and require surface modification to enhance cell interactions.

To overcome the limitations of individual polymers, composite and hybrid materials are increasingly employed [218,219]. These combine the bioactivity of natural polymers with the structural integrity of synthetics. For example, blending gelatin or collagen with PCL can enhance both cell adhesion and mechanical strength [46]. Hybrid systems can also be enhanced with nanoparticles such as silver to provide antibacterial effects, hydroxyapatite to support bone regeneration, and carbon-based nanomaterials to improve electrical conductivity [119]. This material modularity enables the design of multifunctional microfiber systems tailored to specific biomedical challenges.

The choice of material also varies according to application-specific demands. For wound healing, materials that maintain moisture balance, prevent infection, and promote tissue closure are essential. Bone tissue scaffolds require materials with higher stiffness and bioactivity, such as calcium phosphate composites. Nerve and muscle regeneration benefit from elastic materials that support aligned fiber orientation and directional cell growth. In biosensing applications, materials must combine flexibility, conductivity, and chemical stability to ensure signal fidelity and user comfort.

Recent advances in smart and stimuli-responsive polymers have further expanded the material landscape for microfibers [220]. Polymers that respond to temperature, pH, light, or magnetic fields allow for dynamic, on-demand functionalities such as controlled drug release or actuation [221]. Examples include poly(*N*-isopropylacrylamide) (PNIPAM) for temperature responsiveness, poly(acrylic acid) for pH sensitivity, and iron oxide nanoparticle-laden composites for magnetic actuation [222]. These smart materials enable microfibers to transition from passive scaffolds to active, responsive platforms capable of interacting intelligently with their biological environment.

4. Secondary Structures

The structural properties of microfibers play a pivotal role in determining their mechanical behaviour, surface interactions, biological responses, and overall performance in biomedical applications [223]. By tailoring the internal and external morphology of fibers, such as producing solid, porous, hollow, core-shell, helical, bamboo knot, and Janus architectures, researchers can achieve specific functionalities, ranging from controlled drug release to enhanced cell adhesion, electrical responsiveness, and tissue integration (Figure 2) [72,224–226]. The following subsections describe each structural configuration in detail, emphasizing fabrication strategies, physicochemical properties, and biomedical relevance.

Solid microfibers represent the simplest and most common architecture, typically formed through electrospinning, melt electrowriting, wet spinning, or rotary jet spinning (Figure 2A) [5,135]. They consist of a homogeneous cross-section with a uniform polymer matrix, providing high mechanical strength, structural stability, and tunable flexibility depending on polymer type and molecular weight. Their dense morphology ensures durability under mechanical loading, making them ideal for structural scaffolds, sutures, and load-bearing applications such as bone or ligament regeneration. The simplicity of solid fibers allows for easy control of diameter, alignment, and orientation, which can direct cellular responses such as fibroblast elongation or osteoblast differentiation [163]. However, their limited internal surface area has motivated the development of modified variants such as porous or core-shell fibers. Surface functionalization through plasma treatment, coating, or chemical grafting can further improve hydrophilicity and bioactivity, allowing these dense fibers to serve as fundamental building blocks for multifunctional tissue-engineering constructs.

Porous microfibers are characterized by the presence of interconnected pores or cavities within their matrix, resulting in significantly enhanced surface area and permeability (Figure 2B) [217,227]. Porosity can be introduced via phase separation, salt leaching, electrospinning, or gas foaming methods [228,229]. The porous structure promotes nutrient and oxygen diffusion, supports cellular infiltration, and provides additional sites for drug loading. The pore size and distribution can be tuned by varying solvent volatility, polymer concentration, and humidity during spinning [230,231]. For example, electrospinning under high humidity generates breath figure-induced pores, while emulsion electrospinning allows oil-in-water systems to form controlled microcavities [232,233]. Porous

microfibers are especially valuable in wound dressings and regenerative scaffolds where moisture retention, gas exchange, and controlled degradation are critical for healing.

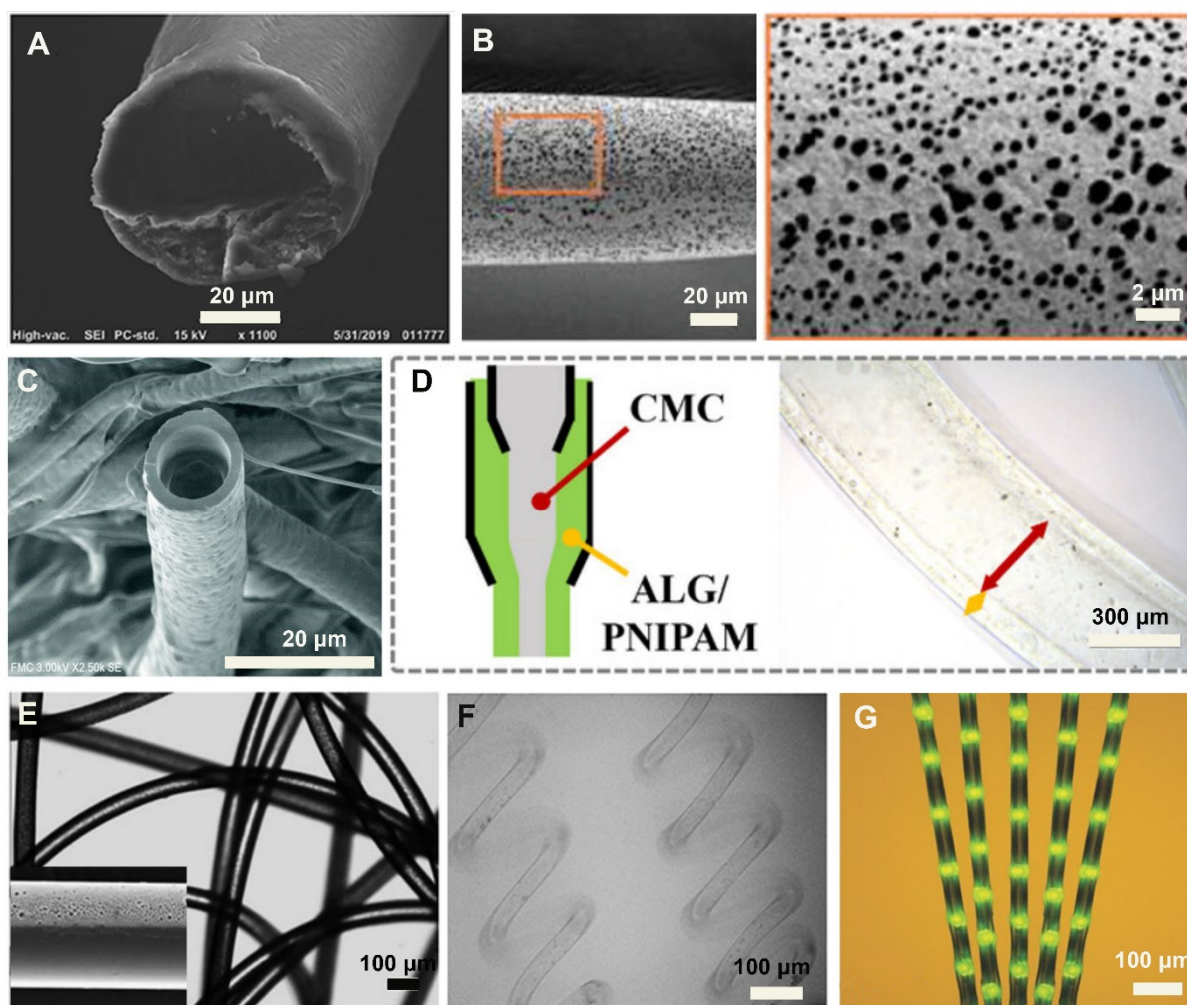


Figure 2. Different structural designs of microfibers. (A) Solid microfibers of alginate. Reproduced with permission [234]. Copyright 2020, Frontiers. (B) Porous prolamin microfibers and its high-magnification view. Reproduced with permission [235]. Copyright 2024, Wiley-VCH. (C) Hollow microfibers of PCL. Reproduced with permission [16]. Copyright 2020, RSC Publishing. (D) Core-shell microfibers, featured as an alginate/poly (N-isopropyl acrylamide) (ALG/PNIPAM) shell encapsulating a carboxymethyl cellulose (CMC) core. Reproduced with permission [236]. Copyright 2023, Wiley-VCH. (E) Janus polyurethane microfibers. Reproduced with permission [216]. Copyright 2009, RSC Publishing. (F) Helical microfibers of Na-alginate. Reproduced with permission [192]. Copyright 2017, Wiley-VCH. (G) Bamboo shaped calcium alginate microfibers. Reproduced with permission [237]. Copyright 2014, Wiley-VCH.

Hollow microfibers contain an empty central lumen surrounded by a solid shell, mimicking natural tubular structures such as blood vessels and nerve conduits (Figure 2C) [238,239]. These fibers are typically fabricated using coaxial electrospinning or microfluidic spinning, where two immiscible fluids are extruded simultaneously to form a core-shell structure, and the core phase is later removed [217,240]. Hollow fibers combine lightweight mechanical behaviour with large internal volume, enabling encapsulation and sustained release of therapeutic agents, growth factors, or even living cells [241]. The internal channel allows fluid transport, making them ideal for vascular grafts, nerve guides, and controlled drug delivery systems [242]. By adjusting core and shell flow rates, shell thickness and lumen diameter can be finely tuned, affecting permeability and mechanical strength [6,243]. Materials such as PCL, PLA, and silk fibroin are often used as shells, while hydrogels or bioactive materials can serve as the core template. Their biomimetic tubular morphology has made hollow microfibers a preferred structure for fabricating artificial blood vessels and nerve guidance conduits.

Core-shell microfibers possess a composite structure with distinct inner (core) and outer (shell) layers, offering superior multifunctionality and controlled release properties (Figure 2D) [224]. Fabrication typically

involves coaxial electrospinning or 3D printing with dual nozzles, where the core and shell are independently formulated to achieve complementary functionalities. The core can encapsulate sensitive bioactive molecules, such as proteins, growth factors, or drugs, shielded by the protective shell that regulates diffusion and degradation. This configuration prevents premature drug loss and preserves bioactivity. The shell often provides mechanical strength, environmental protection, or targeted surface chemistry for cell adhesion. For instance, hydrophilic shells can promote biocompatibility, while hydrophobic shells control drug release kinetics. Core-shell designs have been successfully applied to wound healing (sustained antibiotic release), nerve regeneration (encapsulated neurotrophic factors), and bone repair (dual-phase mineralization). Moreover, the use of stimuli-responsive materials in the core or shell, such as pH-, temperature-, or enzyme-sensitive polymers, enables smart therapeutic systems capable of on-demand release in response to physiological changes.

Janus microfibers feature two distinct sides or compartments with contrasting physical or chemical properties, typically achieved through side-by-side electrospinning, microfluidic co-extrusion, or bicomponent melt spinning (Figure 2E) [197,216]. This asymmetric structure allows simultaneous incorporation of multiple functionalities within a single fiber. One side can be hydrophilic to promote cell adhesion and drug release, while the other remains hydrophobic for barrier or mechanical stability [244]. Alternatively, one hemisphere may contain magnetic nanoparticles or conductive fillers, while the other contains growth factors or biomolecules. Such anisotropy enables directional interactions and programmable responses [245,246]. Janus fibers are ideal for stimuli-responsive or gradient delivery systems, where each phase reacts differently to environmental cues such as temperature, pH, or electric fields.

Helical microfibers exhibit a coiled or spring-like morphology that provides exceptional elasticity, stretchability, and energy dissipation (Figure 2F) [247]. They can be fabricated through mechanical twisting of aligned fibers, controlled jet instabilities during electrospinning, or direct 3D printing using rotating collectors or predesigned helical paths. The helical configuration allows these fibers to withstand cyclic strain and recover their shape, making them suitable for soft tissue engineering (e.g., muscle, tendon, and vascular applications). The coiled structure also enhances surface area, improving interactions with cells and biomolecules. In biomedical devices, helical fibers can function as artificial tendons, stretchable biosensors, or electroactive actuators. When made from conductive or piezoelectric polymers, they can convert mechanical deformation into electrical signals, useful for self-powered sensors and dynamic scaffolds. Additionally, the geometric flexibility of helices provides unique mechanical cues that influence stem cell mechanotransduction and alignment, enhancing muscle and nerve tissue regeneration [248,249].

Bamboo knot microfibers are characterized by a periodic alternation of thick and thin segments along the fiber axis, resembling the joints of bamboo (Figure 2G) [226]. These structures can be created by modulating flow rate, voltage, or collector movement during electrospinning, leading to uneven mass deposition. The “knot” regions serve as mechanical reinforcements and local reservoirs for drug encapsulation. This architecture enables a unique combination of flexibility and localized functionalization. For biomedical applications, bamboo-like fibers are particularly advantageous for sequential or site-specific drug delivery, as the nodal segments can contain different therapeutic agents. Additionally, the varying diameter creates differential mechanical stiffness along the fiber, which can influence cellular behaviour and tissue integration. Bamboo knot structures are also being investigated for hierarchical scaffolds where graded mechanical properties are desirable, such as tendon-to-bone or muscle-to-tendon interfaces [250–252].

In summary, the structural versatility of microfibers, spanning from simple solid configurations to complex Janus or helical architectures, offers powerful means to tailor mechanical performance, surface chemistry, and biological activity. By leveraging advanced fabrication technologies such as coaxial, side-by-side, and microfluidic spinning, researchers can precisely design fiber architectures suited for diverse biomedical applications, including regenerative scaffolds, drug delivery systems, wearable biosensors, and implantable therapeutic devices [253]. The continued evolution of controlled structural engineering at the microscale will enable the next generation of multifunctional, adaptive microfiber systems capable of interacting intelligently with biological environments.

5. Functionalization

Functionalization plays a vital role in modifying the physicochemical and biological properties of microfibers to meet specific biomedical demands. A wide range of physical and chemical methods are available to impart functionality to microfibers post-fabrication, as shown in Figure 3, including dry etching, wet etching, electrospray, sputtering, dip coating, and LbL assembly to tailor surface morphology, reactivity, and bioactivity.

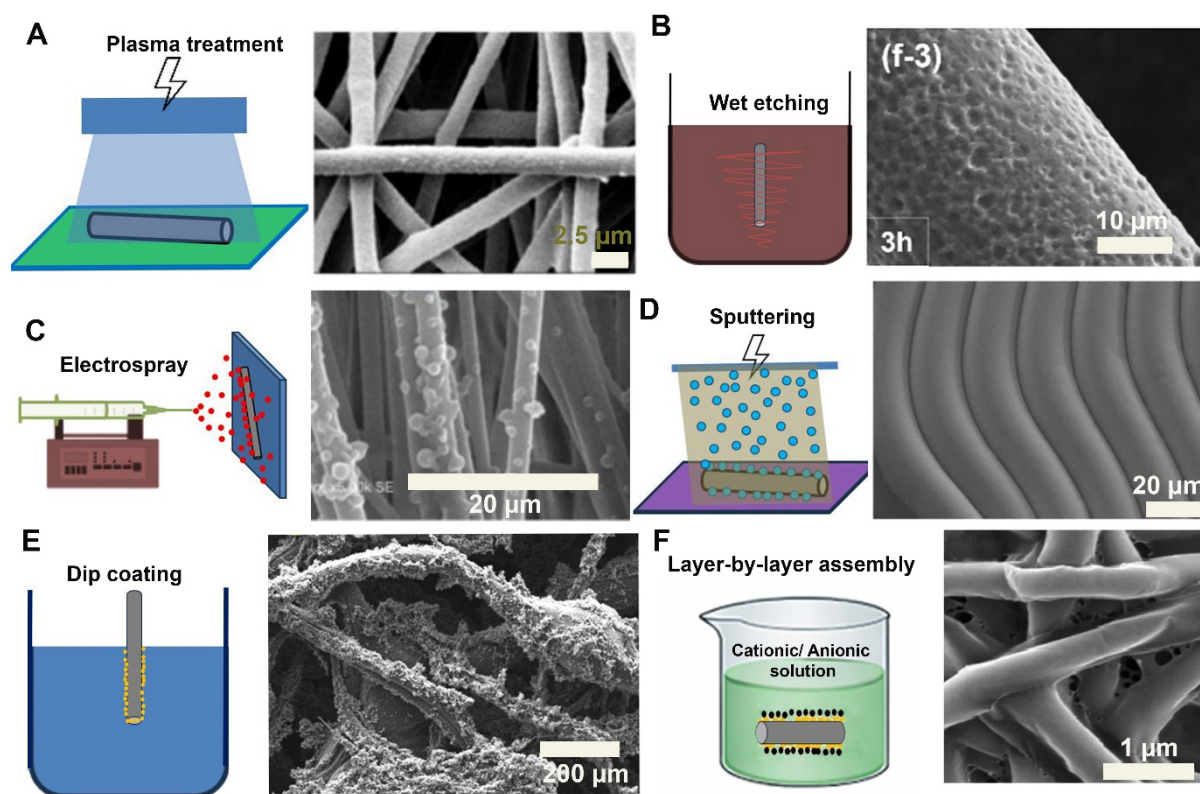


Figure 3. Different methods for functionalization of microfibers. In each case, representative SEM images of postprocessed microfiber scaffolds show the modifications on the fiber surface by the methods described respectively. (A) Dry etching or plasma treatment. Reproduced with permission [254]. Copyright 2013, Wiley-VCH. (B) Wet etching. Reproduced with permission [255]. Copyright 2022, Elsevier. (C) Electrospray coating. Reproduced with permission [256]. Copyright 2022, Sage Journals. (D) Sputtering. Reproduced with permission [257]. Copyright 2023, Springer Nature. (E) Dip coating. Reproduced with permission [84]. Copyright 2025, Wiley-VCH. (F) Layer-by-layer assembly. Reproduced with permission [258]. Copyright 2012, Elsevier.

Plasma treatment or dry-etching is a highly effective surface modification technique used to functionalize microfibers by altering their surface chemistry and topography without affecting their bulk properties (Figure 3A) [259,260]. This method employs ionized gas (plasma) composed of reactive species such as ions, electrons, radicals, and photons to activate or etch the fiber surface. Plasma treatment is particularly valuable for microfibers because it enables the introduction of reactive functional groups, increases surface roughness and energy, and enhances wettability, factors that significantly improve cell adhesion, protein adsorption, coating adherence, and overall biological performance [261,262]. In a typical plasma treatment process, microfiber mats are placed in a vacuum or low-pressure chamber where a gas, commonly oxygen (O_2), argon (Ar), nitrogen (N_2), or air is ionized using radiofrequency (RF) or microwave energy. The resulting plasma interacts with the fiber surface, breaking molecular bonds and forming new polar functional groups such as hydroxyl (-OH), carboxyl (-COOH), amine (-NH₂), or carbonyl (C=O) [254,263]. These groups serve as anchoring sites for subsequent chemical conjugation, such as the attachment of bioactive molecules. Plasma treatment also enhances coating adhesion in post-processing steps. For instance, plasma-treated microfibers exhibit better bonding with drug-loaded polymers, or hydrogel coatings due to the increased density of surface functional groups and roughness [264]. This makes it an essential pre-treatment step in multilayer microfiber systems and sensor-integrated scaffolds. Importantly, plasma treatment is a solvent-free, low-temperature, and rapid process, making it compatible with thermally sensitive polymers and biologically active molecules. The depth of surface modification is typically limited to a few nanometers, which ensures that the mechanical properties of the fibers remain unchanged [259]. In biomedical applications, plasma functionalization has been effectively utilized to enhance the performance of microfiber-based systems. It improves cell adhesion and proliferation in scaffold structures, increases drug loading efficiency by enhancing surface wettability and interactions, and enables site-specific immobilization of biomolecules such as peptides, antibodies, and enzymes for biosensing applications. Additionally, it facilitates the integration of hydrogels, supporting the development of smart wound dressings and moisture-responsive systems.

Wet etching involves immersing fibers in a reactive chemical solution that selectively dissolves specific components of the fiber or modifies the surface (Figure 3B) [265,266]. Acidic or basic solutions such as hydrochloric acid, nitric acid, or sodium hydroxide are commonly used. Wet etching creates increased surface roughness, opens up surface pores, and modifies surface chemistry. This process is valuable for improving hydrophilicity, increasing protein or drug adsorption, and enhancing cell attachment. For instance, alkaline etching of PLA or PCL microfibers exposes carboxyl and hydroxyl groups, which promote collagen adsorption or subsequent chemical grafting. Wet etching is also used to remove sacrificial layers or surface coatings to reveal internal fiber layers or cores.

Electrospray coating is a precise and versatile surface modification technique that enables the deposition of micro- or nanoscale droplets onto a target substrate using an electrically driven spraying process (Figure 3C) [39,267]. In contrast to conventional spray coating, which relies on pneumatic or thermal atomization, electrospray utilizes electrostatic forces to produce a fine mist of charged droplets from a polymer or functional material solution. This technique allows for the uniform and controlled coating of microfiber surfaces with excellent resolution, making it especially suitable for biomedical applications where spatial precision, minimal material waste, and gentle processing conditions are essential. The electrospray process involves applying a high voltage (typically 5–20 kV) to a capillary needle containing the solution to be deposited [268]. Under the influence of this electric field, a Taylor cone forms at the needle tip, and charged droplets are emitted from its apex. These droplets travel toward a grounded collector, which may be a microfiber mat, individual fiber, or rotating substrate. As the solvent evaporates mid-flight, the solute is deposited onto the target surface as a thin film or particulate layer. Key parameters such as solution concentration, flow rate, applied voltage, tip-to-substrate distance, and solvent volatility influence droplet size, deposition rate, and coating morphology. Electrospray coating is particularly advantageous for functionalizing delicate or highly porous microfiber structures, as it provides conformal coverage without fiber bundling or morphological collapse. The technique has been used to deposit a wide range of functional materials including therapeutic drugs, nanoparticles (e.g., silver, hydroxyapatite, magnetic), bioactive polymers, enzymes, and conductive materials. Its ability to deposit material LbL also supports the fabrication of multilayered or gradient coatings with tunable properties. In biomedical applications, electrospray coating has been employed to endow microfibers with antibacterial activity (e.g., electrosprayed silver or zinc oxide nanoparticles), controlled drug release (e.g., coating with hydrophobic or stimuli-responsive drug reservoirs), and bioactivity (e.g., growth factor-loaded coatings for regenerative scaffolds). The low temperature and solvent-based processing also make it suitable for encapsulating and immobilizing sensitive biomolecules such as proteins or peptides without denaturation. Furthermore, electrospray allows selective functionalization by directing the spray to specific areas or patterns on the microfiber surface, which is advantageous in biosensing, wound healing patches, and organ-on-fiber constructs where spatial functionality is required. Despite its advantages, electrospray coating requires careful optimization to ensure consistent deposition, especially on 3D fibrous architectures where charge accumulation and droplet trajectory may affect uniformity. However, with advances in nozzle design, multi-nozzle arrays, and real-time feedback control, electrospray is becoming increasingly scalable and reliable for industrial and clinical translation.

Sputtering, a form of physical vapor deposition (PVD), enables the deposition of ultra-thin metallic or metal oxide layers onto fiber surfaces (Figure 3D) [269]. The process occurs in a vacuum chamber, where a target material (e.g., gold, silver, titanium, zinc oxide) is bombarded by inert gas ions (usually argon), causing ejection of atoms that condense on the fiber surface [270]. The key advantages of sputtering include conformal coverage, high purity, and excellent adhesion. Sputtered coatings are often used to add electrical conductivity, antimicrobial properties, or photothermal activity to microfibers [271]. For example, sputtered silver or copper layers provide antimicrobial effects for wound healing, while gold coatings support biosensor functionality. Titanium and zinc oxide coatings offer biocompatibility and UV protection. The deposition parameters, such as power, time, pressure, and substrate orientation, affect the coating's thickness, crystallinity, and surface energy.

Dip coating is one of the most accessible and widely used methods for surface functionalization (Figure 3E) [272]. In this process, microfiber mats or single fibers are immersed in a solution containing the functional agent (e.g., drug, polymer, nanoparticle) and then withdrawn at a controlled rate [273,274]. Upon drying or curing, a uniform coating forms on the fiber surface. Multiple cycles can be performed to increase coating thickness or to build multilayers. Dip coating is highly adaptable and compatible with a wide range of functional agents and solvents [275]. It is widely used to impart antibacterial, anti-inflammatory, or hemostatic functionality to wound dressings and implantable fibers [276]. For example, chitosan or alginate coatings can provide antimicrobial action, while dip coating with silver nanoparticles or drug-loaded polymers enables therapeutic release [277]. Parameters such as immersion time, withdrawal speed, and solution viscosity determine the coating thickness and uniformity.

LbL assembly is a versatile surface modification technique that relies on the sequential adsorption of oppositely charged polyelectrolytes or biomolecules onto a substrate [278]. In microfiber scaffolds, this method enables the formation of ultrathin multilayer coatings with precise control over composition and thickness [279]. The process typically involves alternating immersion of the fibers in positively and negatively charged solutions, resulting in the gradual buildup of functional layers through electrostatic interactions, hydrogen bonding, or covalent bonding [280]. LbL coatings can incorporate bioactive molecules such as growth factors, enzymes, antimicrobial agents, or nanoparticles, thereby imparting multifunctional properties to microfiber scaffolds [258]. Because the process occurs under mild conditions, it is particularly suitable for loading sensitive biomolecules without compromising their biological activity. Consequently, LbL functionalization has been widely explored to enhance cell adhesion, control drug release, and introduce antibacterial or immunomodulatory properties in microfiber-based biomedical devices [281].

Thus, these functionalization techniques offer a powerful toolkit for tuning the surface and interface properties of microfibers. By selecting and combining these methods, it is possible to endow microfibers with antibacterial, anti-inflammatory, osteoinductive, conductive, or sensing capabilities. The compatibility of these techniques with different fiber materials and structures, whether porous, hollow, aligned, or core-shell, makes them adaptable across a wide range of biomedical applications, from wound care and implants to wearable sensors and organ scaffolds.

6. 3D Assemblies

Three-dimensional (3D) assemblies of microfibers represent a critical structural advancement beyond individual fibers or simple 2D mats, enabling hierarchical architectures that better recapitulate native tissue organization. By organizing microfibers into nonwoven meshes, woven constructs, or precisely patterned 3D frameworks, it is possible to tailor porosity, mechanical anisotropy, interconnectivity, and cell-matrix interactions at multiple length scales. Such assemblies bridge the gap between microscale fiber engineering and macroscale tissue constructs, offering improved structural stability and functional performance.

Nonwoven microfiber meshes are the most widely used 3D assemblies and are typically produced through electrospinning, rotary jet spinning, or air-blowing techniques [11,282,283]. In these systems, fibers are deposited randomly or semi-randomly to form an interconnected network without a defined weaving pattern. The resulting architecture is highly porous, lightweight, and ECM-mimetic, closely resembling the fibrillar structure of natural connective tissues. The key advantages of nonwoven meshes include high surface area, tunable pore size distribution, and ease of fabrication at large scale. Porosity and thickness can be controlled by adjusting spinning time, collector distance, humidity, and fiber diameter [45]. However, conventional electrospun nonwoven mats often suffer from limited cell infiltration due to small pore sizes. To overcome this, strategies such as multilayer stacking, incorporation of sacrificial porogens, gas foaming, or combining micro- and nanofibers have been employed to create thicker, highly porous 3D constructs [284]. Additionally, non-woven meshes can be thermally bonded or chemically crosslinked to improve mechanical stability and structural durability under physiological loading [22,285].

Woven microfiber meshes introduce a higher level of structural organization by interlacing fibers in defined patterns (e.g., plain weave, twill, satin) [286,287]. Unlike nonwoven constructs, woven assemblies provide predictable mechanical behaviour, enhanced tensile strength, and improved load-bearing capacity due to ordered fiber interconnections. Woven architectures allow precise control over pore geometry, anisotropy, and directional stiffness. Fabrication of woven microfiber scaffolds may involve many different techniques such as micro-weaving, textile braiding, knitting, and 3D weaving [288,289]. The interlaced structure enhances resistance to tearing and delamination while maintaining interconnected porosity for cell migration and nutrient diffusion. Moreover, woven scaffolds can be engineered with gradient stiffness by varying fiber density or material composition, enabling the recreation of complex tissue interfaces. However, fabrication complexity and limited flexibility compared to nonwoven meshes can be challenges.

3D patterned meshes represent the most advanced category of microfiber assemblies, where fibers are deposited or organized in predefined spatial geometries to create architecturally precise scaffolds. These structures are typically fabricated using MEW, 3D printing, direct ink writing (DIW), or programmable textile engineering [156,157]. Unlike conventional nonwoven or woven meshes, 3D patterned constructs allow micron-level control over fiber spacing, orientation, stacking sequence, and hierarchical layering [68]. This approach enables the creation of lattice, hexagonal, honeycomb, gradient, or biomimetic patterns tailored to specific tissue requirements [163,290]. Mechanical properties such as stiffness, elasticity, and anisotropy can be finely tuned by adjusting fiber diameter, inter-fiber spacing, and stacking angle. Moreover, pore size can be precisely controlled to enhance cell infiltration,

vascularization, and tissue integration. Multi-material deposition further allows incorporation of bioactive or conductive fibers within specific regions, creating functionally graded scaffolds. Compared to nonwoven and woven systems, 3D patterned assemblies offer superior reproducibility, customization, and integration with digital design platforms [171]. However, scalability and manufacturing speed remain ongoing challenges for widespread clinical translation.

In summary, 3D microfiber assemblies whether nonwoven, woven, or precisely patterned, provide a versatile framework for translating microscale fiber engineering into functional tissue-scale constructs. The strategic selection and combination of these assembly approaches allow microfiber-based systems to address diverse biomedical requirements, ranging from soft tissue repair to mechanically demanding regenerative therapies and advanced bioengineered implants.

7. Biomedical Applications

Microfiber-based systems have demonstrated remarkable versatility across a broad spectrum of biomedical applications, owing to their tunable architecture, surface functionality, mechanical adaptability, and capacity for bioactive integration. In this section, the applications are organized to reflect a progression based on the increasing structural and functional complexity of target tissues, as well as the expanding technological demand. The discussion begins with wound healing, representing surface-level tissue repair, where microfibers primarily act as protective and ECM-mimicking scaffolds. It then advances to bone and muscle regeneration, which require enhanced mechanical strength, and structural anisotropy, followed by nerve and vascular regeneration, where guidance cues and bioelectrical or tubular architectures become essential. Finally, the scope extends beyond regenerative scaffolding to multifunctional biomedical interfaces, including biosensing and diagnostics, and therapeutic systems for cancer treatment. This structured progression highlights how microfiber technologies evolve from passive structural supports to dynamic, smart, and clinically translatable platforms capable of addressing increasingly complex biomedical challenges.

7.1. Wound Healing

Wound healing is a complex, dynamic biological process involving hemostasis, inflammation, proliferation, and remodeling phases [11,291,292]. Effective wound care materials must therefore protect the wound site, manage exudate, prevent infection, and promote tissue regeneration [293]. Microfiber-based scaffolds, due to their high surface area, porosity, mechanical flexibility, and tunable architecture, are increasingly being explored as advanced wound dressings. These materials can mimic the structure of the ECM, support cell migration and proliferation, and deliver therapeutic agents in a controlled manner to accelerate healing [294].

Electrospun microfibers are among the most widely used for wound healing applications, primarily because they can form nonwoven mats with closely resembling native skin ECM [295]. Their high porosity ensures gas exchange while maintaining a moist environment that is critical for re-epithelialization. Additionally, the interconnected pores facilitate nutrient transport and cell infiltration, while the tunable mechanical properties allow for conformity to irregular wound geometries. Microfibers for wound healing are often functionalized with antibacterial, anti-inflammatory, or regenerative agents to enhance therapeutic outcomes [143]. Antibacterial functionalization is particularly important to prevent infection, a common complication in chronic wounds. This can be achieved by directly incorporating silver compounds, silver nanoparticles, zinc oxide nanoparticles, magnesium oxide nanoparticles, antibiotics, or antimicrobial peptides into the fiber matrix via above-mentioned functionalization strategies [32,296]. Anti-inflammatory drugs such as curcumin or corticosteroids can also be loaded into microfibers for sustained release, reducing prolonged inflammation and promoting timely progression to the proliferative phase [206,294]. Another promising approach involves bioactive molecule or growth factor incorporation, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF), to stimulate angiogenesis and cellular proliferation [296,297]. Recent studies have also investigated stimuli-responsive microfibers as intelligent wound-healing systems. These systems, which respond to changes in temperature, pH, or moisture, can release therapeutic agents in a controlled, on-demand manner or dynamically alter their structure to adapt to the wound microenvironment [32,295,298]. Microfibers also support skin tissue regeneration by guiding cell alignment, supporting ECM deposition, and modulating the wound microenvironment [299]. Aligned fibers, for example, promote directional migration of keratinocytes and fibroblasts, which can accelerate re-epithelialization [300,301]. In deeper wounds, microfiber scaffolds serve as dermal substitutes, supporting angiogenesis and integration with surrounding tissue. These scaffolds can be fabricated with multilayered structures to mimic the epidermis and dermis, with different porosity, degradation rates, and bioactive contents in each layer. *In vivo* studies have shown

that microfiber-based wound dressings can significantly enhance the rate and quality of wound closure, reduce inflammation, and promote collagen deposition and neovascularization [302,303]. Some commercial products based on electrospun microfibers have already entered clinical use, highlighting their translational impact to the patients [304].

In addition, hydrogel-forming microfiber mats are gaining traction due to their superior hydration capacity and soft tissue conformity [235]. These systems combine the benefits of fibrous structure with hydrogel swelling and drug release properties, making them especially suitable for treating burn wounds and diabetic ulcers. These hydrogel systems can accelerate wound closure, reduce scarring, and enhance epithelialization. Recent research efforts increasingly emphasize precision fabrication techniques and the utilization of the materials' intrinsic biological activity to enhance therapeutic performance. Shen et al. demonstrated the utility of microfluidic spinning to create an injectable “Fibro-Gel”, an all-aqueous network of entangled polyethylene glycol diacrylate (PEGDA) microfibers (Figure 4A) [305]. This injectable system allows for minimally invasive application to irregularly shaped wounds and provides a key mechanism for accelerating wound closure and vascularization *in vivo* through the tunable and sequential release of distinct therapeutic agents, such as antibiotics and growth factors. Similarly, using a one-step microfluidic spinning technique, Wang et al. developed porous hydrogel microfibers assembled from prolamins (Figure 4B) [235]. They showcased that the porosity and morphology of these microfibers could be precisely tailored, enabling them to serve as flexible delivery vehicles for actives like bacitracin and VEGF, ultimately displaying superior *in vivo* capability in treating diabetic wounds. Expanding on microfiber complexity, Yu et al. reported the use of coaxial capillary microfluidic spinning to create sophisticated core-shell microfibers with an alginate shell and a core containing copper- or zinc-vitamin framework (Figure 4C) [306]. The controlled release of vitamins and metal ions from this system provided combined antibiosis and antioxidation for accelerated tissue healing.

Beyond drug delivery, microfibers are being engineered for active therapy and diagnosis. Jia et al. demonstrated a significant leap by fabricating ultralong conductive silk microfibers (mSFs) via a bioinspired coating of polydopamine (PDA) and Poly(3,4-ethylenedioxythiophene) (PEDOT) (Figure 4D) [206]. This resulted in a flexible patch that acts as a conformable bioelectronic for monitoring physiological signals. Importantly, they showed the patch's active therapeutic role in chronic diabetic wound healing, where the conductive mSFs' intrinsic anti-oxidative activity actively reduced inflammation and regulated oxidative stress. For straightforward, real-time monitoring, Ha et al. created a multifunctional electrospun bilayer-dressing patch (Figure 4E) [307]. The protective layer of this patch incorporated curcumin as a sensing agent, enabling a visible colour change (pH 6–9) for non-invasive, real-time monitoring of wound status, while also providing enhanced antimicrobial protection.

Furthermore, researchers have addressed infection and structural integrity using probiotic therapy [308]. However, probiotic therapy has large obstacle and potential biosafety risks for proper application. Lu et al. reported an effective, probiotic-free strategy for promoting infected wound healing by regulating the wound flora balance (Figure 4F) [271]. They engineered a poly(4-methyl-1-pentene) (PMP) microfiber membrane that exhibited strong bacterial adhesion, allowing for the efficient removal of pathogenic bacteria through dressing changes to maintain a low-concentration, pro-healing environment. Moreover, to address the low structural integrity issues, Xu et al. reported a blended membrane composed of chitosan (CS) and chemically hydrolyzed silk microfibers (mSFs) (Figure 4G) [309]. Their findings demonstrated that incorporating mSFs substantially enhanced the membrane's mechanical strength and decreased its swelling ratio, which in turn improved wound healing performance, as evidenced by increased epithelialization and greater collagen deposition.

In summary, microfiber scaffolds offer a multifunctional platform for wound healing by integrating mechanical protection, moisture regulation, antimicrobial activity, and therapeutic delivery into a single system. With continued advancements in fabrication, functionalization, and clinical validation, microfiber-based wound dressings are poised to become a next-generation solution for managing acute and chronic wounds.

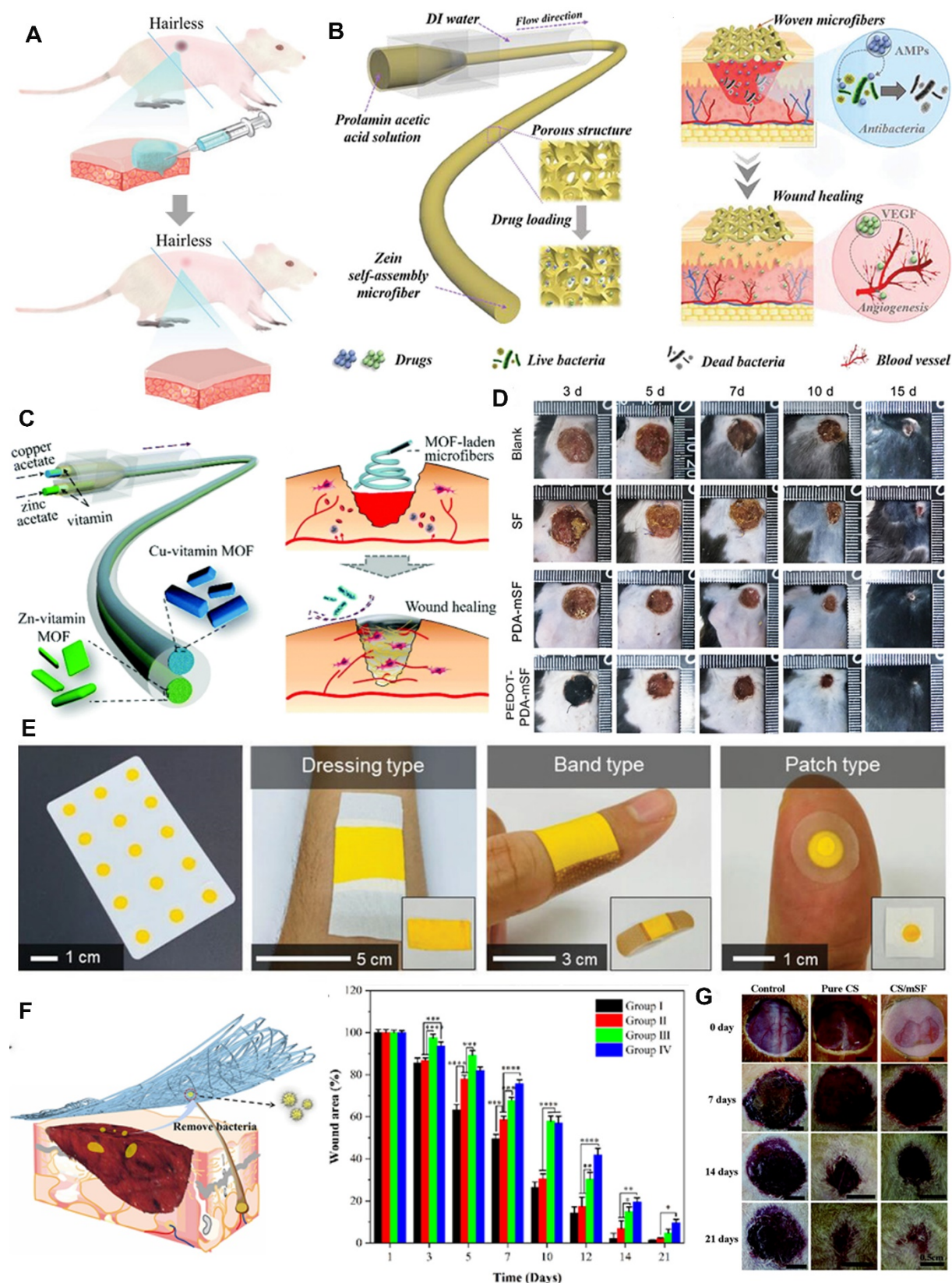


Figure 4. Applications in wound healing. (A) Schematic of the mice excision skin model where the mice were dressed with the PEGDA fibro-gel. Reproduced with permission [305]. Copyright 2023, Wiley-VCH. (B) Schematic diagram of the prolamins-assembled porous hydrogel microfibers from microfluidic spinning technology for wound healing. Reproduced with permission [235]. Copyright 2024, Wiley-VCH. (C) Schematic illustration of the formation of vitamin MOF-laden microfibers using a microfluidic spinning approach and their application in the wound healing. Reproduced with permission [306]. Copyright 2018, RSC Publishing. (D) PEDOT-PDA-silk microfiber patch accelerated dermal wound healing in diabetic mice. Reproduced with permission [206]. Copyright, Wiley-VCH. (E) The multifunctional wound dressing patch containing dexpantenol,

hyaluronic acid, and gelatin (DHG), curcumin and polycaprolactone (C-PCL). Various types of wound treatments (dressings, bands, and patches) suitable for the size of the lesion can be applied. Reproduced with permission [307]. Copyright 2023, Wiley-VCH. (F) Schematic illustrating the regulation of wound flora balance using the poly(4-methyl-1-pentene) microfiber membrane. Right side bar graph shows wound area after therapy *in vivo*. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Reproduced with permission [291]. Copyright 2022, ACS Publications. (G) Digital images of macroscopic wound condition depicting wound size at different durations. Wound size is compared at different days with control (covered with vaseline cream), pure chitosan (CS) membrane and CS/mSF (80% CS/mSF blended membrane). Reproduced with permission [309]. Copyright 2015, Wiley-VCH.

7.2. Bone Repair

Bone tissue engineering aims to restore, repair, or replace damaged bone by creating biomimetic scaffolds that provide structural support and promote cellular activity essential for osteogenesis [310]. Microfiber-based scaffolds have emerged as highly promising platforms for bone regeneration due to their ability to mimic the hierarchical structure of native bone ECM, guide cell behavior, and deliver bioactive cues in a spatially and temporally controlled manner [181,311]. Their tunable mechanical properties, degradability, and functionalization potential make them excellent candidates for load-bearing and non-load-bearing bone defects. The mechanical integrity of microfiber scaffolds is essential in bone applications, especially for supporting load-bearing sites. Aligned or grid-patterned fiber scaffolds produced via melt electrowriting or 3D printing offer high compressive strength and structural anisotropy, which mimic the architecture of trabecular bone [312]. These features not only support mechanical loads but also direct cell alignment and matrix deposition in the direction of applied stress. To enhance osteoinductive and osteoconductive properties, microfiber scaffolds are often functionalized with bioactive molecules or inorganic fillers [87,313]. These fillers can be blended into the polymer prior to spinning or deposited via electrospray coating, sputtering, or dip coating [48,314].

Moreover, microfiber scaffolds can be biofunctionalized with growth factors such as bone morphogenetic protein-2 (BMP-2), VEGF, or transforming growth factor- β (TGF- β) to stimulate osteogenic differentiation, angiogenesis, and matrix remodeling [46,311]. Controlled release of these factors from core-shell or porous microfibers provides a sustained stimulatory environment that mimics natural bone healing processes. Scaffold surfaces can also be modified with RGD peptides or collagen to enhance osteoblast attachment and proliferation [46,227]. Another emerging strategy involves the use of electroactive microfiber scaffolds, incorporating piezoelectric materials such as PVDF or BTO nanoparticles [315,316]. These materials generate electrical signals under mechanical stress, mimicking the endogenous bioelectric cues that naturally stimulate bone regeneration during physical activity. Such scaffolds have shown promise in promoting osteogenic gene expression and enhancing mineral deposition, particularly under dynamic loading conditions. Depending on the design, these scaffolds can degrade over time in synchrony with new tissue formation, eliminating the need for surgical removal.

Despite the progress, challenges remain in translating microfiber scaffolds for clinical bone regeneration. These include achieving optimal load-bearing capacity, matching degradation kinetics to tissue regeneration rates, scaling up fabrication processes, and ensuring regulatory compliance. However, continued advancements in material science, additive manufacturing, and functionalization technologies are rapidly addressing these issues. The current research focuses intensely on developing composite scaffolds that not only provide mechanical support but also actively induce osteogenesis and angiogenesis through smart material design and tailored drug/ion delivery. Zhou et al. reported the formulation of an injectable, composite hydrogel system for the minimally invasive treatment of osteonecrosis (Figure 5A) [313]. This scaffold comprised an alginate (SA)/tricalcium phosphate (β -TCP) hydrogel matrix reinforced with P34HB/MgO+PEG coaxial electrospun microfibers. They demonstrated that by varying the microfiber concentration, the composite's injectability and mechanical strength could be optimized, while the controlled release of magnesium ions (Mg^{2+}) from the MgO-laden core promoted osteogenic differentiation and bone repair. Wu et al. employed dual-nozzle hybrid electrospinning to address the insufficient mechanical properties and biocompatibility of single-polymer systems (Figure 5B) [48]. They fabricated composite scaffolds using a blend of PVA, polyhydroxybutyrate (PHB), and multiwalled CNTs (MWCNTs). This strategy resulted in microfibers with enhanced mechanical properties and improved surface characteristics that significantly promoted the osteogenic differentiation of stem cells *in vitro* and accelerated bone regeneration *in vivo*.

Several studies have aimed to design bone scaffolds that better replicate the natural mineralized structure of bone in order to improve osteogenic activity. In this context, researchers have developed biomineralized and dynamic scaffold systems that combine organic matrices with calcium-based mineral components. Shi et al. developed a self-healing silk fibroin (SF)-based hydrogel for bone regeneration, utilizing a dynamic metal-ligand

coordination chemistry approach (Figure 5C) [311]. Their system involved reversible crosslinking between silk fibroin microfibers (mSF), which were biomaterialized with calcium phosphate, and a polysaccharide binder. This dually crosslinked (DC) hydrogel exhibited shear-thinning and autonomous self-healing properties, allowing it to conform perfectly to irregularly shaped defects. They successfully showed that this scaffold could accelerate bone regeneration in cranial critical size defects without the need for additional exogenous morphogens. Similarly, Wei et al. created a mechanically enhanced biodegradable scaffold based on pure silk fibroin microfibers and mineralized collagen (Figure 5D) [212]. This composite scaffold significantly improved mechanical properties and demonstrated superior efficacy in repairing bone defects in the distal femur of rats.

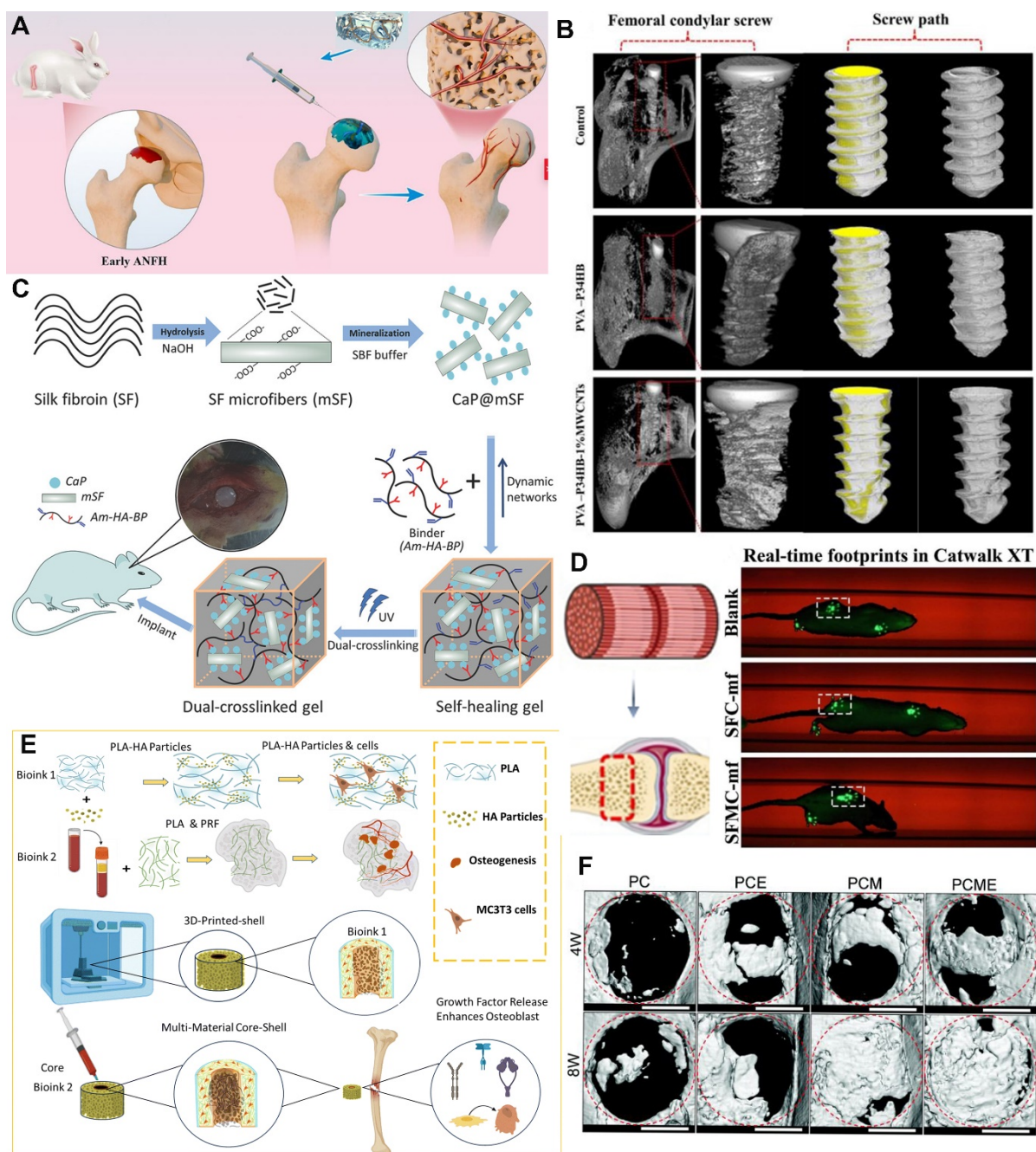


Figure 5. Applications in bone regeneration. (A) Schematic of injectable alginate/ β -TCP hydrogel reinforced with coaxial electrospun P34HB/MgO+PEG microfibers for treating early-stage avascular necrosis of the femoral head (ANFH) in a rabbit model. Reproduced with permission [313]. Copyright 2025, Wiley-VCH. (B) Micro-CT imaging of rabbit femoral condyles at 8 weeks post-implantation of PVA-P34HB based microfiber scaffolds, indicating new bone formation along the screw-bone interface. Reproduced with permission [48]. Copyright 2025, Elsevier. (C) Schematic illustrating the fabrication of mineralized silk fibroin microfiber based hydrogels for repairing rat cranial bone defects. Reproduced with permission [311]. Copyright 2017, Wiley-VCH. (D) Implantation of mechanically enhanced biodegradable silk fibroin microfiber scaffold in rat femoral bone defects

and thereafter gait analysis demonstrating mechanical and functional recovery. Reproduced with permission [212]. Copyright 2024, Elsevier. (E) Schematic illustration of bioink preparation and the fabrication method of multi-material bioactive bone substitutes. Reproduced with permission [213]. Copyright 2024, RSC Publishing. (F) Illustration depicting radiographic reconstruction of cranial bone defects at 4- and 8-weeks after microfiber scaffolds implantation, highlighting new bone growth at surgery sites. Reproduced with permission [46]. Copyright 2019, RSC Publishing.

Furthermore, recent studies have explored the integration of microfibers with advanced biofabrication strategies to improve the structural and biological performance of bone scaffolds. Moghimi et al. reported the development of short microfiber-reinforced printable hydrogels for personalized bone repair (Figure 5E) [213]. By employing 3D bioprinting, they fabricated a core-shell bone graft from a hybrid composite of hydroxyapatite-coated poly(lactic acid) (PLA) fibers embedded within a methacryloyl gelatin (GelMA)/alginate hydrogel matrix. This construct was designed to incorporate platelet-rich fibrin (PRF) to enhance the biological cues for regeneration. Focusing on cell recruitment and differentiation, Chi et al. constructed a dually optimized polycaprolactone (PCL)/collagen I microfiber scaffold using coaxial electrospinning (Figure 5F) [46]. This scaffold was specifically engineered to capture bone marrow stem cells (BMSCs) and deliver BMP2-derived peptides from the core. Their work demonstrated that this design promoted the osteogenic differentiation of the enriched stem cells, ultimately leading to synergistic bone regeneration.

In conclusion, microfiber-based scaffolds offer a customizable, multifunctional, and biomimetic approach to bone regeneration. By tuning fiber structure, surface chemistry, mechanical properties, and biological cues, these systems can effectively guide bone repair and integration, offering a promising alternative to traditional grafts and metallic implants.

7.3. Muscle Regeneration

Skeletal muscle has a remarkable regenerative capacity following minor injuries, however, in cases of severe trauma, volumetric muscle loss (VML), or degenerative diseases such as muscular dystrophy, the endogenous repair mechanisms are insufficient to restore full structure and function [282,317–319]. To address this clinical challenge, microfiber-based scaffolds have emerged as one of promising platforms for muscle tissue engineering due to their ability to replicate the fibrous architecture of native muscle ECM, support cellular organization, and deliver biological or electrical cues to guide regeneration [320].

The structural alignment of muscle fibers plays a fundamental role in skeletal muscle regeneration because the native muscle tissue exhibits a highly organized, anisotropic architecture that enables efficient force generation and transmission [297]. Therefore, biomaterial scaffolds designed for muscle repair must replicate this aligned microenvironment to support proper myoblast orientation and maturation. Microfibers fabricated using techniques such as electrospinning, microfluidics, and 3D printing can be engineered with precise orientation to promote myoblast alignment, fusion into multinucleated myotubes, and anisotropic tissue formation [107,321]. Aligned microfiber scaffolds provide topographical cues that guide cell elongation and cytoskeletal organization, closely mimicking the architecture of native skeletal muscle [317,319]. In addition to structural guidance, muscle tissue relies on electrical signaling to regulate contraction and cellular function, which has motivated the development of conductive microfiber-based scaffolds to better replicate this physiological environment. Incorporating conductive materials such as polypyrrole (PPy), PANI, CNTs, or graphene into fiber matrices enables scaffolds to transmit bioelectrical signals, enhance myogenic differentiation, and improve muscle fiber contractility [322]. These scaffolds can be used with external electrical stimulation setups or integrated with wireless bioelectronics for smart, on-demand stimulation [297]. Microfibrous scaffolds with dynamic mechanical properties or shape-memory behavior can be designed to deliver periodic stretching, which enhances cytoskeletal organization and mimics muscle loading during movement [323,324]. Microfiber scaffolds have been also seeded with myoblasts, MSCs, and induced pluripotent stem cell-derived myogenic progenitors (iPSC-MPs), often in combination with bioactive fibers, to support muscle formation [319,325].

Building on these design principles, several studies have developed biomimetic microfiber-based scaffolds that recreate the hierarchical microfibrillar structure of skeletal muscle. Kim et al. reported a 3D-printed biomimetic scaffold that simulates the microfibril structure of muscle (Figure 6A) [326]. They employed a microfibrillation/leaching process of poly(vinyl alcohol) (PVA) from a PVA/PCL mixture to create a micropatterned PCL microfiber strut. This structure, supplemented with Type-I collagen, successfully promoted C2C12 myoblast proliferation and myogenic differentiation *in vitro*, demonstrating its potential as a platform for realistic 3D muscle tissue modeling. Similarly, for complex tissue structures, Chen et al. developed a flexible, mechanically robust, and well-aligned bi-layered tubular microfiber scaffold based on silk protein (Figure 6B) [327].

This design specifically mimicked the native human intestinal smooth muscle structure, supporting the alignment and elongation of primary human intestinal smooth muscle cells in two orthogonal layers, which is essential for achieving functional innervation in engineered intestinal smooth muscle tissue.

A Mesh structures for mimicking skeleton and cardiac muscle

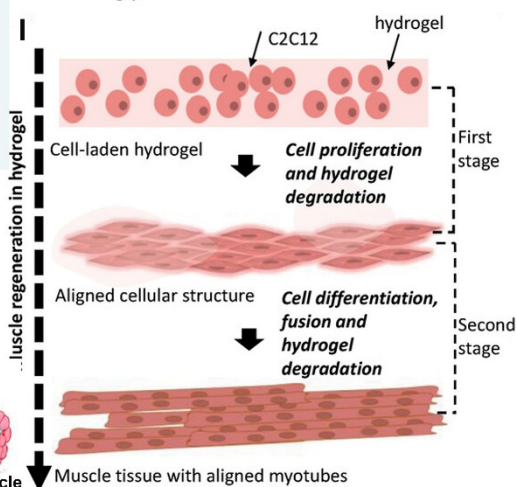
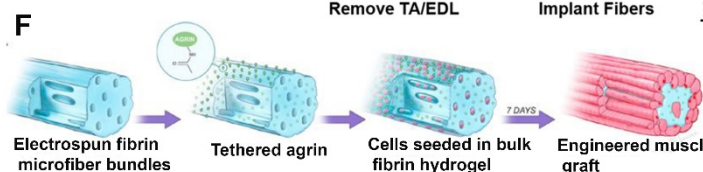
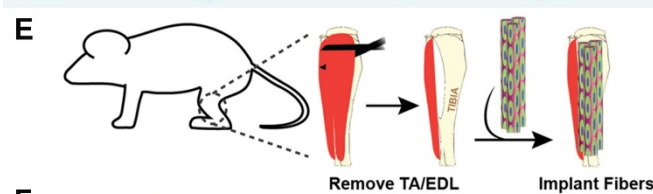
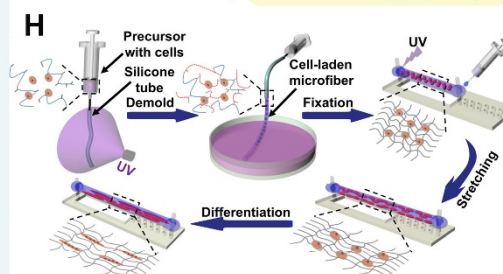
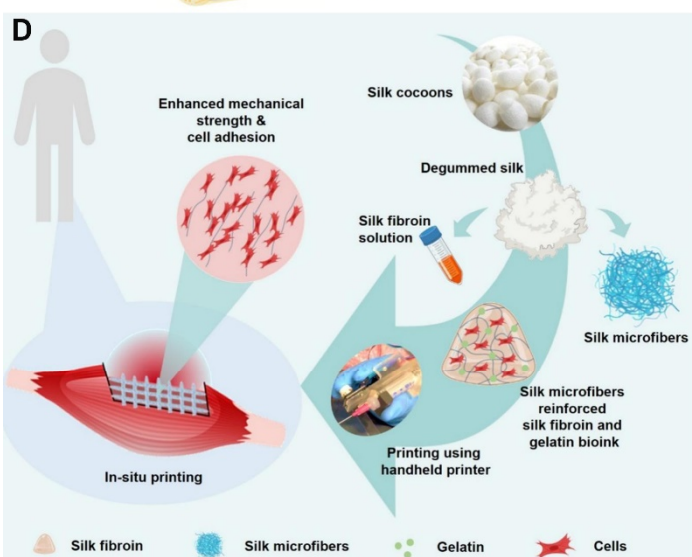
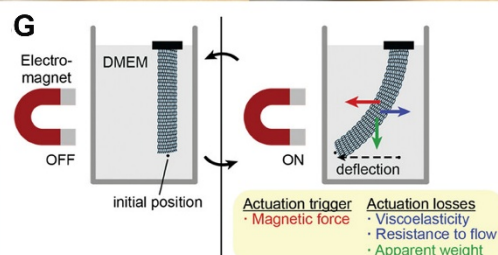
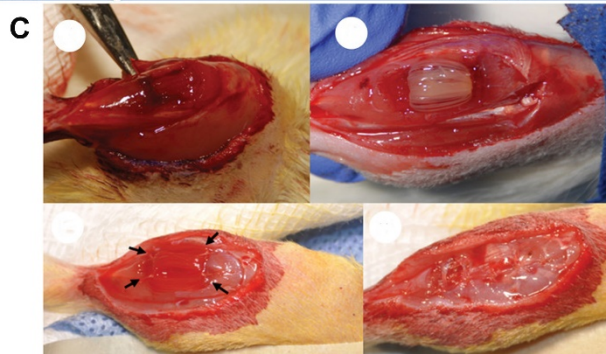
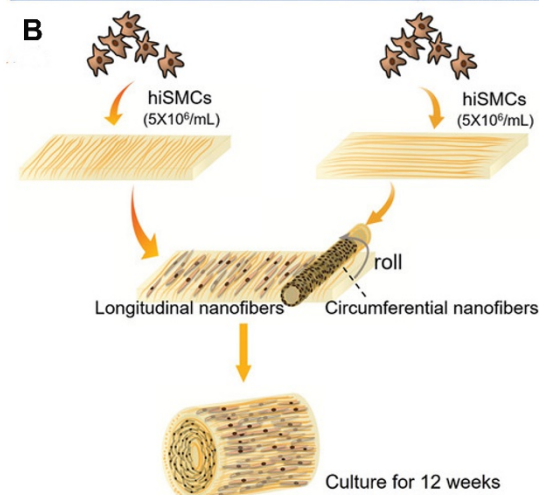
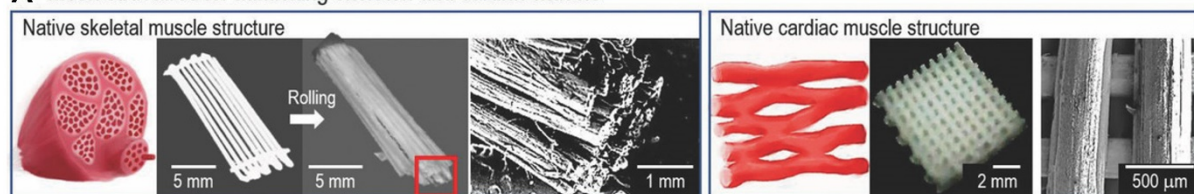


Figure 6. Applications in muscle regeneration and engineering muscle constructs. (A) Skeletal and cardiac muscles mimicked by rolling aligned microfiber membranes and 3D printing. Reproduced with permission [326]. Copyright 2018, Wiley-VCH. (B) Schematic illustrating a cell seeding strategy for making hiSMC-derived 3D intestinal smooth muscle constructs. Reproduced with permission [327]. Copyright 2020, Wiley-VCH. (C) Defect creation,

initial placement of a collagen fiber implant, suture placement for implant attachment, and fascia replacement during functional recovery in a rodent VML model. Reproduced with permission [325]. Copyright 2022, Wiley-VCH. (D) Schematic representation of silk fibroin solution and silk microfibers preparation from silk cocoons and their application as a bioink for in situ 3D bioprinting using custom-made handheld 3D printer for VML repair. Reproduced with permission [318]. Copyright 2025, Elsevier. (E) Schematic illustrating fibrin microfiber implantation into a murine VML defect model. Reproduced with permission [319]. Copyright 2018, Sage Publications. (F) Schematic illustrating engineered muscle graft generated by agrin tethering and cell seeding on electrospun fibrin microfiber bundles. Reproduced with permission [282]. Copyright 2020, Elsevier. (G) Schematic showing magneto-mechanical stimulation of magneto-active skeletal muscle constructs reinforced with magneto-active microfiber scaffolds. Reproduced with permission [156]. Copyright 2024, Wiley-VCH. (H) Schematic representation of the fabrication process, the fixation, and the uniaxial stretching of C2C12 myoblasts laden microfibers. Cells within microfibers are elongated with the application of uniaxial stretching during culture and the differentiation of myoblasts is further promoted. Reproduced with permission [320]. Copyright 2020, ACS Publications. (I) Schematic showing the skeletal muscle regeneration within the microfiber based 3D hydrogel. Reproduced with permission [328]. Copyright 2024, Wiley-VCH.

While such biomimetic microfiber scaffolds are effective for reproducing structural features of muscle tissue *in vitro*, translation toward therapy requires fabrication platforms capable of generating larger, cellularized implants with robust mechanical integrity and reproducible organization. Addressing this need, Christensen et al. invented an advanced biomanufacturing platform called assembled cell-decorated collagen (AC-DC) bioprinting (Figure 6C) [325]. This platform rapidly creates living biomaterial implants using strong, microfluidic wet-extruded collagen microfibers and clinically relevant cells. They showed that AC-DC implants approximate the strength and stiffness of native musculoskeletal tissue and, critically, promoted functional recovery in a critically sized muscle injury model *in vivo*, demonstrating their therapeutic potential for VML. For customized, in situ fabrication, Kamaraj et al. proposed a bioink composed of silk microfiber-reinforced silk fibroin (SF) hydrogel (Figure 6D) [318]. They validated the use of a custom-made handheld 3D printer for in situ printing of these fiber-reinforced scaffolds, which were predesigned to provide the structural support and fiber alignment necessary to promote muscle regeneration for VML treatment.

Beyond fabricating structurally organized implants, another important strategy is to combine aligned microfiber scaffolds with stem cells that may enhance regeneration through direct differentiation or paracrine support. In this regard, Gilbert-Honick et al. assessed the regenerative potential of human adipose-derived stem cells (ASCs) seeded onto electrospun uniaxially aligned fibrin hydrogel microfiber bundles (Figure 6E) [319]. Although the ASCs exhibited alignment and elongation *in vitro*, they failed to fully recapitulate myotube characteristics. However, when implanted *in vivo* in a VML model, the ASC-seeded microfiber bundles enabled moderate muscle reconstruction. In a separate study, Gilbert-Honick et al. also investigated the delivery of the ECM protein agrin from an engineered scaffold (Figure 6F) [282]. They found that agrin, delivered from a scaffold, significantly increased the presence of regenerated neuromuscular junctions (NMJs) and promoted functional recovery following VML.

Furthermore, in addition to static structural design, recent strategies incorporate dynamic, stimuli-responsive features to better mimic the active mechanical environment of skeletal muscle. For example, Cedillo-Servin et al. reported the rational design and fabrication of magneto-active microfiber meshes using melt electrowriting (MEW) of a magnetic PCL-based composite (Figure 6G) [156]. This solvent-free MEW process allowed for the creation of skeletal muscle-inspired hexagonal scaffolds whose zonal distribution of magnetic material enabled out-of-plane reversible deformation triggered by external magnetic fields, providing a platform for remote stimulation and guided organization of 3D muscle models. Chen et al. demonstrated that the uniaxial stretching of cell-laden microfibers, generated via a silicone-tube-based method, effectively promoted the alignment of C2C12 myoblasts and subsequent myofiber formation *in vitro* (Figure 6H) [320]. To efficiently identify optimal culture conditions, Chen et al. introduced a high-throughput method using microfluidic spinning to produce skeletal muscle microphysiological models (microPMs) (Figure 6I) [328]. They developed a micromanipulation-actuated large-scale screening technique to assess the viscoelasticity and contractile force of these microPMs, establishing screening criteria that integrate these parameters to examine the effects of various culture conditions on skeletal muscle regeneration.

In summary, microfiber scaffolds, when rationally designed for anisotropy, mechanical compliance, electrical functionality, and biochemical delivery, offer a comprehensive toolkit for muscle regeneration. Future efforts should emphasize scalable manufacturing, integrated vascular and neural reconstruction, and closed-loop stimulation strategies to translate engineered muscle constructs into clinically practical therapies for VML and other severe muscle pathologies.

7.4. Nerve Regeneration

Peripheral nerve injuries (PNI) and neurodegenerative conditions often result in significant loss of sensory and motor function [329,330]. Although peripheral nerves have some innate regenerative capacity, the outcomes are often suboptimal in cases of large-gap injuries or complex trauma. Autografts, the current clinical gold standard, are limited by donor site morbidity and availability. Consequently, microfiber-based scaffolds have attracted increasing attention as promising alternatives for nerve regeneration, as their fibrous architecture closely resembles the native nerve ECM and can provide physical guidance for axonal extension while also enabling the incorporation of biochemical and biophysical cues that support neural repair [331].

The application of smart polymer microfibers is rapidly advancing the field of neural tissue engineering, particularly for bridging large-gap PNI and addressing spinal cord injury (SCI). The effective design of these scaffolds, which focuses on mimicking the native neural microenvironment by controlling physical, chemical, and electrical properties, is crucial for guiding successful axon regeneration and functional recovery. In this regard, Yu et al. developed the biomimicking microfibers with controllable core-shell structures generated through capillary microfluidic devices (Figure 7A) [200]. Their approach included integrating these microfibers into a multitrack nerve-on-a-chip system, allowing for the systematic *in vitro* assessment of nerve fiber formation before confirming rapid *in vivo* nerve regeneration and functional recovery in a rat sciatic nerve injury model. To further enhance structural and functional guidance for regenerating axons, Fang et al. designed a hierarchical conductive multiscale filled nerve guidance conduit (MF-NGC) using a combination of electrospinning and 3D printing (Figure 7B) [332]. This multiscale conduit consisted of electrospun PCL/collagen nanofibers forming the outer sheath, aligned PCL microfibers providing anisotropic guidance for axonal growth, and reduced graphene oxide (rGO)/PCL microfibers supplying mechanical stability and electrical conductivity. The hierarchical architecture promoted Schwann cell elongation, neurite extension, and vascular recruitment, while *in vivo* studies demonstrated enhanced axon myelination and improved functional recovery in a rat sciatic nerve defect model.

Further, electrical conductivity has also been incorporated into microfiber scaffolds to regulate neuronal behavior and enhance neural differentiation. Wang et al. fabricated three-dimensional conductive scaffolds from PLCL microfibers using near-field electrostatic printing and followed by coating with rGO (Figure 7C) [281]. They demonstrated the capability of these scaffolds to induce oriented neuronal-like network formation in PC-12 cells and primary mouse hippocampal neurons when coupled with external electrical stimulation, with both the electric field strength and microfiber diameter acting as critical regulatory parameters for neurite outgrowth. Building on this concept of electrically active microfiber scaffolds, Guo et al. further demonstrated a self-powered electrical stimulation platform to enhance neural differentiation using conductive hybrid microfibers composed of PEDOT and rGO (Figure 7D) [9]. In this system, the conductive microfiber scaffold served as both the cellular substrate and the electrical signal transmission medium, while pulsed electrical stimulation was supplied by a triboelectric nanogenerator. This integrated system significantly enhanced the neural differentiation of MSCs without the need for additional biochemical factors, highlighting the potential of self-powered electroactive scaffolds for neural regeneration.

The regulatory role of microfibers extends beyond structural and electrical cues to include the modulation of the immune microenvironment. Dong et al. systematically interrogated the topological cues delivered by oriented microfibers, engineering nerve conduits with oriented microfiber cores and random nanofiber sheaths (Figure 8E) [333]. They elucidated a clear cascade where the aligned microfibers facilitated macrophage recruitment and polarization toward a pro-healing M2 phenotype, which consequently promoted Schwann cell migration, myelination, and axonal extension, leading to pronounced functional improvement in a PNI model. Similarly, Kim et al. developed electrospun nerve guidance conduits (NGCs) integrating milk-derived casein protein (MDP) into aligned PCL and PLGA microfibers (Figure 7F) [334]. Their MDP-AF NGC provided both topographical guidance and chemoattraction from the bioactive protein, synergistically promoting Schwann cell migration, macrophage polarization, and enhanced regeneration of injured sciatic nerves *in vivo*.

Moreover, microfibers have proven essential as carriers for cell-based therapies, particularly for central nervous system injuries. In addressing the challenge of low stem cell survival and uncontrollable migration, Yao et al. fabricated three-dimensional MSCs-laden hydrogel microfibers using a hierarchically aligned fibrin hydrogel system for long-distance SCI repair [335]. The implantation of these cell-laden microfibers significantly enhanced the donor MSCs' neural differentiation, encouraged host neuron migration into the injury gap, and promoted nerve fiber regeneration across the injury site, ultimately improving limb motor function. Furthermore, Kim et al. reported a 3D neurosupportive culture system where astrocytes were cytoprotectively encapsulated within hydrogel based microfibers (Figure 7G) [26]. These microfibers significantly enhanced neuronal circuit generation by accelerating

neurite outgrowth, guiding its directionality, and enhancing synaptic formation, even without direct physical contact between the glial cells and the neurons, providing a cell-based therapeutic platform for injuries such as SCI.

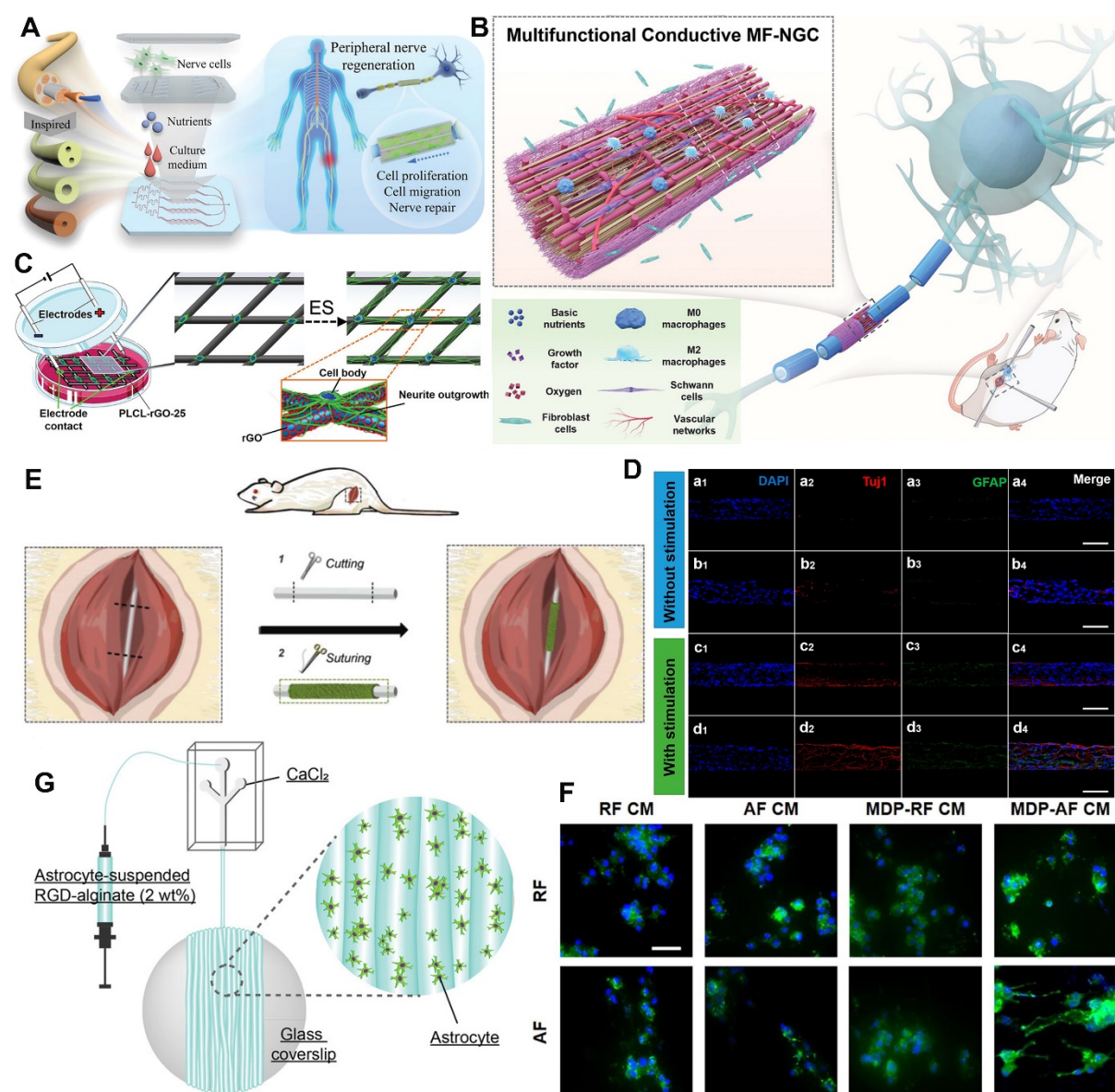


Figure 7. Applications in nerve regeneration. (A) Schematic showing nerve-on-a-chip derived biomimicking microfibers and their applications in peripheral nerve generation. Reproduced with permission [200]. Copyright 2023, Wiley-VCH. (B) Schematic overview of a multifunctional conductive nerve guidance conduit with hierarchical fibers for PNI treatment. Reproduced with permission [332]. Copyright 2023, Wiley-VCH. (C) The culture setup to regulate the neurite outgrowth of PC-12 cells on conductive microfiber patterns under electrical stimulation. Reproduced with permission [281]. Copyright 2020, Wiley-VCH. (D) Neural differentiation of mesenchymal stem cells on reduced graphene oxide (rGO) microfibers (a,c) and rGO-PEDOT hybrid microfibers (b,d) under triboelectric nanogenerator based electrical stimulation. Reproduced with permission [9]. Copyright 2016, ACS Publications. (E) Schematic showing transplanting nerve conduits to the rat sciatic nerve defects. Reproduced with permission [333]. Copyright 2021, Elsevier. (F) Fluorescence image of PC12 cells demonstrating higher neurite outgrowth of PC12 cells for aligned microfiber based nerve guidance conduits. Reproduced with permission [334]. Copyright 2025, ACS Publications. (G) Schematic illustration of astrocyte encapsulation in the RGD-modified alginate microfibers for enhancing neuronal circuit generation. Reproduced with permission [26]. Copyright 2020, Wiley-VCH.

Microfiber-based platforms offer a biomimetic and highly tunable solution for nerve regeneration. By combining aligned architecture, bioactive molecule delivery, electrical conductivity, and cellular support, these

systems can closely replicate the native neural microenvironment and promote long-distance, functional nerve repair. Continued research integrating multifunctional materials, microelectronics, and gene-delivery approaches will further enhance the efficacy of these systems, paving the way for clinically viable, next-generation neural interfaces and repair devices.

7.5. Vascular Regeneration

Vascular regeneration is critical for restoring blood flow in ischemic tissues, healing wounds, and engineering functional tissues and organs [336,337]. Microfibers have emerged as versatile and promising scaffolding platforms for vascular tissue engineering, owing to their ability to mimic the ECM, support endothelialization, and deliver biochemical cues that promote angiogenesis and vascular remodeling [338]. Their high surface-area-to-volume ratio, tunable porosity, and alignment make them ideal for constructing both microvascular and large-diameter vascular grafts. One of the key challenges in vascular regeneration is preventing thrombosis and intimal hyperplasia, particularly in small-diameter vascular grafts [339,340]. To address this, microfibers can be modified with antithrombotic agents (e.g., heparin, nitric oxide donors) or endothelial cell-attracting peptides to accelerate *in situ* endothelialization [341]. Additionally, surface coatings or in-fiber incorporation of antioxidants and anti-inflammatory agents (e.g., curcumin, aspirin, ROS-scavenging materials) help modulate the post-implantation inflammatory response and support long-term graft patency [5]. Bioactive microfiber mats have also been used as angiogenic dressings for ischemic wounds or myocardial infarction, supporting local neovascularization [342].

The advancement of polymer microfibers in vascular regeneration is focused on creating sophisticated scaffolds that mimic the native vessel's structure and function, addressing critical challenges like thrombosis, compliance mismatch, and poor endothelialization, especially in small-diameter applications. The challenge of engineering small-diameter vascular grafts necessitates scaffolds that not only match the mechanical compliance of native vessels but also orchestrate the complex biological processes of immunomodulation, cell recruitment, and anti-thrombogenicity. Federici et al. developed a multicomponent vascular graft using MEW technique to produce a tubular scaffold with vascular-mimetic fiber architecture (Figure 8A) [343]. This MEW framework was combined with a lyophilized fibrinogen matrix for tailored degradation, further functionalized with heparin for anti-thrombogenicity, and encased in an outer electrospun layer to mimic the tri-layered structure of the vessel wall and reduce permeability. Further, Wang et al. utilized a bio-inspired 4D printing strategy to design Janus-structured scaffolds capable of programmable multi-step transformations (Figure 8B) [344]. Inspired by the curling behavior of leaves, these scaffolds transition from a flattened 2D construct into a tubular 3D structure upon dehydration, followed by a crucial secondary transformation *in vivo* to adapt to the local intravascular geometry, thus enabling on-demand vascular reconstruction. In parallel, Liu et al. focused on enhancing vascular regeneration by creating immunomodulatory hybrid scaffolds composed of PCL microfibers and human placental ECM nanofibers via co-electrospinning, demonstrating a method to leverage natural ECM components within a mechanically robust synthetic framework while modulating the host immune response (Figure 8C) [339].

In addition to structural design approaches, biofunctionalization has been explored to enhance endogenous regenerative responses. Wang et al. demonstrated improved vascular regeneration by developing anti-Sca-1 antibody-functionalized vascular grafts (Figure 8D) [345]. This surface modification allows the selective capture of endogenous vascular stem/progenitor cells (SPCs), effectively utilizing the host's innate regenerative capacity to improve tissue outcomes. In addition to promoting regenerative cell recruitment, improving hemocompatibility and controlling inflammatory responses are critical for maintaining long-term graft patency. Shi et al. reported an inflammation-responsive PCL/gelatin microfiber scaffold that provided sustained release of both nitric oxide (NO) and heparin (Figure 8E) [341]. This scaffold not only improved blood compatibility but also suppressed the excessive proliferation of smooth muscle cells, thus preventing intimal hyperplasia and promoting desirable endothelialization. For fabricating microscale vascular structures, Wu et al. reported the microfluidic printing of tunable hollow microfibers using a composite bioink comprised of alginate, gelatin methacrylate, and silk fibroin (Figure 8F) [346]. This approach allowed for the rapid seeding and perfusion of human umbilical vein endothelial cells (HUVECs) into the hollow chambers, leading to the formation of vessel-like tissue structures with high cell viability within three days. Further, Sun et al. used magnetic alginate microfibers as scaffolding elements (Figure 8G) [347]. By loading these microfibers with cells and applying a controlled magnetic field, they were able to induce the self-assembly of cells into micro-scale toroidal cellular modules, which can serve as versatile building blocks for the bottom-up construction of complex microvascular-like structures.

In summary, microfiber-based scaffolds provide a versatile platform for vascular regeneration due to their ability to mimic the structural features of native ECM and support endothelialization and tissue integration. Their architecture, material composition, and surface functionalization can be tailored to regulate cellular responses and

7.6. Biosensing and Diagnosis

Biosensing and diagnostic technologies play a crucial role in modern healthcare by enabling early disease detection, continuous monitoring, and personalized medicine [348,349]. The confluence of polymer science, textile engineering, and microfabrication has ushered in a new era for biosensing and diagnostics, fundamentally redefining the interface between electronic devices and the human body [36]. This burgeoning field, largely anchored by the development of polymer microfibers, represents a paradigm shift from rigid, planar electronics to soft, textile-integrated systems capable of seamless, continuous physiological monitoring [15,350]. The sophisticated control over fiber geometry and material composition now permits the creation of functional architectures that are not only highly sensitive to biological signals but also mechanically compliant with human tissue, overcoming the pervasive issues of signal instability and wearer discomfort inherent in first-generation wearable sensors [351].

For a notable example, Yu et al. first demonstrated a significant advancement in functional material integration by introducing a dynamic liquid metal-microfiber interlocking strategy, enabling the realization of highly conductive and strain-insensitive metastructured fibers for wearable electronics (Figure 9A) [352]. Recognizing that embedding solid conductive materials severely compromises electrical pathways under strain, their approach utilized a mixture of Copper and Eutectic Gallium-Indium (Cu-EGaIn) partially embedded within a porous polymer microfiber mat. This composite structure was then rolled up into a spiral-layered, metastructured fiber. This dynamic solid-liquid interfacial interlocking mechanism enabled self-compensating conductive pathways, resulting in fibers that maintained an ultra-stable conductance, exhibiting only a 16% relative resistance change even at 100% strain, far exceeding theoretical predictions for similar composites. Crucially, the fiber boasted a high conductivity of $1.5 \times 10^6 \text{ S m}^{-1}$ and considerable stretchability up to 629%, making it an exceptionally stable platform for applications such as temperature-visualizing electrothermal fabrics and stretchable smart sensing displays. Similarly, to address the challenges of achieving both mechanical robustness and high sensitivity, Gao et al. engineered ultra-robust and extensible conducting microfibers for wearable smart healthcare (Figure 9B) [353]. Their fabrication process yielded fibers with a remarkable tensile strength of 17.6 MPa and extensibility up to 700%. This mechanical superiority translated directly into a high-performance mechanical sensor capable of detecting strains across an unprecedented range, from 0.0075% to 400%, while also demonstrating the capacity to monitor low-frequency biological vibrations in the 0 to 40 Hz range, effectively capturing tremors and micro-movements pertinent to neurological health monitoring. This work underscored that mechanical resilience is not merely a durability feature but a prerequisite for stable and wide-range biosensing performance.

Inspired by the need to match the mechanical behavior of biological tissues, researchers have increasingly explored biomimetic fiber designs to improve interface stability and functional performance. In this context, Hanif et al. developed a bio-inspired nanofiber-reinforced microfiber designed to replicate the mechanical behavior of natural biological tissue [354]. They constructed a core-shell system using a soft polyurethane (PU) microfiber, which mimics the elasticity of elastin, wrapped with PVDF nanofibers, which mimic the strain-limiting function of collagen. The resulting microfiber exhibited a J-shaped stress-strain curve, characterized by elasticity at low strains and a rapid increase in modulus at high strains, effectively providing a strain-limiting mechanism. This mechanical profile is critical for wearable electronics, offering both comfort at rest and structural protection during vigorous activity, thus ensuring continuous and accurate data acquisition without sensor damage or detachment. Similarly, Guo et al. introduced an innovative electronic skin based on an elastic MXene hydrogel microfiber for effective joint monitoring (Figure 9C) [207]. Using the microfluidic technology, they fabricated a double-network hydrogel microfiber composed of covalently cross-linked polyacrylamide and ionically cross-linked alginate, reinforced by highly conductive MXene nanosheets. The resulting microfiber possessed superior superelasticity and high electrical conductivity, allowing it to conform perfectly to dynamic, large-scale body movements, such as the bending of fingers and wrists. This MXene-hydrogel electronic skin demonstrated excellent stability and sensitivity for monitoring large-scale joint motions, providing a precise digital readout for human posture tracking and rehabilitation diagnostics.

Furthermore, achieving high sensitivity for detecting subtle physiological signals remains a major challenge in biosensing applications. In this direction, Jiang et al. developed a coaxial heterogeneous microfiber for ultrasensitive pressure monitoring of living organisms [355]. Their design featured a core of rGO-PPy aerogel encased in a flexible polymer sheath. The unique three-dimensional porous structure of the aerogel core maximized the piezoresistive effect by creating a vast network of inter-fiber contact points. This coaxial structure enabled the microfiber sensor to achieve exceptional sensitivity, making it capable of comprehensive surveillance and monitoring of living organisms through the detection of minute micropressure changes associated with organ activity, a capability previously unachievable by traditional e-skin systems. In addition to the sensitivity of the biosensor, wearability and comfort is also crucial factors for continuous monitoring. In this context, Mondal et al.

presented an all-organic rotary jet-spun textile piezoelectret dressing for biosignal-monitored wound healing (Figure 9D) [84]. This work elegantly combined sensing and therapeutic functions. The textile, fabricated through a scalable spinning method, exhibited outstanding piezoelectric properties, yielding a sensitivity of 400 mV kPa^{-1} . The enhanced piezo-potential was attributed to trapped polarized charges within the fiber matrix, confirmed by finite element modeling. Functionally, the textile seamlessly monitored vital physiological signals, including pulse and respiratory rate, while also acting as a pressure-mapping array with 98% accuracy. Crucially, the piezoelectric output, which generates micro-electrical stimulation, was demonstrated to accelerate the proliferation and migration of L929 cells, highlighting its potential for active, signal-guided wound healing and regeneration. Similarly, Kim et al. introduced a wireless breathable face mask sensor designed for spatiotemporal 2D respiration profiling and respiratory diagnosis (Figure 9E) [356]. Developed in response to the growing need for comfortable and quantitative pulmonary monitoring post-pandemic, their sensor array was integrated into a porous, breathable substrate. Unlike single-pixel sensors, this flexible pressure-sensing mask allowed for the 2D mapping of airflow distribution and pressure over the nasal and oral regions, providing rich spatiotemporal data for detailed respiratory pattern recognition and diagnosis. This level of detail is invaluable for diagnosing conditions like obstructive sleep apnea or tracking the progression of pulmonary diseases. Furthermore, the complexity in sensor adhesion to the skin's irregular surface was tackled by Drotlef et al., who proposed bioinspired composite microfibers to significantly improve skin adhesion and signal amplification for wearable sensors [7]. Their films consisted of elastomeric microfibers decorated with conformal, mushroom-shaped vinylsiloxane tips, mimicking the adhesive structures found in certain biological systems. Strong adhesion, up to 18 kPa, was achieved through the controlled crosslinking of the viscous tips directly onto the skin surface, allowing excellent shape adaptation to the skin's multiscale roughness. When integrated with a strain sensor, this microfibrillar adhesive system dramatically improved the signal-to-noise ratio to 59.7, demonstrating a considerable signal amplification effect that is critical for accurately monitoring subtle vital signs like heart rate and respiration under real-world conditions.

Moreover, the advanced control over fiber mechanics is not limited to passive sensing but extends to active components. For example, the work by Hou et al. on bioinspired liquid crystalline spinning to fabricate high-performing fibrous artificial muscles represents a foundational advance for future fiber-based diagnostic and therapeutic devices (Figure 9F) [357]. By drawing inspiration from the dragline silks of spiders, which utilize multiple drawdowns for alignment, they employed internal drawdown via a tapered-wall-induced-shearing mechanism and external mechanical stretching to shape liquid crystal elastomers (LCEs) into thin, aligned microfibers. This process enabled the continuous, high-speed production (up to 8400 m h^{-1}) of LCE microfibers with unprecedented actuation metrics, including a rapid deformation strain rate of $810\% \text{ s}^{-1}$ and powerful actuation stress of 5.3 MPa. Such high-performance active fibers could be integrated into smart micro-robotics or fiber-based soft actuators for *in vivo* manipulation or targeted drug delivery systems that are intrinsically linked to a fiber-optic diagnostic interface. This concept of intrinsic functionality was mirrored in the design of proprioceptive sensors by Lee et al., who developed an inherently integrated microfiber-based flexible proprioceptive sensor for feedback-controlled soft actuators (Figure 9G) [358]. In their system, two parallel conductive microfibers were embedded around an elastomeric chamber of a pneumatic soft actuator. These microfibers simultaneously served two purposes: they acted as the radial expansion limiting fibers, which are essential for the actuator's mechanical function, and also as a capacitive bending sensor to provide real-time proprioceptive feedback on the actuator's bending and expansion state. By leveraging the essential structural components for sensing, they eliminated the need for separate, bulky, or rigid sensing devices, significantly reducing structural complexity and manufacturing costs while enhancing interfacial stability, a strategy highly relevant to creating multi-functional, fiber-integrated diagnostic probes for endoscopy or surgical robotics.

In summary, microfibers offer a highly versatile platform for biosensing and diagnostics owing to their structural tunability, large surface area, and compatibility with functional nanomaterials and biomolecules. By enabling continuous monitoring of biochemical and physiological signals, microfiber-based sensors hold significant promise for wound monitoring, wearable health devices, implantable diagnostics, and theranostic systems. Future directions will focus on improving sensitivity and selectivity, integrating multiplexed detection, ensuring long-term stability in physiological environments, and coupling with wireless communication and AI-driven data analysis to create robust, real-world diagnostic tools.

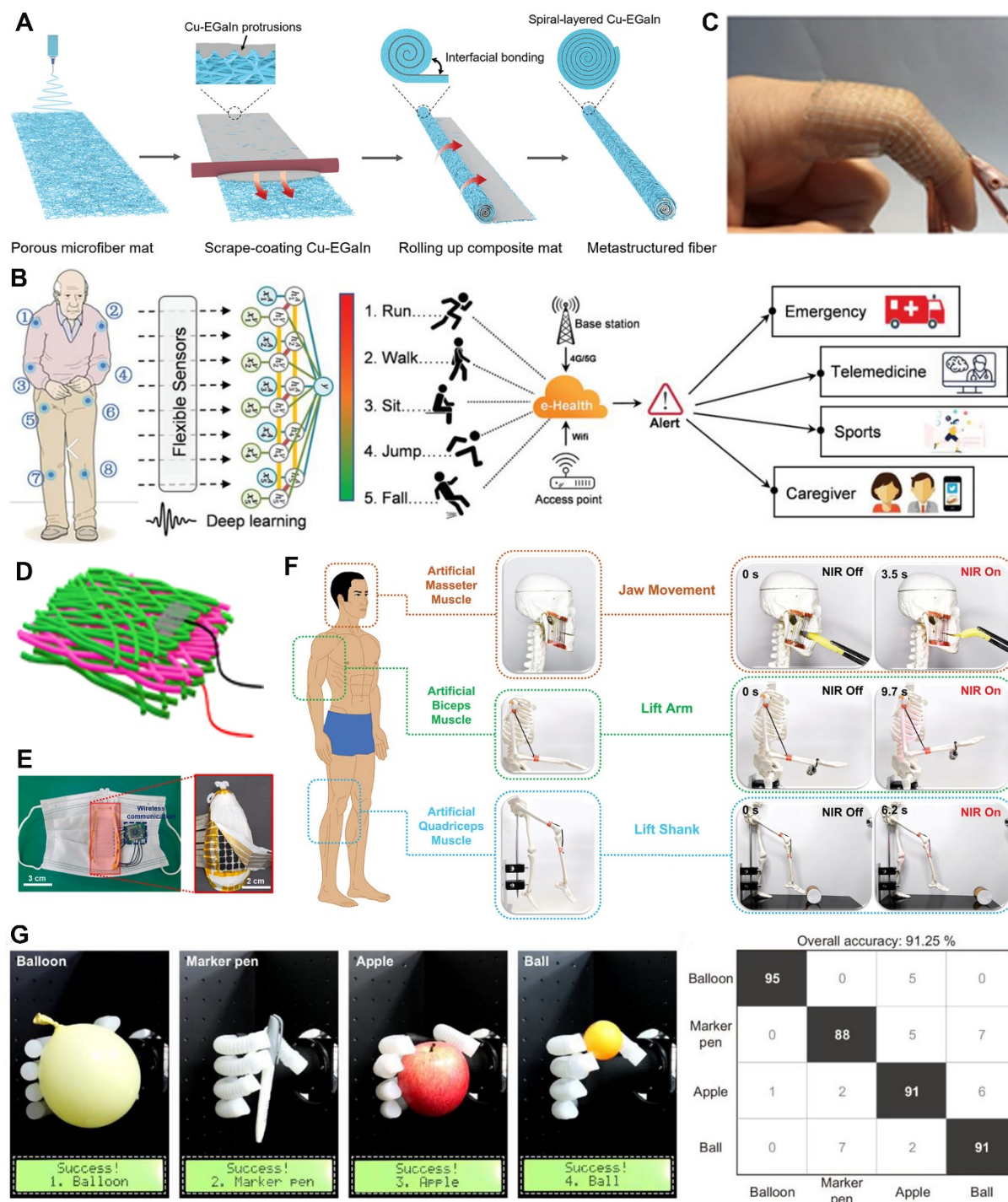


Figure 9. Applications in biosensing. (A) Schematic illustrating the preparation of the metastructured fibers for wearable electronics. Reproduced with permission [352]. Copyright 2025, Wiley-VCH. (B) Schematic showing the wireless wearable health monitoring and early warning system assisted by microfibers and artificial intelligence. Reproduced with permission [353]. Copyright 2021, Wiley-VCH. (C) Optical images of the mesh hydrogel during the finger bending. Reproduced with permission [207]. Copyright 2021, ACS Publications. (D) Schematic showing wearable microfiber piezoelectric sensor. Reproduced with permission [84]. Copyright 2025, Wiley-VCH. (E) Digital images of the microfiber-based array sensor equipped inside a commercial face mask. Reproduced with permission [356]. Copyright 2024, Elsevier. (F) Schematic illustrating the liquid crystal elastomer microfibers as masseter artificial muscle, biceps artificial muscle and quadriceps artificial muscle on a human skeleton. Reproduced with permission [357]. Copyright 2023, Wiley-VCH. (G) Photographs showing the object recognition and classification with the soft prosthetic hand, integrated with microfiber-based sensor. Reproduced with permission [358]. Copyright 2024, Springer Nature.

7.7. Cancer Treatment

The integration of polymer microfibers into advanced biomedical strategies has heralded a new epoch for cancer treatment, moving beyond conventional systemic limitations toward site-specific, stimuli-responsive, and highly localized therapies and diagnostic platforms [359,360]. The inherent structural versatility and biomimetic cues offered by fibrous scaffolds, particularly those fabricated via techniques like electrospinning, 3D printing and microfluidic spinning, provide an exceptional foundation for optimizing drug delivery, enhancing immunotherapy, and establishing high-fidelity *in vitro* tumor models [361]. A significant focus in recent years has been on applying these materials to address challenging clinical scenarios, such as preventing postoperative recurrence and developing personalized drug screening assays [362].

The postsurgical management of tumors remains a critical hurdle in oncological practice, as residual cancer cells often lead to recurrence. To combat this, Ye et al. explored the application of electrospun composite microfibers designed for dual-modal therapy, specifically for use in the postoperative niche (Figure 10A) [215]. Their work involved incorporating molybdenum disulfide (MoS₂) nanosheets, a photothermal agent, and the widely used anticancer drug doxorubicin (DOX) into a poly(lactic-co-glycolic acid) (PLGA) microfiber matrix. This durable photothermal agent exhibited excellent photothermal transformation and stability in both the near-infrared I (NIR I) and near-infrared II (NIR II) biowindows, which are crucial for deeper tissue penetration. Importantly, they demonstrated that the synergistic effect of photothermal therapy (PTT) and chemotherapy, delivered directly by the microfibers at the surgical site, was capable of completely prohibiting postoperative tumor reoccurrence *in vivo*. In addition to develop localized postsurgical care, Chen et al. further developed a strategy using microfluidic spinning to produce microfibers derived from natural origin materials for breast tumor postsurgical treatment (Figure 10B) [363]. The scaffold incorporated extracts from the traditional Chinese herb *Premna microphylla* alongside curcumin, a compound celebrated for its anti-inflammatory and anti-cancer properties. By utilizing the mild, biocompatible characteristics of natural polymers, this work provides a blueprint for effective, localized drug delivery platforms that seek to minimize systemic toxicity while maximizing therapeutic efficacy against residual tumor cells.

Beyond drug delivery systems, microfiber scaffolds are also being explored as platforms for cancer immunotherapy by acting as potent vaccination scaffolds. Huo et al. detailed the creation of Astragaloside IV (AS-IV) microfibers that spontaneously assemble into injectable 3D-scaffolds with intrinsic immunoactivity, serving as an injectable tumor vaccine platform [364]. This approach ingeniously minimizes the reliance on expensive synthetic adjuvants by utilizing a traditional Chinese herb component (AS-IV) as both a physical scaffold and a biological stimulant. The self-assembled microfiber network acts as a crucial reservoir for tumor antigens and simultaneously stimulates dendritic cells (DCs) within the tumor microenvironment (TME), leading to enhanced DC maturation and subsequent robust T-cell activation. This dual-role as an immunoadjuvant and physical cell-recruitment niche significantly boosts the overall efficacy of the tumor vaccine. Similarly, Sharma et al. developed an inorganic-organic hybrid nanocomposite using poly-L-lactide (PLLA) microfibers decorated with radially grown zinc oxide (ZnO) nanowires (Figure 10C) [365]. This 3D complex was then loaded with a tumor antigen to function as a therapeutic cancer vaccine. This hybrid microfiber system efficiently delivered the tumor antigen into DCs, a critical step for initiating a robust anti-tumor immune response, showcasing a promising new avenue for cancer immunotherapy based on scaffold-mediated antigen presentation.

Besides therapeutic delivery and vaccination strategies, microfiber systems are also valuable for constructing biomimetic *in vitro* tumor models that can improve drug screening accuracy. Since tumor stiffness strongly influences cancer progression and treatment resistance, Song et al. engineered mechanically tunable hydrogel microfibers for a biomimetic drug testing platform focused on neuroblastoma (Figure 10D) [236]. The core innovation lay in fabricating alginate/poly(N-isopropyl acrylamide) (PNIPAM) shell microfibers that exhibit a rapid temperature response. This thermal sensitivity allowed for the precise and quick manipulation of the hydrogel's mechanical stiffness, thereby mimicking the changing stiffness of the ECM surrounding neuroblastoma cells. The resultant platforms successfully regulated the cellular pressure environment and rapidly formed highly active 3D tumor spheroids, which, crucially, displayed varying sensitivities to different chemotherapeutic agents, thus validating the model's predictive power for personalized treatment selection.

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VCH. (E) CLSM images of A549 cells on different PCL microfibers based scaffolds after 1, 4, and 8 days of culture, Scale bar, 100 μm . Reproduced with permission [366]. Copyright 2021, ACS Publications. (F) Schematic illustrating a bionic esophageal cancer model featuring a hollow microfiber structure with esophageal squamous cell carcinoma (ESCC) cells lining the internal wall and HUVECs covering the surface, enabling the study of malignant progression following *Porphyromonas gingivalis* infection. Reproduced with permission [241]. Copyright 2025, Cell Press. (G) Schematic depicting the cell seeding and cell aggregation on the gelatin microfibers. Reproduced with permission [367]. Copyright 2021, IOP Publishing.

Beyond mechanical regulation, the topographical characteristics of the tumor microenvironment also play a crucial role in tumor behavior. Jing et al. engineered scaffolds with precise nanotopography on microfibers of 3D-printed PCL scaffolds to modulate cellular responses and establish a superior *in vitro* tumor model (Figure 10E) [366]. They achieved tunable nanoscale features by simply leaching gliadin from PCL/gliadin composites using an ethanol solution. This approach provides a level of architectural control not possible with simpler 3D scaffolds, offering an optimized platform for drug screening. To further capture the complexity of tumor progression, Shi et al. engineered hydrogel-based hollow microfibers to functionally remodel esophageal carcinoma (esophageal squamous cell carcinoma or ESCC) (Figure 10F) [241]. This platform was specifically designed to study the malignant progression of ESCC under *Porphyromonas gingivalis* (Pg) infection, a known risk factor, providing a promising and high-fidelity *ex vivo* model for pathological research and the advancement of new tumor therapies. In addition to biochemical and structural cues, physical forces within the TME are also critical regulators of tumor growth and metastasis. To quantify these biomechanical interactions, Lee et al. detailed a method using suspended hydrogel microfibers for the guided assembly of a cancer ellipsoid (Figure 10G) [367]. This innovative platform allows for the estimation of multi-cellular traction force, a key biophysical parameter that regulates tumor growth, invasion, and metastasis, thus providing crucial quantitative data for understanding tumor-matrix interactions.

In summary, microfiber-based platforms hold significant promise for cancer treatment by enabling localized drug delivery, gene therapy, photothermal/photodynamic therapy, immunomodulation, and theranostic applications. Their structural versatility and ability to integrate multiple therapeutic functions make them superior to conventional delivery systems. With continued innovation in stimuli-responsive designs, multifunctional composites, and bioinspired architectures, microfiber technologies are poised to contribute to more effective, personalized, and minimally invasive cancer therapies in the near future.

8. Microfiber-Based Medical Products and Translational Challenges

Microfiber-based materials have transitioned from laboratory-scale research to a wide range of clinically approved biomedical products [368]. Over the past few decades, advances in fiber spinning technologies have enabled the large-scale manufacturing of fibrous medical devices with controlled fiber diameter, mechanical properties, and structural architecture. These technologies have facilitated the commercialization of various microfiber-based medical products, including surgical meshes, wound dressings, sutures, and vascular grafts, summarized in Table 3 [369].

Table 3. Clinically used microfiber products.

Materials	Brand Name (Company)	Fabrication Technique	Fiber Diameter (μm)	Final Structure	Application	Ref.
PP	Prolene® (Ethicon)	Melt extrusion	150	Knitted monofilament	Hernia mesh	[370,371]
Polyester	Acticoat® Flex7 (Smith & Nephew)	Melt extrusion	9–11	knitted mesh	Wound dressing	[372]
P4HB	GalaFLEX (Becton Dickinson)	Melt extrusion	154	Knitted monofilament	plastic and reconstructive surgery	[373,374]
P4HB	Phasix™ mesh (Becton Dickinson)	Melt extrusion	150–180	Wrap-knitted monofilament	Hernia repair	[375,376]
PVDF/ PP	DynaMesh® (FEG Textiltechnik mbH)	Melt extrusion	120–145	Interwoven knitted monofilament	abdominal wall repair	[377]
PGA	Neoveil® Sheet (Gunze)	Melt extrusion	15–17	Nonwoven felt/mesh	Reinforcement of soft tissue	[378]
Ca-Na alginate	Kaltostat® (Convatec)	-	14–19	Nonwoven mat	Wound dressing	[379]
Na-CMC	Durafiber® (Smith & Nephew)	-	9–13	Nonwoven sheet	Wound dressing	[380]
PVA	Exufiber® (Mölnlycke Health Care)	-	16–24	Nonwoven sheet	Wound dressing	[379]

Table 3. *Cont.*

Materials	Brand Name (Company)	Fabrication Technique	Fiber Diameter (μm)	Final Structure	Application	Ref.
Na-CMC	Aquacel® Extra (Convatec)	-	10–13	Nonwoven pad	Wound dressing	[379,381]
Calcium alginate	Suprasorb® A (Lohmann & Rauscher)	-	9–14	Nonwoven sheet	Wound dressing	[380]
Na-CMC	Kliniderm® Super Fiber (Klinion)	-	13–18	Nonwoven sheet	Wound dressing	[380]
PET	Mersilene® (Ethicon)	Melt extrusion	15	Woven/knitted mesh	Wound dressing	[382]
PET	Uni-Graft® K DV (B Braun)	Melt extrusion	15–20	Knitted graft	Vascular graft	[383,384]
Nylon	Supramid® Extra II (S JACKSON INC)	Melt extrusion	23	Multifilaments enclosed in outer sheath	Non-absorbable suture	[382]
PDO	PDS Plus® (Ethicon)	Melt extrusion	270	Monofilament	Absorbable suture	[385]
Polyglactin 910	Vicryl® (Ethicon)	Melt extrusion	10–15	Braided multifilament	Absorbable suture	[386]
PP	Surgipro® (Medtronic)	Melt extrusion	185	Monofilament	Non-absorbable suture	[387]
Poliglecaprone 25	Monocryl® (Ethicon)	Melt extrusion	295	Monofilament	Absorbable suture	[388,389]
PGA	Dexon® (Medtronic)	Melt extrusion	-	Braided multifilament	Absorbable suture	[390]
Natural collagen	Chromic Gut® (Medtronic)	-	196	Monofilament	Absorbable suture	[388]

A large proportion of commercial microfiber medical devices are fabricated using material extrusion process followed by textile processing techniques, such as knitting, weaving, or braiding, to produce mechanically robust fibrous constructs [5,370]. Synthetic polymers such as PP, PET, PVDF, poly-4-hydroxybutyrate (P4HB), and polydioxanone (PDO) are commonly used because of their excellent mechanical strength, stability, and biocompatibility [368]. For example, polypropylene meshes such as Prolene® are widely used for hernia repair, while P4HB-based scaffolds such as GalaFLEX® and Phasix™ meshes are biodegradable alternatives designed for soft-tissue reinforcement and reconstruction [373,382]. Similarly, PVDF-based meshes (e.g., DynaMesh®) are employed for abdominal wall repair due to their long-term mechanical stability and chemical resistance [377]. In addition to implantable surgical meshes, microfiber architectures are extensively utilized in wound care products. Hydrophilic fiber-based wound dressings produced through wet spinning techniques provide excellent fluid absorption, moisture retention, and conformability to irregular wound surfaces. Materials such as calcium alginate, sodium carboxymethyl cellulose (Na-CMC), and polyvinyl alcohol (PVA) are frequently used in commercial wound dressings including Kaltostat®, Durafiber®, Aquacel® Extra, and Exufiber® [379,380]. These fibrous dressings form highly absorbent nonwoven matrices that promote wound healing by maintaining a moist environment and facilitating exudate management. Microfiber are also widely employed in surgical sutures, where mechanical reliability and controlled degradation are essential. Both absorbable and non-absorbable sutures are produced using melt-extruded polymer filaments that are subsequently braided or formed into monofilaments [387]. Examples include polyglactin-based Vicryl® sutures, polydioxanone-based PDS Plus® sutures, and polypropylene-based Surgipro® sutures [377,386]. These materials are designed to provide temporary mechanical support during tissue healing while minimizing tissue reaction and maintaining high tensile strength. Furthermore, microfiber-based materials are used in vascular grafts and soft-tissue reinforcement devices, where fibrous structures provide both mechanical integrity and controlled porosity for tissue integration. The fibrous design of vascular grafts, such as Uni-Graft® K DV facilitates tissue ingrowth while maintaining sufficient mechanical stability to support physiological loads [383].

Thus, though there is the significant progress in microfiber based products, translation from laboratory-scale prototypes to clinically approved products remains challenging. The clinical success of microfiber-based systems depends not only on biological performance but also on regulatory classification, manufacturing reproducibility, biocompatibility, and degradation. From a regulatory perspective, microfiber-based products may follow different approval pathways depending on their intended use, composition, risk level, and mechanism of action. Relatively simple products such as sutures, hernia meshes, and some vascular grafts are generally regulated as medical devices. In the United States, many moderate-risk devices may require a 510 (k) submission to demonstrate substantial equivalence to a legally marketed predicate device [17,374]. High-risk implantable systems that support or sustain life, present significant risk, or lack sufficient safety history may require premarket approval, which involves more extensive evidence of safety and effectiveness. Regulatory translation also requires robust quality

control [391]. In the United States, manufacturers of finished medical devices must comply with the Quality Management System Regulation, which governs design, manufacturing, packaging, labeling, storage, installation, and servicing of finished devices intended for human use. The regulatory pathway becomes more demanding when microfiber scaffolds incorporate therapeutic agents, biologics, or living cells. Therefore, multifunctional microfiber systems must establish not only device safety but also drug release behavior, biological activity, dose control, pharmacokinetics, toxicity, and the interaction between components.

Manufacturing scalability is another major barrier [149]. Techniques such as melt extrusion, melt blowing, rotary jet spinning, and conventional electrospinning are more compatible with large-scale production because they can generate continuous fibers or large-area nonwoven mats. However, they often provide limited control over pore geometry, fiber placement, and 3D architecture. In contrast, melt electrowriting, microfluidic spinning, and direct-write 3D printing offer precise spatial control, defined pore size, and complex architectures, but they typically suffer from lower throughput, higher equipment cost, and more difficult process standardization. For clinical translation, key manufacturing requirements include batch-to-batch reproducibility, control of fiber diameter distribution, mechanical uniformity, residual solvent removal, sterilization compatibility, and quality management during scale-up.

Long-term biocompatibility and degradation behavior are especially important for implantable microfiber systems [255,327]. FDA guidance on ISO 10993-1 emphasizes a risk-based biological evaluation of medical devices based on the nature and duration of body contact, and the FDA notes that biocompatibility assessment should consider the device in its final finished form, including sterilization when applicable [392]. Nondegradable materials such as polypropylene, PET, and PTFE provide mechanical stability but may cause chronic foreign body responses, thrombosis, or long-term mechanical mismatch [17,203,365]. Biodegradable polymers such as PLA, PLGA, PCL, PDO, and PLCL can gradually transfer mechanical load to regenerating tissues, but their degradation rate, acidic byproducts, crystallinity changes, and loss of mechanical integrity must be carefully matched to the healing timeline [198,393].

Overall, successful clinical translation of microfiber-based biomedical systems requires an integrated design strategy that considers regulatory classification, scalable manufacturing, quality control, sterilization, degradation kinetics, and long-term biological response from the beginning of material development. Future progress will depend on establishing standardized testing protocols, improving scalable precision manufacturing, reducing variability, and generating long-term preclinical and clinical evidence. Addressing these translational barriers will be essential for moving advanced microfiber platforms from experimental studies toward clinically reliable regenerative, therapeutic, and diagnostic products.

9. Conclusions

The development of microfibers has opened new frontiers in biomedical engineering, offering platforms that combine biomimetic architecture, tunable material properties, and multifunctional performance. As this review highlights, advances in fabrication techniques, from electrospinning and melt electrowriting to microfluidic spinning, rotary jet spinning, and emerging methods like micro-adhesion guided spinning, have greatly expanded the design space for tailoring microfiber structure, composition, and functionality. By careful selection of materials and processing parameters, researchers can engineer fibers with diverse morphologies such as solid, hollow, porous, core-shell, and Janus structures, each serving distinct biomedical purposes.

Beyond structural engineering, microfiber functionalization strategies, including surface coating, plasma treatment, nanoparticle incorporation, and bioactive ligand conjugation, have transformed simple fibers into smart systems capable of dynamic interactions with their biological environment. Such modifications allow the integration of sensing, actuation, drug delivery, and regenerative functions within a single platform. This versatility has positioned microfibers as powerful tools across a broad spectrum of biomedical applications, ranging from wound healing and tissue regeneration to biosensing, diagnostics, and targeted cancer therapy. The unique combination of biocompatibility, high surface-to-volume ratio, and customizable properties enables microfibers not only to mimic natural ECM but also to surpass them by delivering controlled biochemical, mechanical, and electrical cues. Their adaptability further supports the convergence of regenerative medicine with precision diagnostics and theranostics, paving the way for next-generation personalized healthcare solutions.

Despite these advances, several challenges remain before microfiber technologies achieve widespread clinical translation. Key hurdles include scaling up fabrication processes while ensuring reproducibility, integrating multifunctionality without compromising mechanical integrity, achieving long-term biocompatibility and stability *in vivo*, and addressing sterilization and regulatory concerns. Furthermore, real-time monitoring and adaptive feedback within microfiber systems remain underexplored but are critical for safe and effective clinical use. Looking ahead,

the integration of microfiber fabrication with emerging technologies such as additive manufacturing, artificial intelligence-driven design optimization, and bioelectronic interfaces holds immense potential. Stimuli-responsive and multi-material microfiber systems capable of synergistically combining regenerative, diagnostic, and therapeutic functions are expected to play a transformative role in future biomedical devices.

In brief, microfibers represent a versatile and rapidly evolving class of biomaterials with profound implications for medicine. Continued interdisciplinary efforts bridging materials science, biology, engineering, and clinical research will be essential to unlock their full potential and translate laboratory innovations into clinically relevant therapies that improve patient outcomes.

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High-Entropy Intermetallic Nanocrystals: Formation Principles, Synthetic Strategies, and Electrocatalytic Applications

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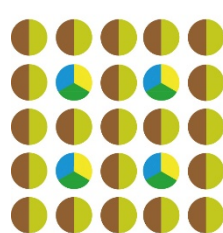
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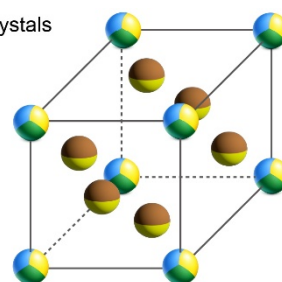
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Abstract: High-entropy intermetallic (HEI) nanocrystals have recently emerged as a promising class of multicomponent catalysts that combine the compositional complexity of high-entropy alloys with the structural precision of ordered intermetallics. This unique combination offers new opportunities to regulate atomic arrangement, surface structure, and catalytic behavior beyond what is achievable in conventional alloys or disordered high-entropy systems. In this Review, we discuss HEI nanocrystals

High entropy intermetallic nanocrystals



Ordered intermetallic lattice



from the perspectives of formation principles, synthetic strategies, and catalytic applications. We first outline the thermodynamic and kinetic factors that govern ordering in multicomponent nanoscale systems, and then summarize the emerging synthetic approaches for controlling phase selection, atomic ordering, and surface structure. We next examine representative electrocatalytic applications and discuss how ordering, multielement interactions, and structural evolution collectively influence activity and stability. Finally, we highlight four critical directions for future research, including growth mechanisms, facet control, atomic-scale structural identification and interatomic interactions, and electrocatalytic mechanisms. We anticipate that deeper insights into ordering, surface structure, and catalytic mechanisms will further expand the potential of HEI nanocrystals for a wide range of catalytic applications.

Keywords: high-entropy intermetallic nanocrystals; high-entropy alloy nanocrystals; atomic ordering; synthetic strategies; electrocatalysis

1. Introduction

High-entropy alloys (HEAs) have emerged as a distinctive class of multicomponent materials that extend alloy design beyond the conventional host-dominated framework [1,2]. In traditional alloys, one principal metal is typically modified by minor alloying additions to tailor structure and functionality. By contrast, HEAs are generally composed of multiple principal elements in near-equiatomic or comparable proportions, thereby creating a much broader compositional space and a more complex thermodynamic landscape [3–7]. This concept has opened access to metallic systems with unusual phase behavior, local atomic environments, and coupled physicochemical properties that are difficult to achieve in simpler alloys. Since their introduction, HEAs have attracted broad attention across materials science, initially for structural applications and more recently for energy-related and catalytic systems. The distinctive behavior of HEAs is often interpreted in terms of four core effects, namely the high-entropy effect, lattice distortion effect, sluggish diffusion effect, and cocktail effect [1,3,8]. The high-entropy effect refers to the configurational entropy contribution associated with multicomponent mixing, which can reduce the free energy of compositionally complex phases and, under appropriate conditions, favor



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solid-solution formation. The lattice distortion effect arises from differences in atomic size, bonding characteristics, and electronic structure among constituent elements, leading to local strain and coordination heterogeneity. The sluggish diffusion effect describes the kinetically hindered atomic transport often observed in multielement systems, which can affect phase transformation and segregation behavior. The cocktail effect highlights that the overall functionality of a multicomponent alloy may differ substantially from a simple average of its individual constituents because emergent properties can arise from their collective interactions. Together, these effects provide a useful conceptual basis for understanding why HEAs often exhibit unusual combinations of structural stability, surface reactivity, and functional tunability.

With continued advances in nanoscale synthesis, the HEA concept has been successfully extended from bulk materials to HEA nanocrystals. This size reduction is not merely geometric. At the nanoscale, the high surface-to-volume ratio, the abundance of undercoordinated atoms, and the increased importance of surface and interface energetics make compositional and structural complexity even more consequential [9,10]. As a result, HEA nanocrystals have become highly attractive for catalysis, where surface atoms and their local environments largely determine reactivity. In these systems, multiple principal elements can generate a broad spectrum of adsorption sites with distinct coordination motifs and electronic structures, offering opportunities to simultaneously regulate activity, selectivity, and durability. Recent studies have therefore rapidly expanded the use of HEA nanocrystals in electrocatalysis, thermocatalysis, and photocatalysis [5,6,11–24]. Their promise lies not only in the ability to tune adsorption energetics and reaction pathways, but also in the possibility of discovering catalytic behaviors that cannot be readily accessed in monometallic or low-component alloy nanocrystals.

Despite this progress, most studies on HEA nanocrystals have focused on disordered solid-solution structures, in which constituent atoms are randomly distributed throughout the lattice. Although such disorder provides diverse local coordination and adsorption environments, it also limits precise control over atomic arrangement and active-site definition. In this context, high-entropy intermetallic (HEI) nanocrystals have recently emerged as a particularly intriguing direction because they introduce long-range atomic ordering into compositionally complex multielement systems [25–33]. Unlike disordered HEA nanocrystals, HEI nanocrystals combine multicomponent complexity with ordered crystallographic sublattices, enabling more defined site occupation, heteroatomic connectivity, and surface ensembles. This structural ordering is catalytically significant because it can provide more predictable local motifs and clearer correlations between atomic structure and catalytic function. Accordingly, HEI nanocrystals should not be regarded simply as ordered derivatives of HEA nanocrystals, but rather as a distinct class of nanoscale catalysts in which compositional complexity and crystallographic ordering coexist.

More importantly, atomic ordering can provide catalytic advantages that are difficult to achieve in disordered HEA nanocrystals. In disordered HEAs, the random distribution of elements generates a broad spectrum of local coordination environments, which is beneficial for creating diverse adsorption sites but may also obscure the relationship between atomic structure and catalytic function. By contrast, the ordered sublattices in HEI nanocrystals can produce more reproducible surface ensembles and better-defined active sites, enabling more systematic regulation of adsorption energetics and reaction pathways. Such ordering may also enhance catalytic selectivity by constraining the geometry of key intermediates and suppressing undesired adsorption configurations. In addition, stronger heteroatomic bonding and sublattice-specific coordination in ordered intermetallic frameworks can improve structural stability and mitigate elemental segregation or surface reconstruction under reaction conditions. Therefore, HEI nanocrystals offer a unique platform in which high-entropy compositional complexity and crystallographic ordering work together to optimize catalytic activity, selectivity, and durability.

In addition, the difference between HEI nanocrystals and conventional ordered intermetallic nanocrystals is equally significant. Traditional intermetallic compounds typically involve two or three elements arranged in ordered lattices with relatively fixed stoichiometry or narrow compositional windows. Their catalytic properties often arise from defined atomic ensembles, directional heteroatomic bonding, and characteristic electronic interactions. HEI nanocrystals retain the advantages of ordering while greatly expanding compositional complexity. By incorporating multiple principal elements into ordered frameworks, they offer a richer chemical landscape for tuning local coordination, charge distribution, and adsorption behavior. At the same time, they introduce a much more difficult ordering problem because several elements with distinct bonding preferences and site occupancies must be accommodated simultaneously. HEI nanocrystals thus represent a new frontier in which the structural precision of intermetallics intersects with the compositional versatility of high-entropy design.

Recent breakthroughs have demonstrated that ordered multicomponent nanocrystals can indeed be realized, highlighting the promise of HEI nanocrystals in catalysis [25–27,29–33]. Their ordered lattices can regulate local coordination geometry, orbital hybridization, and heteroatomic bonding in ways distinct from both conventional intermetallics and disordered HEA nanocrystals [30–32]. Nevertheless, the field remains at an early stage, and several key challenges remain unresolved. One major challenge is the formation of ordered HEI phases, because

configurational entropy tends to stabilize the disordered state, whereas ordering requires sufficient enthalpic driving force and accessible kinetic pathways for atomic rearrangement. At the nanoscale, this balance becomes even more complicated because particle size, surface energy, and diffusion limitations strongly influence phase evolution. Another challenge lies in synthetic control. Because several elements with different reduction behaviors, bonding preferences, and mobilities must be coordinated simultaneously, it remains difficult to reproducibly synthesize HEI nanocrystals with targeted compositions, uniform particle sizes, controlled morphologies, and well-defined ordering. In addition, the surface structure and catalytic roles of individual elements remain poorly understood. Even when bulk ordering is achieved, the active surface may deviate from the ideal lattice because of facet dependence, defects, local relaxation, or segregation. Under catalytic conditions, HEI nanocrystals may also undergo reconstruction or partial disordering, meaning that the catalytically relevant structure can differ substantially from the as-synthesized one.

In view of these opportunities and challenges, this Review focuses on HEI nanocrystals from the perspectives of formation principles, synthetic strategies, and catalytic applications. We first discuss the thermodynamic and kinetic foundations that govern ordering in multicomponent nanoscale systems and highlight how these principles differ from those of conventional alloys and simpler intermetallic nanocrystals. We then summarize the emerging synthetic approaches developed to access HEI nanocrystals, with emphasis on the key factors that control phase selection, atomic ordering, and surface structure. Next, we examine representative electrocatalytic applications and discuss how ordering, multielement interactions, and structural evolution collectively influence activity and stability. Finally, we highlight four critical directions for future research: growth mechanisms, facet control, atomic-scale structural identification and interatomic interactions, and electrocatalytic mechanisms. By integrating these aspects, this Review aims to provide a framework for understanding HEI nanocrystals and guiding the rational design of next-generation multicomponent catalysts.

2. Intermetallic Nanocrystals: Formation Principles, Synthetic Strategies, and Structural Advantages

Intermetallic nanocrystals are a structurally ordered class of materials that differ fundamentally from random alloy nanocrystals. Unlike disordered solid solutions, intermetallic phases exhibit long-range chemical ordering, in which constituent elements occupy specific crystallographic sublattices. Such atomic ordering modifies orbital hybridization and charge distribution, thereby tuning d-band characteristics, adsorption energetics, catalytic activity, selectivity, and stability [34–37]. The formation of intermetallic nanocrystals is governed by the interplay between thermodynamics and kinetics [38–40]. While negative mixing enthalpy associated with favorable heteroatomic bonding promotes ordering, configurational entropy and nanoscale surface energy tend to stabilize the disordered state. Achieving ordered structures therefore requires sufficient enthalpic driving force together with atomic diffusion within an appropriate temperature-time window. This chapter first introduces the thermodynamic and kinetic principles underlying intermetallic formation. It then discusses synthetic strategies including thermal annealing, rapid thermal shock, post-synthetic treatments, and epitaxial growth. Finally, the effects of atomic ordering on electronic structure and catalytic behavior are summarized.

2.1. Thermodynamic and Kinetic Framework for Intermetallic Nanocrystal Formation

The formation of ordered intermetallic nanocrystals from disordered alloys can be described within a coupled thermodynamic and kinetic framework, as illustrated in Figure 1a [38]. In thermodynamic terms, the free-energy landscape of a binary alloy features a metastable disordered state and a stable ordered intermetallic state separated by an energy barrier. The disorder-to-order transition is driven by the negative mixing enthalpy associated with favorable heteroatomic bonding, as specific atomic pairs in the ordered lattice maximize bond strength and coordination preference, thereby lowering the internal energy relative to the disordered state. The thermodynamic balance governing this transformation can be expressed as

$$\Delta G_{\text{mix}} = \Delta H_{\text{mix}} - T\Delta S_{\text{mix}} \quad (1)$$

where ΔH_{mix} represents the enthalpic stabilization of ordered bonding and ΔS_{mix} denotes the configurational entropy contribution. During ordering, $\Delta H_{\text{mix}} < 0$ due to strengthened heteroatomic interactions, while $\Delta S_{\text{mix}} < 0$ because atomic positions become constrained to specific sublattices. Thus, ordering becomes thermodynamically favorable when the enthalpic gain outweighs the entropy penalty. However, at elevated temperatures, the $T\Delta S$ term increases, reducing the net driving force and stabilizing the disordered state. This competition defines a temperature-dependent thermodynamic driving force, here denoted as $F(T)$, which decreases with increasing temperature as entropy stabilization becomes dominant.

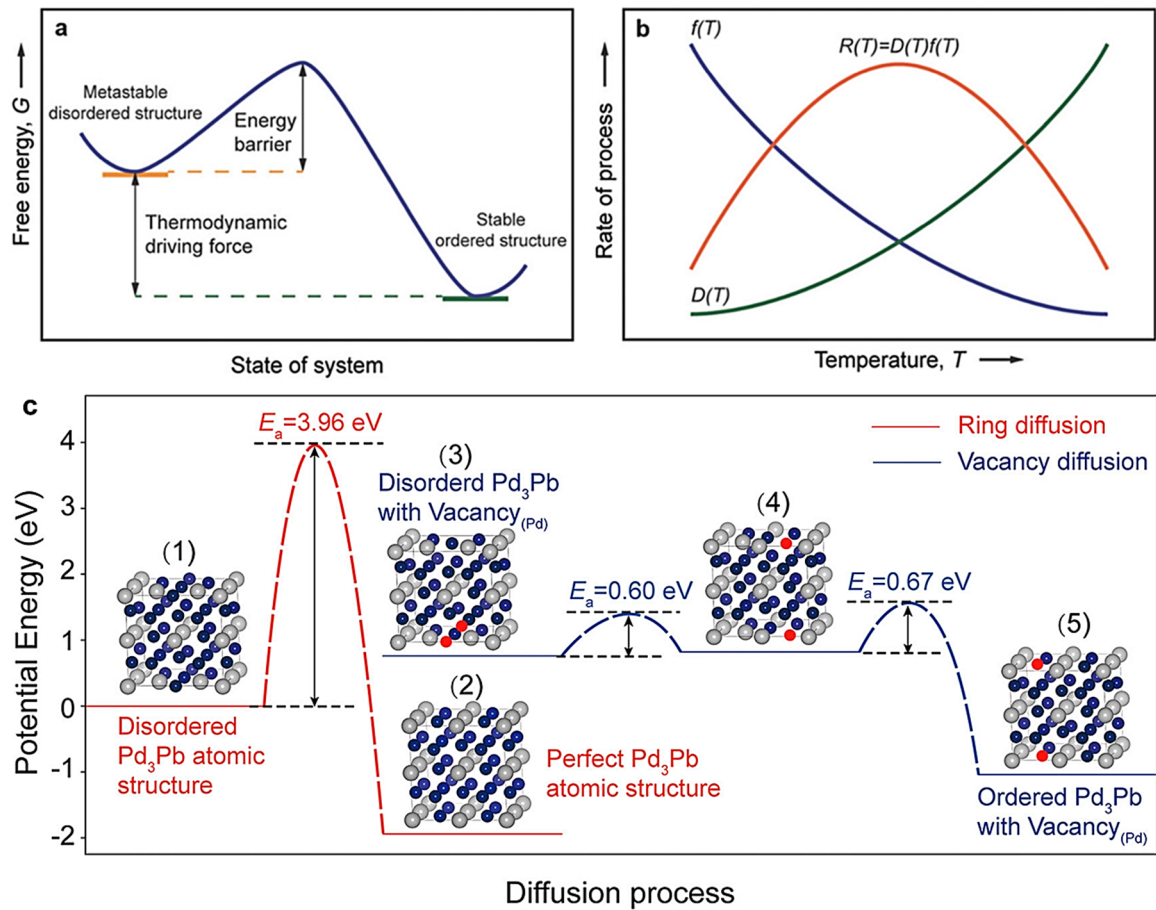


Figure 1. Thermodynamic and kinetic principles governing ordering in intermetallic systems. (a) Free-energy landscape illustrating the transformation from a metastable disordered structure to a stable ordered phase. (b) Temperature dependence of the ordering rate determined by the interplay between atomic diffusion and thermodynamic driving force. Reproduced with permission [38]. Copyright 2017, John Wiley and Sons. (c) Representative diffusion pathways during ordering in Pd₃Pb, comparing ring diffusion and vacancy-mediated diffusion. Reproduced with permission [40]. Copyright 2022, American Chemical Society.

At the nanoscale, surface energy contributions must also be incorporated into the free-energy description:

$$\Delta G = \Delta H_{\text{mix}} - T\Delta S_{\text{mix}} + \Delta\gamma A \quad (2)$$

where $\Delta\gamma$ is the difference in surface energy between ordered and disordered structures, and A is the particle surface area. Because surface atoms constitute a significant fraction of total atoms in nanocrystals, the $\Delta\gamma A$ term can substantially shift the effective disorder-order transition temperature. This size dependence can be estimated by

$$\frac{T_{\text{nano}}}{T_{\text{bulk}}} = 1 + \frac{A/V}{\Delta H_V} \Delta\gamma \quad (3)$$

where ΔH_V is the volume-specific enthalpy change and surface-to-volume ratio (A/V) scales inversely with particle size. As a result, surface energy effects become increasingly significant at the nanoscale, potentially destabilizing the ordered phase to the disordered state. Consequently, smaller nanocrystals may require more stringent thermal conditions to achieve ordering, even when the bulk phase is thermodynamically stable.

While thermodynamics defines the stability of the ordered state, the actual transformation pathway is governed by kinetics. The rate of the disorder-to-order transition can be expressed as

$$R(T) = D(T) \cdot F(T) \quad (4)$$

where $D(T)$ is the temperature-dependent diffusion coefficient and $F(T)$ represents the thermodynamic driving force defined above. As illustrated in Figure 1b [38], $D(T)$ increases exponentially with temperature due to enhanced atomic mobility, typically following an Arrhenius relationship:

$$D = D_0 \exp\left(-\frac{E_a}{RT}\right) \quad (5)$$

where E_a is the activation energy for diffusion. In contrast, $F(T)$ decreases with increasing temperature as entropy stabilization becomes significant. The product of these two competing factors results in a characteristic temperature window where $R(T)$ reaches a maximum, defining the optimal regime for intermetallic ordering. At low temperatures, the driving force for ordering may be strong, but atomic diffusion is kinetically limited. At excessively high temperatures, diffusion is rapid, but entropy suppresses the overall tendency toward ordering.

The atomistic diffusion mechanism governing the disorder-to-order transition is illustrated in Figure 1c using Pd₃Pb as a representative system [40]. Ordering requires long-range atomic rearrangement, which is primarily governed by atomic diffusion pathways. In ordered alloys, diffusion typically proceeds through two mechanisms: cooperative ring diffusion and vacancy-mediated diffusion. Ring diffusion involves the concerted displacement of multiple atoms and is therefore associated with a high activation barrier of about 3.96 eV. By contrast, vacancy-mediated diffusion has a much lower activation barrier of about 0.60–0.67 eV, enabling stepwise atomic exchange and the gradual development of long-range order. Vacancy-mediated diffusion therefore serves as the dominant kinetic pathway for disorder-to-order transformation in intermetallic nanocrystals. Nevertheless, sufficient thermal energy is still required to overcome migration barriers and activate atomic rearrangement. Taken together, the free-energy landscape of the disorder-to-order transition (Figure 1a), the temperature-dependent interplay between thermodynamic driving force and atomic diffusion (Figure 1b), and the atomistic diffusion pathways that enable atomic rearrangement (Figure 1c) collectively define the kinetic window for intermetallic nanocrystal formation. This coupled thermodynamic and kinetic framework explains why activation strategies such as high-temperature annealing and rapid thermal shock are often required to overcome diffusion barriers and promote long-range ordering. Representative synthetic approaches are discussed in the following sections.

2.2. Thermally Activated Routes to Intermetallic Nanocrystals

Building on the thermodynamic and kinetic framework discussed in Section 2.1, the synthesis of ordered intermetallic nanocrystals requires strategies that can overcome diffusion barriers and promote atomic rearrangement within nanoscale structures. In practice, such ordering can be achieved through several thermally activated routes, including rapid high-temperature activation, conventional annealing, diffusion-mediated phase evolution, and atomic reorganization revealed under in situ conditions. Because each approach regulates atomic mobility and thermodynamic driving force differently, they strongly influence particle size, ordering kinetics, and structural evolution. Representative examples are summarized in Figure 2, including rapid Joule heating and conventional annealing as thermally driven routes for intermetallic formation (Figure 2a), annealing-induced ordering pathways (Figure 2b), diffusion-controlled phase evolution (Figure 2c), and atomic-scale ordering mechanisms revealed by in situ electron microscopy (Figure 2d) [40–43].

One strategy for promoting rapid atomic ordering is ultrafast high-temperature activation. Rapid Joule heating has been demonstrated to synthesize intermetallic nanocrystals within extremely short time scales. By applying heating pulses to temperatures up to ~1600 K with ramp rates on the order of 10^5 K s⁻¹, disordered Pd-Pb precursors transform into ordered L1₂ Pd₃Pb nanocrystals within ~60 s (Figure 2a) [40]. Ultrafast heating generates a high concentration of vacancies in the transient alloy structure, markedly lowering diffusion barriers for atomic rearrangement. Density functional theory (DFT) calculations show that vacancy-mediated diffusion has a much lower activation energy than collective ring diffusion, thereby accelerating ordering. At the same time, the short thermal exposure suppresses particle coarsening and yields uniformly distributed nanocrystals of about 6 nm. In contrast, conventional furnace annealing at 1273 K can also produce ordered Pd₃Pb intermetallics, but prolonged heating typically results in much larger particles (~85 nm) due to enhanced coarsening. Despite this limitation, conventional annealing remains a widely used route to thermodynamically stable intermetallic phases because it provides sufficient atomic mobility for long-range ordering.

Under thermally activated conditions, conventional high-temperature annealing enables detailed observation of ordering pathways in alloy nanocrystals. In situ electron microscopy studies show that Pt-Fe alloy nanocrystals annealed at ~700 °C undergo a sequential ordering process (Figure 2b) [41]. The transformation begins with the rapid formation of an L1₀ PtFe surface layer, followed by conversion into an L1₂ Pt₃Fe phase that propagates inward. This process is governed by counter-diffusion of Pt and Fe atoms, where outward Pt diffusion and inward Fe diffusion drive compositional redistribution. Subsequent nucleation of the thermodynamically stable L1₀ PtFe phase in the particle core is followed by outward growth through the consumption of the surrounding Pt₃Fe region. The diffusion-mediated restructuring ultimately produces a PtFe@Pt core-shell configuration consisting of an L1₀ PtFe core and a thin Pt-rich shell. The respective high-resolution transmission electron microscope (HRTEM) image, representing the final diffusion-mediated state (iv) illustrated in Figure 2b, confirms the formation of the PtFe@Pt core-shell structure (Figure 2c).

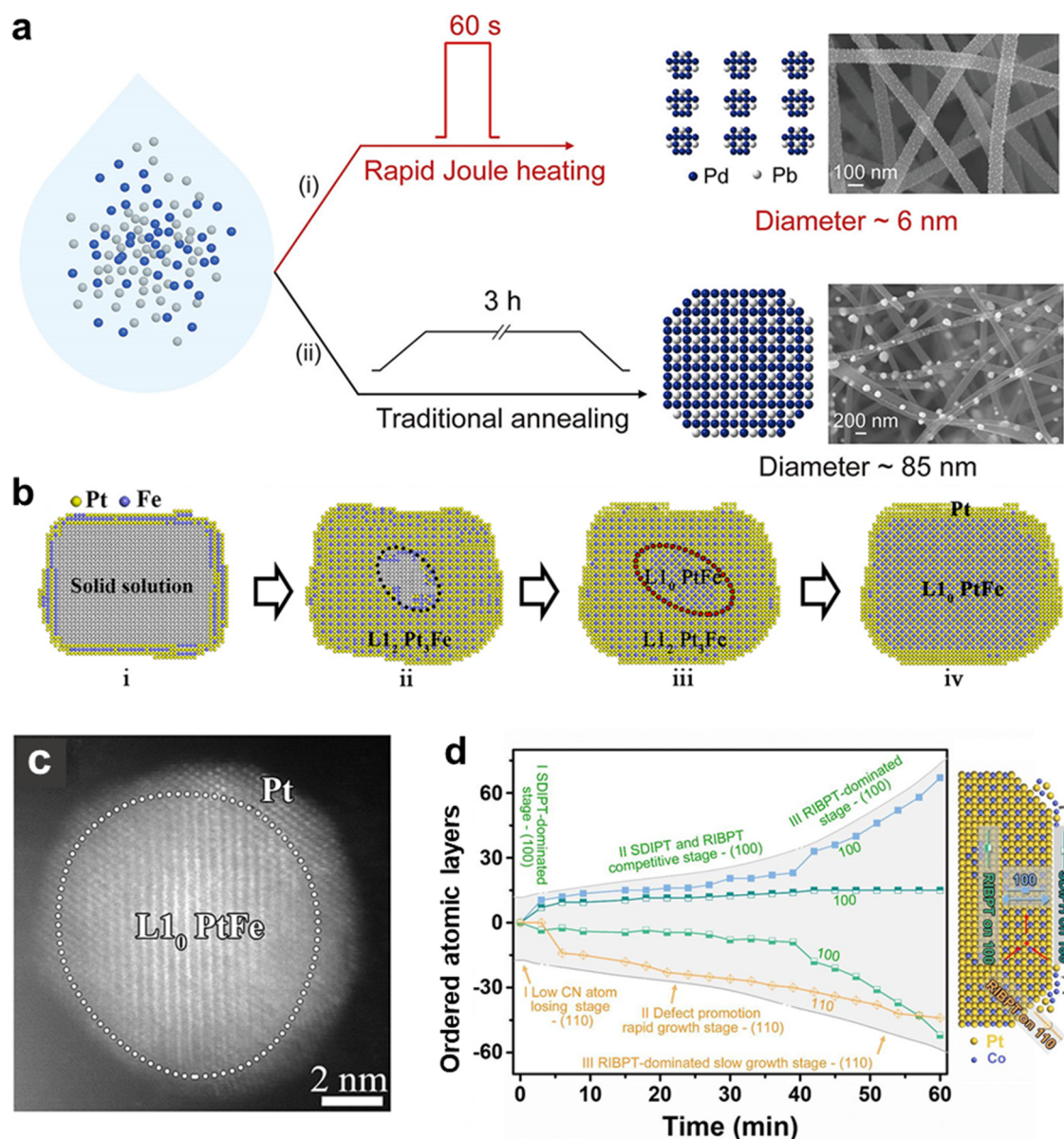


Figure 2. Representative thermally activated formation pathways of intermetallic nanocrystals. (a) Ultrafast ordering of Pd₃Pb nanocrystals via rapid Joule heating. Reproduced with permission [40]. Copyright 2022, American Chemical Society. (b,c) Thermally induced ordering pathway in Pt-Fe nanocrystals during annealing and HRTEM image of the well annealed PtFe@Pt core shell structure. Reproduced with permission [41]. Copyright 2022, PNAS. (d) In situ visualization of atomic ordering dynamics in Pt₃Co nanocrystals involving SDIPT and RIBPT mechanisms. Reproduced with permission [43]. Copyright 2021, American Chemical Society.

While high-temperature annealing enables thermodynamically driven ordering pathways (Figure 2b), intermetallic formation can also proceed through diffusion-controlled phase evolution when the migration of the second element is kinetically regulated [42]. In Pt-Sn systems, hydrogen reduction of Pt/SnO₂@mSiO₂ at moderate temperatures (~300 °C) enables controlled diffusion of Sn into Pt nanocrystals, leading to sequential phase transformations from Pt to Pt₃Sn and ultimately to PtSn. When the reduction time is limited to ~5 min, Sn diffusion remains incomplete, resulting in Pt₃Sn-enriched surfaces. Extending the reduction time to ~30 min produces Pt₃Sn/PtSn structures with coherent interfaces, whereas prolonged reduction (~4 h) leads to complete transformation into the PtSn intermetallic phase. These results highlight elemental diffusion control as an effective strategy for regulating phase evolution and structural development in intermetallic nanocrystals.

Beyond diffusion-controlled phase evolution, advanced in situ microscopy techniques have enabled direct visualization of atomic-scale ordering processes in intermetallic nanocrystals. In situ HRTEM studies of Pt₃Co nanocubes reveal that the transformation from a disordered face-centered cubic (FCC) alloy to an ordered L1₂ structure proceeds through two distinct mechanisms (Figure 2d) [43]. One pathway involves surface diffusion of

atoms from high-energy corner sites toward terrace regions, referred to as surface diffusion-induced phase transition (SDIPT). The other corresponds to reconstruction-induced body phase transition (RIBPT), where atomic rearrangement occurs within the nanocrystal interior through short-range reconstruction. The interaction between these processes governs the overall ordering dynamics and structural evolution of the nanocrystal, with surface diffusion dominating early stages and bulk reconstruction completing the ordering transformation.

Taken together, these representative studies show that the formation of ordered intermetallic nanocrystals is governed by the interplay between thermodynamic driving forces and kinetic pathways. Rapid thermal activation, conventional annealing, diffusion-mediated transformation, and atomic-scale reconstruction all demonstrate how atomic mobility, diffusion behavior, and defect formation regulate ordering dynamics and structural evolution in alloy nanocrystals. These findings are consistent with the framework described in Section 2.1, in which the accessible ordering window is determined by the balance between diffusion kinetics and thermodynamic driving force. Although the examples above mainly involve thermally activated processes, wet-chemical routes have also been developed to construct ordered intermetallic nanocrystals under solution-phase conditions. Representative solution-phase strategies are discussed in the following section.

2.3. Solution-Phase and Non-equilibrium Synthetic Strategies

In contrast to thermally driven approaches that rely on high-temperature diffusion to induce atomic ordering, solution-phase synthesis offers an alternative route for constructing ordered intermetallic nanostructures under milder conditions. Wet-chemical methods provide precise control over nucleation, precursor supply, and surface reactions, enabling the regulation of nanocrystal morphology, composition, and atomic distribution at the nanoscale [44–46]. These features are particularly advantageous for preserving well-defined nanostructures while introducing heteroatoms in a controlled manner.

A representative example is the seed-mediated synthesis of Cu@CuAu core-shell nanocubes, in which controlled precursor injection and interfacial diffusion enable the formation of single-atom-alloy (SAA) surfaces and ordered intermetallic shells [45]. In this system, Au precursors are introduced onto preformed Cu nanocubes, where galvanic replacement and atom interdiffusion driven by the Kirkendall effect leads to the initial dispersion of Au as isolated surface atoms, forming a dilute Cu@CuAu SAA structure. Increasing the Au precursor concentration induces further atomic rearrangement and ordering, resulting in the formation of a tetragonal CuAu intermetallic shell epitaxially grown on the Cu core. Atomic-resolution HAADF-STEM characterization reveals well-defined lattice features of the Cu core together with superlattice ordering in the CuAu shell, confirming the formation of the ordered intermetallic phase. Overall, solution-phase synthesis provides an effective route for regulating atomic distribution and interfacial ordering in nanoscale alloys under relatively mild conditions.

2.4. Structural Features and Catalytic Advantages of Intermetallic Nanocrystals

Beyond the synthetic approaches described above, the significance of intermetallic nanocrystals lies in their structurally defined atomic arrangements and the resulting catalytic advantages. Unlike random alloys, they exhibit long-range chemical ordering, in which constituent elements occupy specific crystallographic sublattices. This ordering creates well-defined coordination environments and surface ensembles, enabling systematic regulation of electronic structure, adsorption energetics, reaction pathways, and catalytic stability [47–49].

In this context, the catalytic behavior of multimetallic systems is commonly interpreted in terms of four fundamental effects: ligand, strain, geometric, and bifunctional effects (Figure 3a–d) [47]. When a second metal is introduced into a host metal lattice, the coordination environment of surface atoms is altered, leading to orbital hybridization and partial charge transfer between neighboring atoms. This electronic perturbation shifts the d-band center relative to the Fermi level and changes the adsorption strength of reaction intermediates, which is referred to as the ligand effect (Figure 3a). Lattice mismatch between different atomic layers can also generate tensile or compressive strain, thereby modifying interatomic distances and d-band dispersion and ultimately tuning adsorption energetics and catalytic activity (Figure 3b). In addition to these electronic effects, ordered intermetallic structures can influence catalytic behavior through geometric effects, in which constituent elements occupy specific lattice positions and form well-defined surface ensembles. Such arrangements can isolate active atoms or constrain adsorption configurations, thereby altering reaction pathways (Figure 3c). Furthermore, cooperative interactions between different metals may give rise to bifunctional mechanisms, in which one component preferentially adsorbs oxygen-containing species, such as OH*, that subsequently react with intermediates bound on neighboring sites. This cooperation lowers the energy barriers of key reaction steps and enhances catalytic performance (Figure 3d).

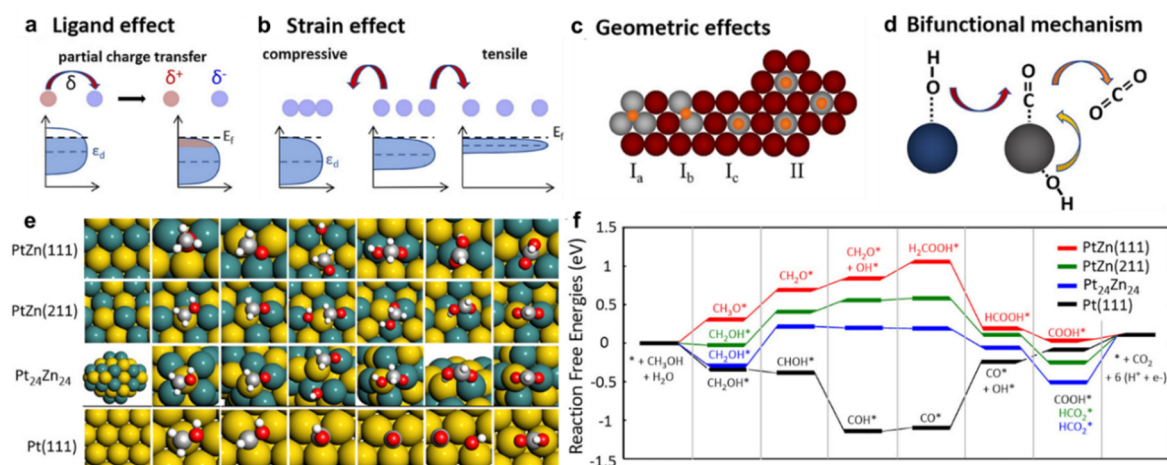


Figure 3. Structural and electronic factors governing catalysis in intermetallic nanocrystals. (a–d) Schematic illustrations of ligand, strain, geometric, and bifunctional effects. Reproduced with permission [47]. Copyright 2019, American Chemical Society. (e,f) Structure model and DFT-derived methanol oxidation pathways on PtZn and Pt surfaces. Reproduced with permission [49]. Copyright 2017, American Chemical Society.

These electronic and geometric effects can be further rationalized by DFT calculations. Projected density of states analysis reveals that alloying can significantly shift the d-band center relative to the Fermi level [48]. For example, incorporation of a second metal component causes the d-band center to downshift from -1.587 to -2.591 eV, indicating weakened metal-adsorbate interactions that facilitate the desorption of reaction intermediates. This electronic redistribution directly influences catalytic energetics. Consistent with this trend, free-energy calculations show that the ordered intermetallic structure exhibits a lower energy barrier for the potential-determining step than its disordered counterpart, suggesting more favorable reaction kinetics. These results highlight how heteroatomic coordination and structural ordering regulate electronic structure and adsorption energetics, thereby optimizing catalytic pathways.

Further insight into catalytic mechanisms can be gained by comparing DFT-derived reaction pathways (Figure 3e,f) [49]. For example, intermetallic PtZn preferentially follows a non-CO pathway during methanol oxidation, in which CH_2O^* is converted into H_2COOH^* through interaction with surface hydroxyl species before being further oxidized to CO_2 . By contrast, pure Pt typically proceeds through a CO-mediated pathway involving $CHOH^*$, COH^* , and CO^* intermediates. The strong stabilization of CO-related species on Pt creates a thermodynamic sink that hinders further oxidation and promotes catalyst deactivation through CO poisoning. In PtZn intermetallics, however, the presence of Zn modifies the adsorption environment and stabilizes surface hydroxyl species, thereby facilitating OH-assisted oxidation of intermediates and suppressing the formation of strongly bound CO species. As a result, intermetallic PtZn exhibits improved poisoning resistance and more favorable reaction energetics than pure Pt. In addition, site-dependent calculations suggest that undercoordinated surface atoms, such as corner and edge sites, can further lower the apparent reaction barriers, helping explain the enhanced catalytic activity often observed in intermetallic nanocrystals.

In addition to tuning adsorption energetics, structural ordering also enhances the stability of surface ensembles. In disordered alloys, local compositional fluctuations can promote surface segregation or atomic rearrangement under reaction conditions, leading to changes in active-site identity. By contrast, the periodic bonding network of intermetallic phases constrains atomic mobility through sublattice-specific coordination, thereby preserving structural integrity and well-defined active motifs during catalysis. Structural ordering therefore provides both electronic modulation and structural stability, reinforcing the structure-property relationships that distinguish intermetallic nanocrystals from random alloys. This understanding further motivates the extension of intermetallic design to compositionally complex high-entropy intermetallic nanocrystals, as discussed in the next section.

3. High-Entropy Intermetallic Nanocrystals: Formation Principles, Synthetic Strategies, and Experimental Realization

The extension from conventional intermetallic nanocrystals to multicomponent HEI nanocrystals introduces a much more complex ordering landscape. In HEIs, multiple principal elements participate simultaneously in lattice ordering, so configurational entropy, cooperative enthalpic interactions, and nanoscale effects collectively raise the thermodynamic threshold for ordered-phase formation [2,50–52]. Meanwhile, lattice distortion and

chemically heterogeneous local environments create distributed diffusion barriers, leading to more intricate and pathway-dependent atomic rearrangement than in binary intermetallic systems. HEI formation should therefore be understood as a multicomponent process governed by the interplay between thermodynamic stabilization and kinetic pathway selection. This chapter discusses the thermodynamic and kinetic design principles of HEIs and then summarizes the synthetic strategies and in situ characterization approaches that have enabled their formation and direct observation.

3.1. Thermodynamic and Kinetic Principles of HEI Nanocrystal Formation

While the thermodynamic and kinetic framework established for conventional intermetallic systems remains broadly applicable to multicomponent alloys, the presence of multiple principal elements substantially reshapes the energetic and structural landscape governing atomic ordering. The most immediate distinction arises from configurational entropy. For an N-component equiatomic alloy, the configurational entropy is given by

$$\Delta S_{\text{conf}} = R \ln N \quad (6)$$

which increases monotonically with the number of constituent elements. As illustrated in Figure 4, this entropy amplification significantly enlarges the $T\Delta S_{\text{conf}}$ contribution in the free-energy balance [2]. Although Figure 4 is presented in the context of mixing thermodynamics, the same configurational entropy term governs the stability of disordered states and thus directly influences the disorder-to-order transformation in multicomponent systems. In high-entropy systems, configurational entropy is the dominant entropy contribution and therefore represents the primary entropy term in the free-energy expression. Accordingly, the disorder-to-order transformation can be expressed as

$$\Delta G_{\text{ord}} = \Delta H_{\text{ord}} - T\Delta S_{\text{conf}} + \Delta \gamma A \quad (7)$$

where ΔH_{ord} represents the enthalpic stabilization associated with ordered bonding, while ΔH_{mix} describes the overall enthalpic tendency for multicomponent mixing relative to the pure elemental state (Equation (8)). The term $\Delta \gamma A$ accounts for surface energy contributions that become non-negligible at the nanoscale. Compared with binary intermetallic systems, the larger configurational entropy of multicomponent alloys more strongly stabilizes the disordered state. Consequently, HEI nanocrystal formation requires a sufficiently strong enthalpic driving force to overcome this entropy effect and enable ordering.

The formation of HEI nanocrystals is governed by the coupled effects of amplified configurational entropy, cooperative enthalpic stabilization, nanoscale size effects, and heterogeneous diffusion pathways [52]. Compared with conventional binary intermetallic systems, HEI formation proceeds under a significantly elevated thermodynamic threshold and follows a more complex structural evolution pathway. In multicomponent systems, achieving atomic ordering requires not only sufficiently negative ΔH_{mix} but also cooperative interactions among multiple elemental pairs, highlighting the critical role of compositional design in stabilizing ordered HEI phases.

This enthalpic contribution can be further quantified by expressing ΔH_{mix} as the summation of pairwise interaction enthalpies among constituent elements:

$$\Delta H_{\text{mix}} = \sum_{i \neq j} x_i x_j \Delta H_{ij} \quad (8)$$

where ΔH_{ij} denotes the mixing enthalpy between elemental pairs. As shown in Figure 5a, strongly negative ΔH_{ij} values favor chemical ordering, interactions with small magnitude stabilize entropy-dominated solid solutions, whereas positive values promote immiscibility and phase separation [2]. Unlike binary intermetallic systems, where a single strongly negative interaction may be sufficient to stabilize an ordered phase, HEI formation requires a cooperative enthalpic stabilization arising from multiple elemental pairs. In other words, long-range ordering can only emerge when the cumulative negative interactions collectively overcome the amplified configurational entropy. This requirement significantly narrows the compositional window capable of supporting HEI formation.

The two-dimensional interaction mapping therefore provides a practical guideline for compositional design (Figure 5b) [50]. By visualizing the enthalpic landscape, it becomes possible to identify element combinations in which dominant negative interactions promote ordering while minimizing strongly positive interactions that would otherwise drive phase separation. Importantly, the stability of HEIs is not dictated by a single pair interaction, but by the collective balance among all pairwise contributions. This cooperative enthalpic effect represents a defining characteristic of high-entropy systems and establishes a fundamental distinction from binary intermetallics, where ordering can often be traced to a single dominant interaction channel.

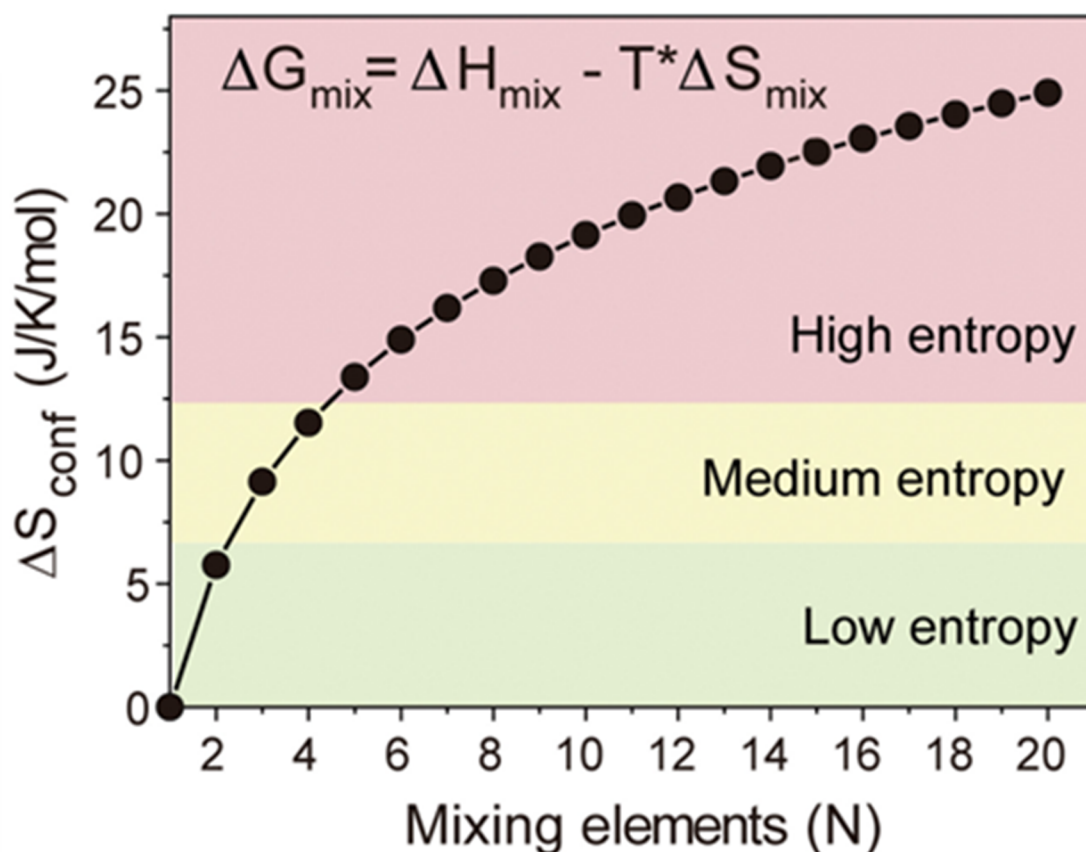


Figure 4. Configurational entropy (ΔS_{conf}) versus the number of mixing elements (N), highlighting the transition from low- to high-entropy regimes and its role in stabilizing HEIs. Reproduced with permission [2]. Copyright 2017, The American Association for the Advancement of Science.

Beyond thermodynamic elevation, the practical realization of HEIs is critically governed by kinetic pathway engineering, as illustrated in Figure 5c,d [2]. As described above, the ordering rate can be expressed by Equation (4), with the diffusion coefficient following an Arrhenius-type relationship:

$$D = D_0 \exp \left(-\frac{E_a^{\text{eff}}}{RT} \right) \quad (9)$$

In binary systems, atomic diffusion is usually governed by a relatively well-defined activation barrier, resulting in more predictable ordering behavior. By contrast, HEIs exhibit a distribution of migration barriers arising from lattice distortion and chemically heterogeneous local environments. Consequently, diffusion pathways become both spatially and elementally dependent, giving rise to a more complex kinetic landscape in which multiple metastable states may be accessed.

The rapid thermal shock pathway illustrated in Figure 5c shows how nonequilibrium processing can access entropy-stabilized structures in high-entropy systems. Ultrafast heating greatly enhances atomic mobility, enabling rapid elemental intermixing and the formation of a highly disordered state with maximized configurational entropy. Subsequent rapid quenching sharply suppresses long-range diffusion and freezes the atomic configuration, thereby kinetically trapping metastable states such as entropy-stabilized solid solutions or partially ordered structures, despite the thermodynamic driving force for ordering or phase separation. This decoupling between thermodynamic driving force and kinetic accessibility is particularly pronounced in high-entropy systems because of their intrinsically heterogeneous diffusion behavior. The temperature-time ordering diagram in Figure 5d further shows that structural evolution in HEIs is strongly governed by diffusion time. Rapid quenching preserves disordered or amorphous-like states by suppressing atomic rearrangement, whereas increasing diffusion time enables short-range ordering and the gradual emergence of intermetallic phases through vacancy-mediated diffusion. With further thermal exposure, long-range diffusion drives the system toward thermodynamic equilibrium and may ultimately lead to phase separation, depending on the enthalpic interaction landscape.

These results show that structural outcomes in HEIs are governed not only by thermodynamics but also by the extent of diffusion allowed during processing. The interplay between diffusion kinetics and thermodynamic

driving forces therefore determines structure selection across multiple metastable states, making kinetics a key design parameter in high-entropy systems. This pathway-dependent evolution distinguishes HEIs from conventional binary intermetallic nanocrystals and highlights the need for synthetic strategies that precisely control mixing, diffusion, and ordering.

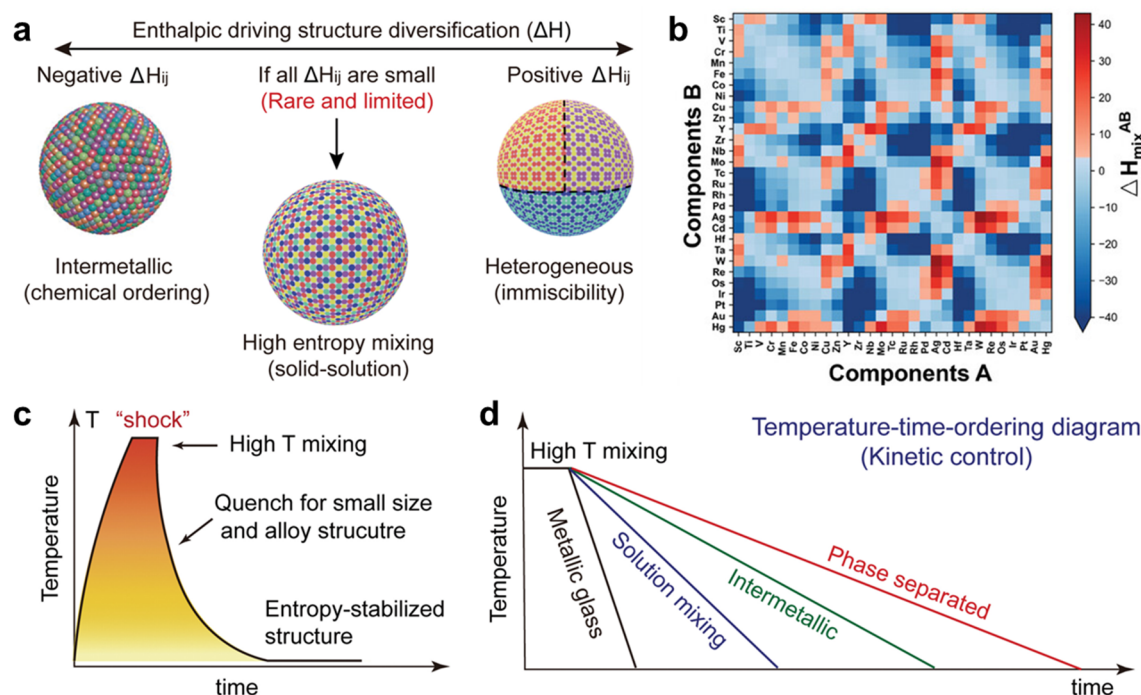


Figure 5. Enthalpic design and kinetic pathways in HEI formation. (a) Structure diversification determined by pairwise mixing enthalpy (ΔH_{ij}). (b) Pairwise interaction map (ΔH_{mix}^{AB}). (c) Rapid thermal shock pathway. (d) Temperature-time ordering diagram illustrating kinetic selection among disordered, ordered, and phase-separated states. The images in (a,c,d) were reproduced with permission [2]. Copyright 2017, The American Association for the Advancement of Science. The image in (b) was reproduced with permission [50]. Copyright 2024, John Wiley and Sons.

3.2. Synthetic Strategies of HEI Nanocrystals

This section discusses the synthetic strategies and mechanistic understanding of HEI formation, with emphasis on the interplay between thermodynamics and kinetics in multicomponent systems. Unlike conventional intermetallics, HEI formation requires not only homogeneous mixing of multiple elements but also controlled atomic ordering within a limited kinetic window. Accordingly, several synthetic routes have been developed, including direct thermally driven HEI formation, thermally driven HEA-to-HEI conversion, and solution-phase synthesis [17,19,53–57]. In parallel, advanced in situ characterization techniques provide direct insight into the dynamic structural evolution during HEI formation, revealing temperature-dependent transitions from disordered solid solutions to ordered intermetallic phases and, ultimately, phase-separated states. These findings show that HEI formation is governed by kinetically accessible pathways rather than equilibrium thermodynamics alone, providing a framework for understanding and designing multicomponent intermetallic nanostructures.

3.2.1. Direct Thermally Driven Synthesis of HEI Nanocrystals

In multicomponent systems, direct thermally driven HEI formation typically involves high-temperature reduction of metal precursors followed by annealing to activate atomic diffusion and promote ordering. However, these conditions also accelerate particle sintering, causing coarsening and loss of active surface area. Excessive particle growth can further disrupt diffusion pathways and hinder atomic rearrangement, making direct HEI formation at the nanoscale more difficult. Strategies that suppress sintering while preserving sufficient atomic mobility are therefore essential. A representative strategy uses sulfur-anchored carbon supports to stabilize intermetallic nanocrystals during high-temperature ordering [17]. As illustrated in Figure 6a, metal precursors are first immobilized on sulfur-doped carbon through wet impregnation, followed by high-temperature H_2 reduction at 800–1100 °C. The strong metal-sulfur interaction anchors metal species and suppresses particle migration,

coalescence, and Ostwald ripening during thermal treatment. As a result, atomically ordered intermetallic nanocrystals smaller than 5 nm can be obtained even under conditions that would normally induce severe sintering. X-ray diffraction (XRD) and atomic-resolution imaging further confirm the formation of ordered intermetallic phases through characteristic superlattice reflections and well-defined atomic arrangements (Figure 6b–d). These results show that particle-size control is not only important for suppressing sintering, but also critical for balancing diffusion kinetics and thermodynamic stability during intermetallic formation at the nanoscale.

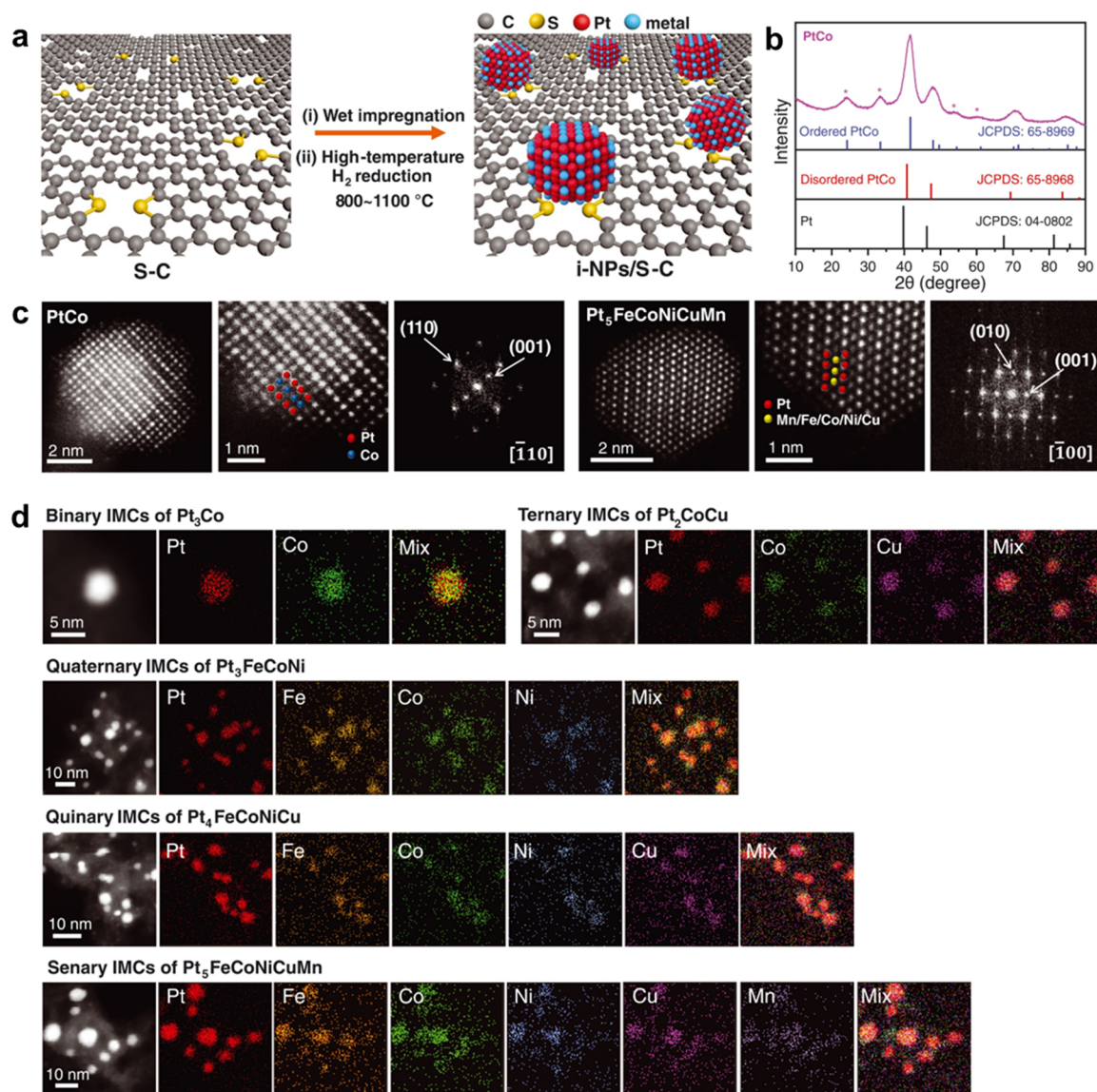


Figure 6. Direct thermally driven synthesis of HEI nanocrystals on sulfur-doped carbon supports. (a) Schematic of wet impregnation and high-temperature H_2 reduction on sulfur-doped carbon (S-C). (b) XRD patterns showing superlattice reflections of ordered intermetallic phases. (c) Atomic-resolution HAADF-STEM images and fast fourier transform (FFT) patterns confirming ordered structures. (d) Energy-dispersive X-ray spectroscopy (EDS) elemental maps demonstrating homogeneous distribution of multiple elements. Reproduced with permission [17]. Copyright 2021, The American Association for the Advancement of Science.

To elucidate the formation pathway of ordered intermetallics during annealing, temperature-resolved characterization provides direct and quantitative insight into both structural and chemical evolution [53,54]. In the hollow-carbon confinement system (Figure 7a–c), X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) analysis shows a clear coordination transition, where Pt-N/O coordination decreases from 6.0 to 0, accompanied by the formation of Pt-Pt/Pt-TM bonds with coordination numbers of ~3–5, indicating reduction of metal precursors and the emergence of metallic bonding [53]. Consistent with this evolution, XRD shows the formation of an FCC alloy phase at intermediate temperatures between 500 and 700 °C, followed by the appearance of superlattice reflections such as (100) and (110) at 1000 °C, confirming the transition from a disordered alloy to an ordered intermetallic structure. More direct mechanistic evidence is

obtained from quasi-simultaneous in situ PXRD/XANES measurements (Figure 7d,e), where noble metals (Pt, Pd) reduce rapidly within ~ 150 – 175 °C, while 3d elements reduce gradually via intermediate states (~ 100 – 300 °C) [54]. This asynchronous reduction is reflected in the reduced metal fraction and correlates with the emergence of the FCC phase (~ 150 – 200 °C), followed by continuous lattice contraction from ~ 3.90 to ~ 3.76 – 3.80 Å due to progressive incorporation of smaller 3d elements. Together, these results reveal a stepwise formation mechanism in which rapid noble-metal reduction initiates nucleation, incorporation of 3d elements promotes compositional homogenization, and further thermal activation drives atomic ordering into intermetallic phases. This also highlights the unique power of in situ techniques to capture transient intermediates and kinetic pathways that cannot be resolved by ex situ analysis.

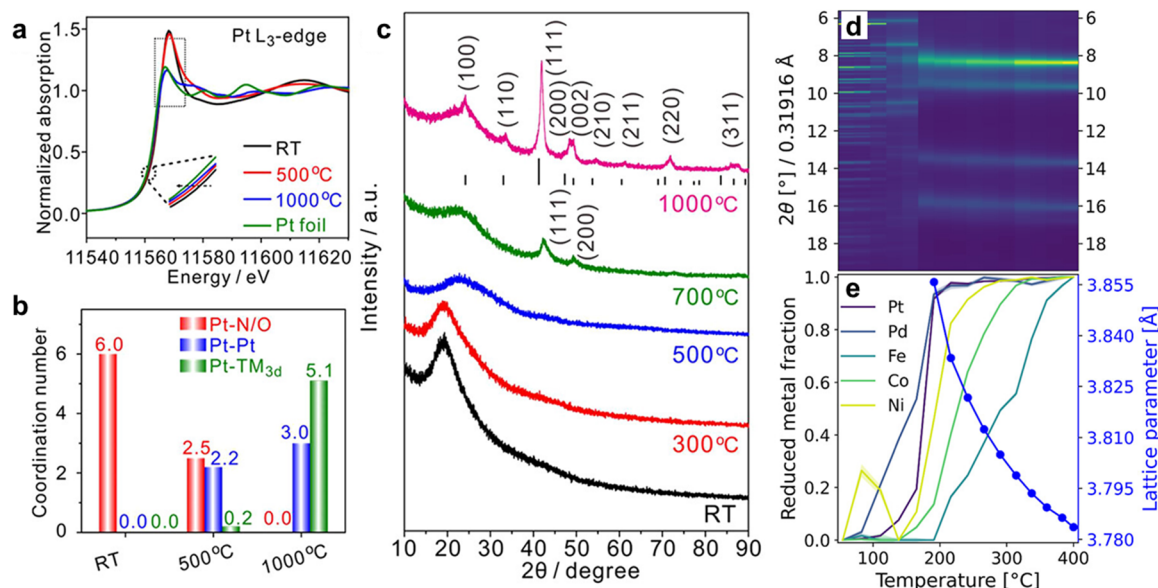


Figure 7. Temperature-resolved evolution of coordination, phase structure, and reduction behavior during HEI formation. (a) In situ Pt L₃-edge XANES. (b) EXAFS-derived coordination numbers showing Pt-N/O to Pt-Pt/Pt-TM transition. (c) XRD patterns indicating FCC formation between 500–700 °C and ordering at 1000 °C. Reproduced with permission [53]. Copyright 2023, American Chemical Society. (d) In situ PXRD contour plot. (e) Reduced metal fraction and lattice parameter evolution, revealing asynchronous reduction and lattice contraction. Reproduced with permission [54]. Copyright 2025, American Chemical Society.

3.2.2. Thermally Driven HEA-to-HEI Conversion

A representative route for thermally driven HEA-to-HEI conversion is to decouple the reduction of metal precursors and the nucleation and growth of HEA nanocrystals from the subsequent atomic ordering process [19]. As illustrated in Figure 8a,b, ultrafast Joule heating first generates chemically homogeneous HEA nanocrystals, after which a time-limited annealing step drives the disorder-to-order transition into a multiprincipal-element intermetallic phase (MPEI), followed by rapid cooling to preserve the ordered nanostructure. This two-step process sequentially addresses two key challenges: establishing uniform atomic mixing among multiple principal elements and enabling long-range ordering without severe particle coarsening or phase separation. Compared with conventional annealing, brief and controlled thermal exposure provides a practical route for converting HEAs into ordered HEIs while retaining ultrasmall particle size.

The structural evolution shown in Figure 8c,d further confirms the success of this approach. In the quinary Pt(Fe_{0.7}Co_{0.1}Ni_{0.1}Cu_{0.1}) system, aberration-corrected HAADF-STEM and elemental mapping reveal the coexistence of long-range atomic ordering and homogeneous multielemental distribution (Figure 8c), while the emergence and progressive sharpening of superlattice reflections in XRD demonstrate the continuous conversion from a disordered HEA precursor to an ordered intermetallic phase as annealing time increases (Figure 8d). These results are particularly important for HEI synthesis because they show that ordering can be achieved without sacrificing multicomponent compositional uniformity, which is often the major difficulty in extending intermetallic design beyond binary or ternary systems.

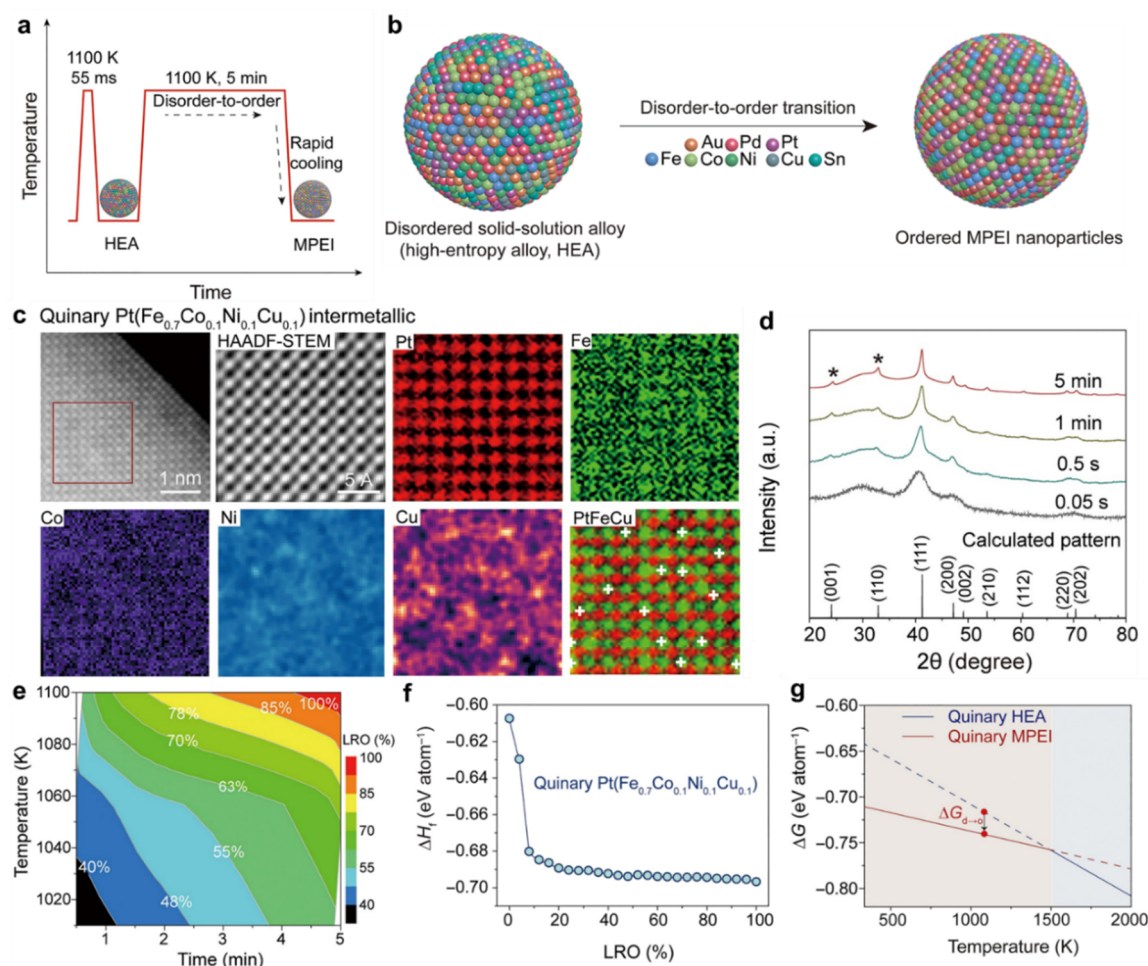


Figure 8. Rapid Joule heating enabled thermally driven HEA-to-MPEI conversion. **(a)** Schematic illustration of the two-step thermal process consisting of rapid Joule heating and subsequent annealing. **(b)** Atomic transition from a disordered HEA to an ordered MPEI structure. **(c)** HAADF-STEM image and corresponding elemental maps of quinary Pt(Fe_{0.7}Co_{0.1}Ni_{0.1}Cu_{0.1}) intermetallic nanocrystals. **(d)** Time-resolved XRD patterns showing the evolution of superlattice peaks during annealing. **(e)** Temperature-time contour map of LRO. **(f)** Relationship between enthalpy change and ordering degree. **(g)** Temperature-dependent Gibbs free energy profiles of the HEA and MPEI phases. Reproduced with permission [19]. Copyright 2022, The American Association for the Advancement of Science.

This case provides direct experimental support for the coupled thermodynamic and kinetic framework of HEI formation. The successful formation of an ordered quinary intermetallic phase indicates that the cumulative enthalpic stabilization among multiple elemental pairs can overcome the amplified configurational entropy that stabilizes the disordered HEA phase. The temperature-time contour map of long-range ordering (LRO) (Figure 8e) further reveals a well-defined processing window, in which increasing temperature and dwell time enhance atomic diffusion and promote ordering, highlighting the importance of kinetic accessibility. Consistently, the monotonic decrease in enthalpy with increasing ordering degree (Figure 8f) confirms that the disorder-to-order transition is driven by cumulative enthalpic gain, while the calculated Gibbs free energy profiles (Figure 8g) show that the disordered HEA phase is favored at higher temperatures because of configurational entropy, whereas the ordered MPEI phase becomes more stable at lower temperatures where enthalpic stabilization dominates. Importantly, the ordered phase can only be retained when particle growth is effectively suppressed, whereas prolonged annealing leads to substantial coarsening and phase separation. These results show that successful HEI synthesis requires not only sufficient enthalpic driving force for ordering but also precise control of the thermal budget to balance atomic diffusion and structural stability. Accordingly, this work establishes a practical synthetic framework in which precursor homogenization, limited-time ordering, and rapid quenching are integrated to access ordered multicomponent intermetallic nanocrystals that are difficult to obtain under near-equilibrium conditions.

Constructing ordered multicomponent intermetallic nanocrystals via thermal annealing remains challenging due to concurrent particle coarsening, compositional segregation, and complex diffusion pathways in multicomponent systems. To address these challenges, a template-assisted thermal annealing strategy is employed, as illustrated in Figure 9a. PtCu nanosheets first serve as structural templates, enabling the incorporation of

additional metal species, such as Pd, Ag, Ru, and Fe, to form multicomponent nanosheets with homogeneous elemental distribution [55]. Subsequent annealing at elevated temperature (650 °C) induces atomic diffusion and rearrangement, leading to the transformation from disordered multicomponent nanosheets into structurally ordered intermetallic nanocrystals. TEM and HAADF-STEM analyses (Figure 9b–d) confirm that the nanocrystals maintain a uniform size (~5 nm) without obvious agglomeration after thermal treatment, indicating that the template effectively suppresses coarsening during ordering.

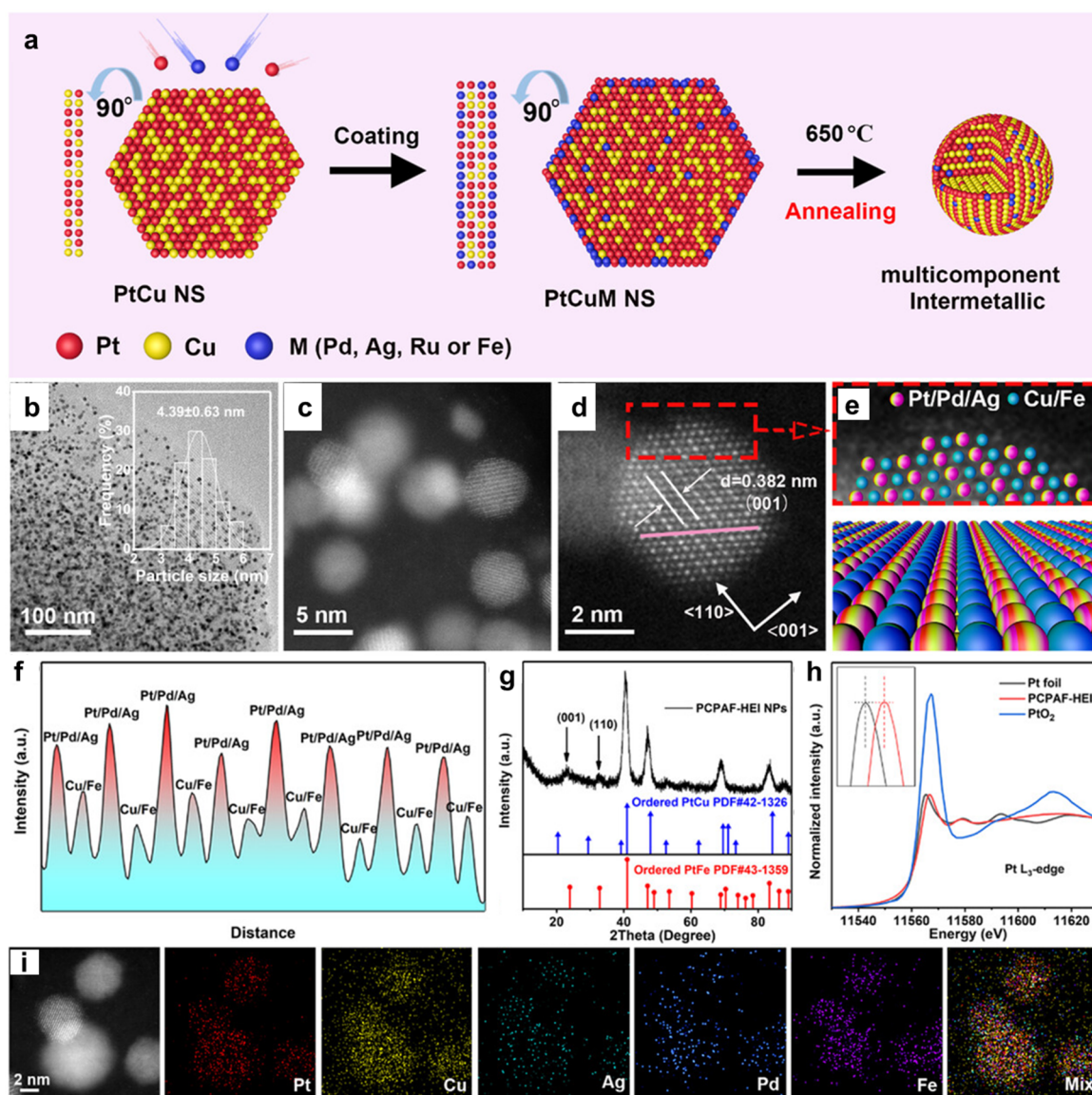


Figure 9. Template-assisted thermally driven HEA-to-HEI conversion and structural characterization of HEI nanocrystals. (a) Schematic illustration of the formation process of intermetallic nanocrystals. (b–d) TEM and HAADF-STEM images of nanocrystals. (e,f) Atomic-resolution HAADF-STEM and corresponding intensity profile revealing an ordered L₁₀-type structure with alternating high-Z and low-Z atomic columns. (g) XRD pattern of ordered intermetallic phases, located between L₁₁-PtCu and L₁₀-PtFe. (h) Pt L₃-edge XAFS spectra. (i) STEM-EDS elemental mappings. Reproduced with permission [55]. Copyright 2024, John Wiley and Sons.

At the atomic scale, aberration-corrected HAADF-STEM reveals clear alternating contrast between high-Z (Pt/Pd/Ag) and low-Z (Cu/Fe) atomic columns along the <001> direction, consistent with the formation of a chemically ordered L₁₀-type structure (Figure 9d,e). The corresponding intensity profile observed in HRTEM and XRD further verify the successful transition to intermetallic phases (Figure 9f,g). Notably, the introduction of Fe significantly alters the crystal structure, shifting from an L₁₁-like PtCu-based phase to an L₁₀-type structure resembling PtFe, as evidenced by both lattice fringe variation and XRD peak positions. This indicates that compositional modulation can effectively tune the final ordered phase through thermally activated atomic redistribution. X-ray absorption fine structure (XAFS) analysis further supports this structural evolution, where

the Pt L₃-edge white-line intensity remains close to that of metallic Pt and distinctly different from PtO₂, confirming that Pt maintains a metallic state after annealing (Figure 9h). Meanwhile, the observed shift in white-line features suggests a modification in the d-band electronic structure of Pt, indicative of electron redistribution among neighboring elements within the ordered lattice. Furthermore, elemental mapping and line-scan analyses confirm a uniform distribution of all constituent elements without phase segregation, suggesting that high-temperature annealing promotes not only ordering but also compositional homogenization (Figure 9i). Overall, this template-assisted thermally driven HEA-to-HEI conversion enables the controlled formation of multicomponent HEI nanocrystals with well-defined atomic ordering, tunable crystal structures, and corresponding electronic-structure modulation for tailored catalysis.

To further elucidate the thermally driven ordering pathway and phase stability, in situ HAADF-STEM is used to directly track the structural evolution of multicomponent PtRuFeCoNi HEA atomic layers on Pd nanocubes with {100} facets during annealing (Figure 10) [56]. Below 200 °C, the atomic layers retain a disordered FCC solid-solution structure with randomly distributed elements (Figure 10a–o). Upon heating to 300 and 400 °C, a progressive disorder-to-order transition occurs, leading to the emergence of L1₀ and subsequently L1₂ intermetallic phases, as evidenced by the appearance of characteristic superlattice reflections and atomic-scale intensity modulation (Figure 10p–y). This transformation is attributed to thermally activated interdiffusion between PGMs and IGMs, where the relatively faster inward diffusion of smaller IGM atoms induces sublattice rearrangement and ordering. Notably, further increasing the temperature to 500 °C results in phase separation, accompanied by morphological evolution from cubic to pseudospherical particles and the formation of a Pt-rich surface layer (Figure 10z–ad). FFT analysis reveals the coexistence of multiple phases, including FCC Pt, Pd, and L1₂ intermetallic domains, indicating the breakdown of the ordered structure at elevated temperature. These observations collectively reveal a temperature-dependent evolution from high-entropy solid solution to ordered intermetallic phases and ultimately to phase-separated states, highlighting that the ordered intermetallic structure formed during annealing represents a metastable state governed by kinetic constraints in this case (Figure 10ae,af).

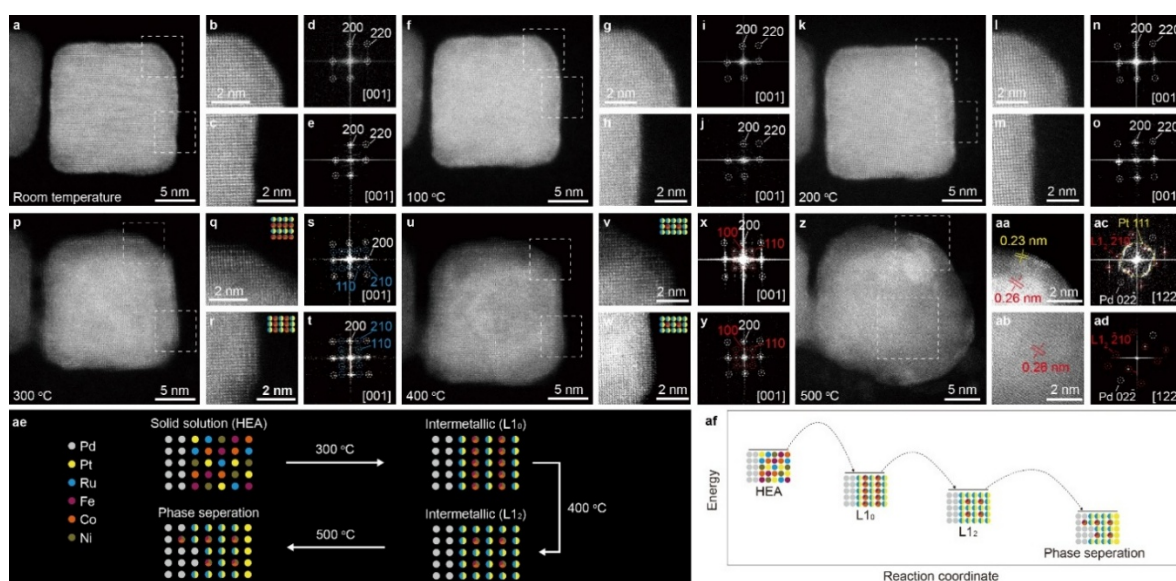


Figure 10. Temperature-dependent HEA-to-HEI conversion and metastability of multicomponent atomic layers revealed by in situ HAADF-STEM. HAADF-STEM images and corresponding FFT patterns of Pd@PtRuFeCoNi core-shell nanocubes at different temperatures: (a–e) room temperature, (f–j) 100 °C, (k–o) 200 °C, (p–t) 300 °C, (u–y) 400 °C, and (z–ad) 500 °C. (ae) Schematic illustration of the temperature-induced phase evolution from solid-solution PtRuFeCoNi atomic layers to ordered L1₀ and L1₂ intermetallic phases, followed by phase separation. (af) Schematic illustration highlighting the kinetically controlled nature of PtRuFeCoNi atomic layers as a metastable phase. Reproduced with permission [56]. Copyright 2024, The American Association for the Advancement of Science.

3.2.3. Solution-Phase Synthesis of HEI Nanocrystals

In contrast to thermally driven synthesis at elevated temperatures, direct solution-phase synthesis provides an alternative route for constructing intermetallic nanostructures under comparatively mild conditions. Although the reaction temperature is substantially lower, the formation of ordered intermetallic phases can still be achieved when sufficient atomic diffusion and rearrangement are enabled during crystal growth. A representative example

is the one-pot synthesis of PtRhBiSnSb HEI nanoplates at 220 °C, where the combined use of suitable precursors, coordinating solvents, surfactants, and reducing agents allows prolonged atomic exchange and structural reorganization in the solution phase [57]. Under these conditions, the product evolves into well-defined hexagonal nanoplates with a single-phase hcp structure, as confirmed by TEM and XRD analyses (Figure 11a–g). Aberration-corrected HAADF-STEM further reveals well-ordered atomic stacking along multiple zone axes, consistent with a hexagonal close packing (HCP) PtBi-type intermetallic framework. The measured lattice spacing and distinct atomic-column contrast indicate that the five constituent elements are incorporated into a chemically ordered lattice rather than a random solid solution. In addition, elemental mapping suggests a spatially regulated elemental distribution within the nanoplate, further reflecting that low-temperature wet-chemical synthesis can still support progressive atomic redistribution and ordering (Figure 11h). This example highlights that, beyond high-temperature annealing, intermetallic formation may also be realized through kinetically accessible diffusion pathways in solution, provide that crystal growth and atomic reorganization are sufficiently sustained.

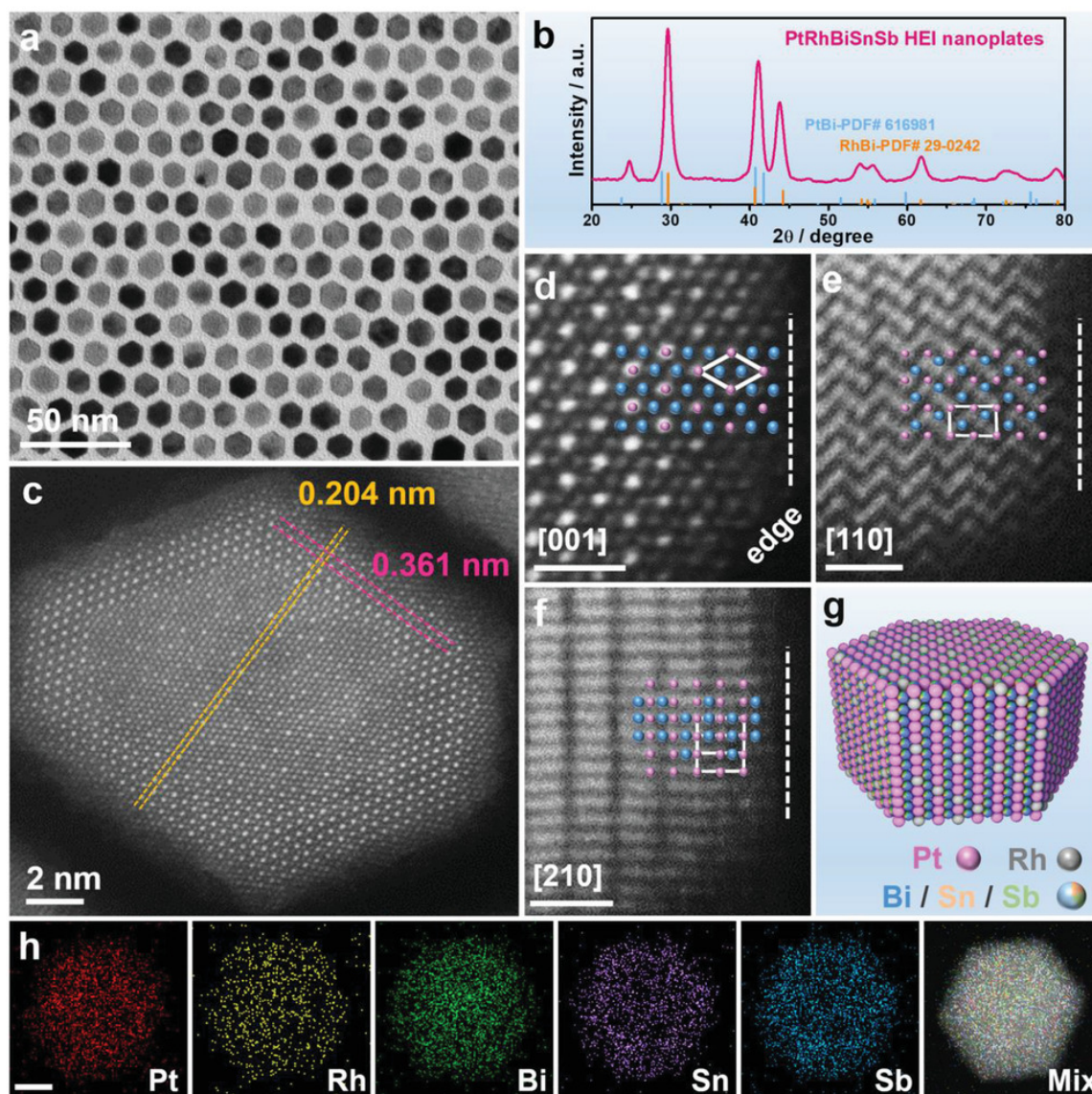


Figure 11. Solution-phase synthesis of HEI nanocrystals. (a) TEM image; (b) XRD pattern; and (c–f) Typical aberration-corrected HAADF-STEM images of the surface of PtRhBiSnSb HEI nanoplates. The insets in (d–f) show the corresponding PtBi unit cells viewed along different zoom axes. The pink and blue spheres represent Pt and Bi atoms, respectively. Scale bars: 1 nm. (g) The schematic illustration showing the atomic arrangement of Pt/Rh/Bi/Sn/Sb atoms on the surface of the PtRhBiSnSb HEI nanoplate. (h) EDS mapping images of a PtRhBiSnSb HEI nanoplate. Scale bar: 5 nm [57]. Reproduced with permission [57]. Copyright 2022, John Wiley and Sons.

Taken together, these three synthetic routes highlight that HEI nanocrystals can be accessed through distinct yet complementary pathways, including direct thermally driven synthesis, thermally driven HEA-to-HEI conversion, and solution-phase synthesis. Although these approaches differ in processing conditions, they share a

common requirement: the need to balance multielement mixing, atomic diffusion, and ordering within an appropriate kinetic window. Direct thermally driven synthesis relies on suppressing sintering while maintaining sufficient mobility for ordering, HEA-to-HEI conversion separates multicomponent mixing from subsequent ordering to improve structural control, and solution-phase synthesis offers a milder route in which crystal growth and atomic reorganization can proceed simultaneously. Together, these studies show that HEI formation is governed not only by thermodynamic driving forces but also, more importantly, by how synthetic pathways regulate diffusion, ordering, and structural stabilization across nonequilibrium conditions.

In summary, the three major synthetic routes to HEI nanocrystals exhibit complementary strengths and limitations. Direct thermally driven synthesis provides sufficient atomic mobility to achieve high ordering degree and thermodynamically stable intermetallic phases, but it often suffers from particle coarsening, sintering, and possible phase segregation, making particle-size control difficult without anchoring or confinement strategies. Thermally driven HEA-to-HEI conversion improves compositional homogeneity by separating multielement mixing from subsequent ordering, thereby offering better control over ordering evolution and phase purity. However, this approach is highly sensitive to thermal budget, because insufficient annealing causes incomplete ordering whereas excessive annealing may lead to coarsening or phase separation. Solution-phase synthesis offers superior control over particle size, morphology, and surface structure under milder conditions and can effectively suppress sintering, but the lower reaction temperature may limit atomic diffusion and ordering degree. In addition, precursor compatibility, ligand effects, phase purity, and large-scale reproducibility remain important challenges. The advantages and limitations of these representative synthetic strategies are comparatively summarized in Table 1, with emphasis on particle-size control, ordering degree, phase purity, sintering resistance, and scalability. Therefore, future HEI synthesis may benefit from combining the high ordering capability of thermal methods with the size, morphology, and surface control enabled by solution-phase approaches.

Table 1. Advantages and limitations of representative synthetic strategies for HEI nanocrystals.

Synthetic Strategy		Advantages	Limitations
Direct thermally driven synthesis	(1)	High atomic mobility	(1) Particle coarsening
	(2)	High phase crystallinity	(2) Sintering
	(3)	Access to thermodynamically stable phases	(3) Possible phase segregation
Thermally driven HEA-to-HEI conversion	(1)	Decouples multielement mixing and ordering	(1) Sensitive to annealing temperature and time
	(2)	Improved phase purity and compositional homogeneity through separated mixing and ordering steps	(2) Incomplete ordering if under-treated
	(3)	Tunable ordering degree	(3) Coarsening or phase separation if over-treated
Solution-phase synthesis	(1)	Better morphology/size control	(1) Limited atomic diffusion
	(2)	Reduced sintering	(2) Ordering degree may be lower
	(3)	Suitable for shape and facet engineering	(3) Phase purity is sensitive to precursor reactivity and ligand coordination.
	(4)	Milder reaction conditions	(4) Large-scale reproducibility remains challenging

4. Electrocatalytic Applications of HEI Nanocrystals

As a structurally ordered class of multicomponent catalysts, HEI nanocrystals have opened new opportunities for electrocatalyst design because of their highly tunable electronic structures and unique ordered lattices. Unlike conventional binary intermetallic compounds and disordered HEA nanocrystals, HEI nanocrystals incorporate multiple elements into specific crystallographic sublattices, thereby generating well-defined atomic arrangements. Such structural features not only enable more precise isolation of active sites but also strengthen coordination interactions between noble and non-noble metals [58,59]. As a result, HEI nanocrystals can effectively regulate surface electronic structure and adsorption behavior, optimize the adsorption energetics of key reaction intermediates, and reduce competitive adsorption, thereby enhancing catalytic activity and selectivity [2]. Although catalytic studies of HEI nanocrystals remain less extensive than those of HEA nanocrystals, HEIs have already been explored in several important electrocatalytic reactions, such as the hydrogen evolution reaction (HER), oxygen reduction reaction (ORR), and ethanol oxidation reaction (EOR), as shown in Table 2. Existing studies have shown that HEI catalysts can exhibit higher activity and improved stability than conventional binary

intermetallic compounds or their corresponding HEA counterparts, highlighting the critical role of structural ordering in governing catalytic behavior. This chapter therefore reviews recent progress in the electrocatalytic applications of HEI nanocrystals in these three representative electrocatalytic reactions and aims to provide a basis for the rational design of next-generation multifunctional and high-performance catalysts.

Table 2. Representative electrocatalytic applications of HEI-based catalysts.

Synthetic Strategy	Composition	Structure	Electrocatalytic Application	Ref.
Thermally Driven HEA-to-HEI Conversion	PtPdAgFeCu	L1 ₀	HER	[55]
Arc-melting	FeCoNiAlTi	L1 ₂	HER	[60]
Thermally Driven HEA-to-HEI Conversion	(IrRh) ₂ PrNdTb	TCP	HER	[61]
Direct Thermally Driven Synthesis	Pt ₄ FeCoNiCu	L1 ₀	HER, ORR	[59]
Direct Thermally Driven Synthesis	PtFeCoNiCuZn	FCT	ORR	[29]
Direct Thermally Driven Synthesis	Pt(FeCoNiCuZn) ₃	L1 ₂	ORR	[62]
Direct Thermally Driven Synthesis	PtCrFeCoNiZn	FCT	ORR	[33]
Direct Thermally Driven Synthesis	PtIrFeCoNiCu	FCC	ORR	[63]
Direct Thermally Driven Synthesis	Pt(FeCoNiCu) ₃	L1 ₂	ORR	[64]
Direct Thermally Driven Synthesis	PtIrFeCoCu	L1 ₀	ORR	[30]
Direct Thermally Driven Synthesis	Pt ₄ FeCoNiCu, Pt ₅ MnFeCoNiCu	L1 ₀	ORR	[17]
Solution-phase synthesis	PdFeCoNiCu	L1 ₂	ORR	[65]
Thermally Driven HEA-to-HEI Conversion	Pt _{0.45} Fe _{0.18} Co _{0.12} Ni _{0.15} Mn _{0.10}	L1 ₀	ORR	[53]
Thermally Driven HEA-to-HEI Conversion	PtRhFeNiCu	FCC	EOR	[25]
Direct Thermally Driven Synthesis	PtPdAuFeCoNiCuSn	L1 ₀	EOR	[19]
Solution-phase synthesis	PtRhBiSnSb	HCP	EOR, Methanol oxidation reaction (MOR), Glycerol oxidation reaction (GOR)	[57]
Thermally Driven HEA-to-HEI Conversion	(PtPdIrRu) ₂ FeCu	L1 ₀	Formic acid oxidation reaction (FAOR)	[66]
Electrospinning	(PtIr)(FeMoBi)	L1 ₀	Ethylene glycol oxidation reaction (EGOR)	[67]
Solution-phase synthesis	Pt _{0.8} Fe _{0.2} Co _{0.2} Ni _{0.2} Cu _{0.2} Pt _{0.8} Fe _{0.2} Co _{0.2} Ni _{0.2} Zn _{0.2} Pt _{0.8} Fe _{0.2} Co _{0.2} Ni _{0.2} Mn _{0.2}	L1 ₀	Nitrate reduction reaction (NO ₃ ⁻ RR)	[50]
Arc-melting	(Fe _{0.25} Co _{0.25} Ni _{0.25} Cu _{0.25}) ₆ Sn ₅	Monoclinic	NO ₃ ⁻ RR	[68]
Direct Thermally Driven Synthesis	FeNiCuGaGe	BCC	Acetylene Semihydrogenation	[58]
Direct Thermally Driven Synthesis	(PtCoNi)(SnInGa)	Hexagonal NiAs-type	Propane dehydrogenation	[69]

4.1. Hydrogen Evolution Reaction (HER)

Amid the growing depletion of non-renewable energy resources, hydrogen has attracted increasing attention as a clean energy carrier with the potential to replace fossil fuels [70]. Among the various hydrogen-production technologies, the electrocatalytic HER is regarded as an efficient and sustainable route for large-scale hydrogen generation powered by renewable energy [71]. In acidic media, the HER process initiates with the Volmer step ($H^+ + e^- \rightarrow H^*$). H₂ is then released via either the Heyrovsky step ($H^+ + H^* + e^- \rightarrow H_2$) or the Tafel step ($H^* + H^* \rightarrow H_2$) [72]. According to the Sabatier principle [73], optimal activity requires moderate binding affinity: weak adsorption limits the Volmer step, while excessive binding hinders H₂ desorption. Thus, the hydrogen adsorption free energies (ΔG_{H^*}) ≈ 0 is the primary descriptor for peak performance, ensuring neither adsorption nor desorption becomes the rate-determining step (RDS) [73,74]. Unlike acidic systems, alkaline HER is fundamentally limited by sluggish water dissociation kinetics (the Volmer step), which imposes a high energy barrier for H-OH bond cleavage [75]. At low H* coverage, the reaction follows the Heyrovsky step ($H^* + H_2O + e^- \rightarrow H_2 + OH^-$), while high coverage favors the Tafel step ($H^* + H^* \rightarrow H_2$). Because of the additional water-dissociation barrier, even Pt shows HER activity in alkaline media that is several orders of magnitude lower than in acid. Nevertheless, alkaline electrolysis remains attractive for industrial applications because metallic electrodes generally exhibit better corrosion tolerance under alkaline conditions [76].

Owing to these intricate kinetic demands, traditional monometallic catalysts struggle to optimize the adsorption of multiple intermediates simultaneously. HEIs, characterized by their highly tunable electronic structures and multi-component synergistic effects, provide an ideal platform for the precise modulation of ΔG_{H^*} and hydroxide adsorption free energies (ΔG_{OH^*}). Saleem et al. reported PtPdAgFeCu HEI nanocrystals with an $L1_0$ intermetallic phase that exhibited excellent HER activity in 0.5 M H_2SO_4 , with an overpotential of 24 mV at 10 mA cm^{-2} , a Tafel slope of 29 mV dec^{-1} , and a mass activity of $9.531\text{ A mg}_{Pt}^{-1}$ at -0.05 V vs. RHE, about 12 times higher than that of commercial Pt/C (Figure 12a–c) [55]. The catalyst also showed outstanding durability, retaining nearly 100% of its initial current density after 140 h at 100 mA cm^{-2} and 10000 CV cycles (Figure 12d,e). DFT calculations indicated that PtPdAgFeCu HEI nanocrystals possess a smaller absolute ΔG_{H^*} value, suggesting a more favorable HER environment (Figure 12f). The partial density of states (PDOS) analysis further reveals a high density of states for the Pt d-orbital near the Fermi level, which facilitates efficient charge transfer and enhances electrical conductivity (Figure 12g). Compared with pristine Pt, the redistribution of electronic states within the HEI lattice leads to a significant downshift of the d-band center. This electronic modulation effectively weakens the hydrogen binding affinity on the Pt surface, shifting the ΔG_{H^*} toward a near-thermoneutral value to boost HER kinetics.

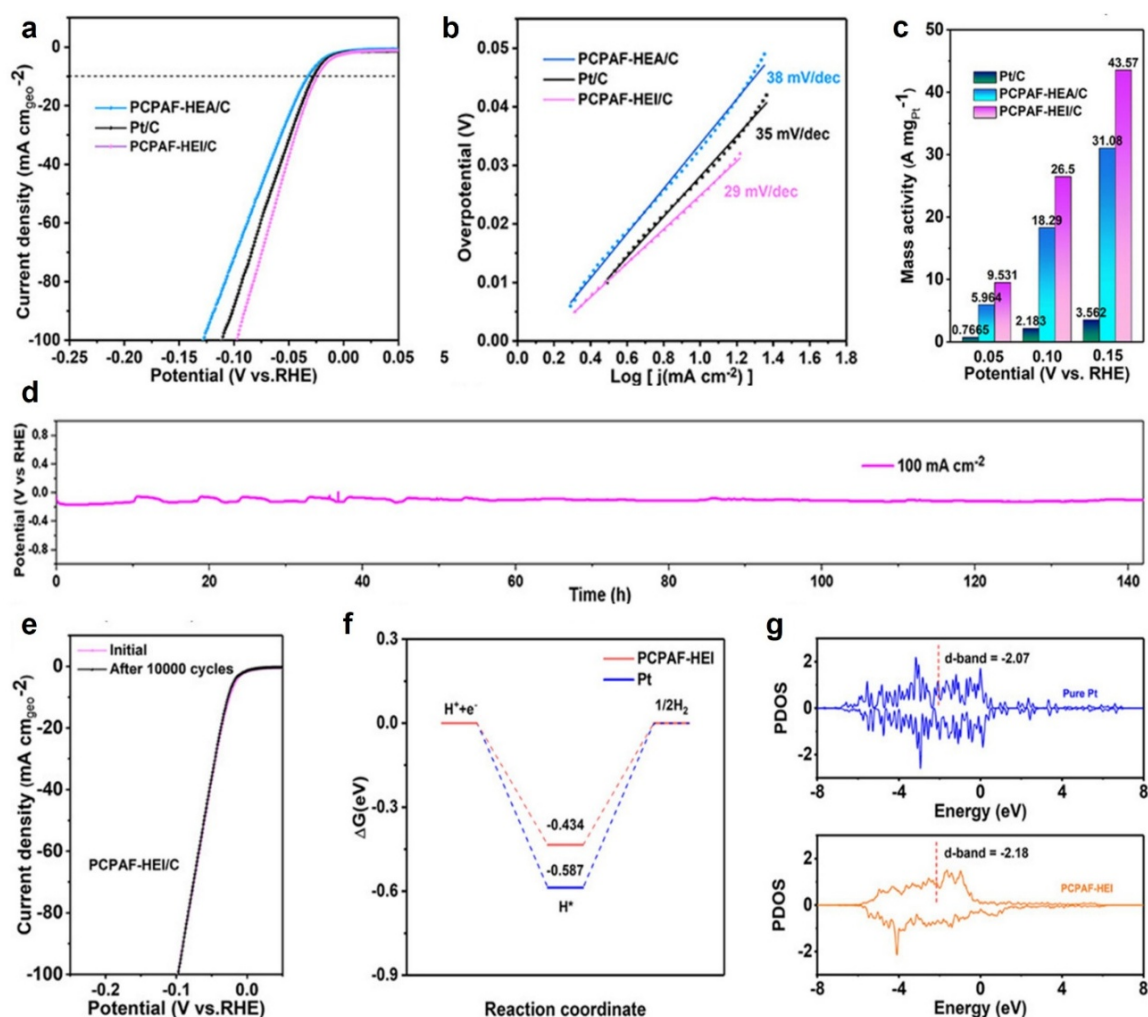


Figure 12. Electrocatalytic acidic HER performance of PtPdAgFeCu HEI nanocrystals in 0.5 M H_2SO_4 . (a) HER polarization curves. (b) Tafel slopes. (c) Quantitative comparisons of HER mass activity. (d) Time-dependent overpotential curve at constant current density of 100 mA cm^{-2} . (e) Stability test recorded before and after 10000 cycles. (f) Calculated ΔG_{H^*} at the equilibrium potential. (g) PDOS of the d-band for the surface Pt atoms in the pure Pt (111) and HEI (111) slab system. Reproduced with permission [55]. Copyright 2024, John Wiley and Sons.

For alkaline HER, simultaneously optimizing the adsorption and desorption of OH^* and H^* on a single active site remains a major challenge [77]. To overcome this limitation, several high-performance catalysts have adopted dual-site strategies that separate the functions of water dissociation and hydrogen adsorption. These catalysts achieve superior activity by optimizing the synergistic balance between H_2O dissociation and the adsorbed hydrogen atom (H_{ad}) [78,79]. Building upon these principles, the multicomponent nature of HEI systems provides

a vast array of active sites and diverse configurations, enabling precise tuning of the adsorption strengths for various intermediate species. Wu et al. reported $(\text{IrRh})_2\text{PrNdTb}$ HEI nanocrystals with a topologically close-packed (TCP) C15 structure [61]. Compared to disordered $\text{A1-(IrRh)}_2\text{PrNdTb}$ HEA nanocrystals and $\text{C15-Ir}_2\text{Pr}$, the resulting catalyst exhibited superior alkaline HER activity in 1 M KOH, which can be attributed to its larger ECSA and excellent intrinsic activity (Figure 13a,b). Besides, the HEI nanocrystals showed excellent stability with negligible degradation after a 200h stability test, confirming the high stability and minimal degradation of catalyst (Figure 13c). DFT calculations also demonstrate that the ΔG_{H^*} values of the HEI nanocrystals close to zero, indicating an optimal balance for hydrogen adsorption and desorption, which is essential for high catalytic efficiency (Figure 13d). Moreover, $\text{C15-(IrRh)}_2\text{PrNdTb}$ HEI nanocrystals exhibit stronger OH^* binding energy, which facilitates the polarization and activation of adsorbed H_2O molecules, thereby accelerating the Volmer step (Figure 13e). The calculated water dissociation barriers of HEI nanocrystals exhibit lower values, ensuring a sufficient supply of active hydrogen during the Volmer step (Figure 13f). Figure 13g,h demonstrates that the strengthened d - f orbital coupling between noble metals (Ir, Rh) and rare earth elements (Pr, Nd, and Tb) in $\text{C15-(IrRh)}_2\text{PrNdTb}$ HEI nanocrystals not only facilitates water dissociation but also establishes a kinetically favorable hydrogen spillover pathway, ensuring sufficient active hydrogen supply during the Volmer step.

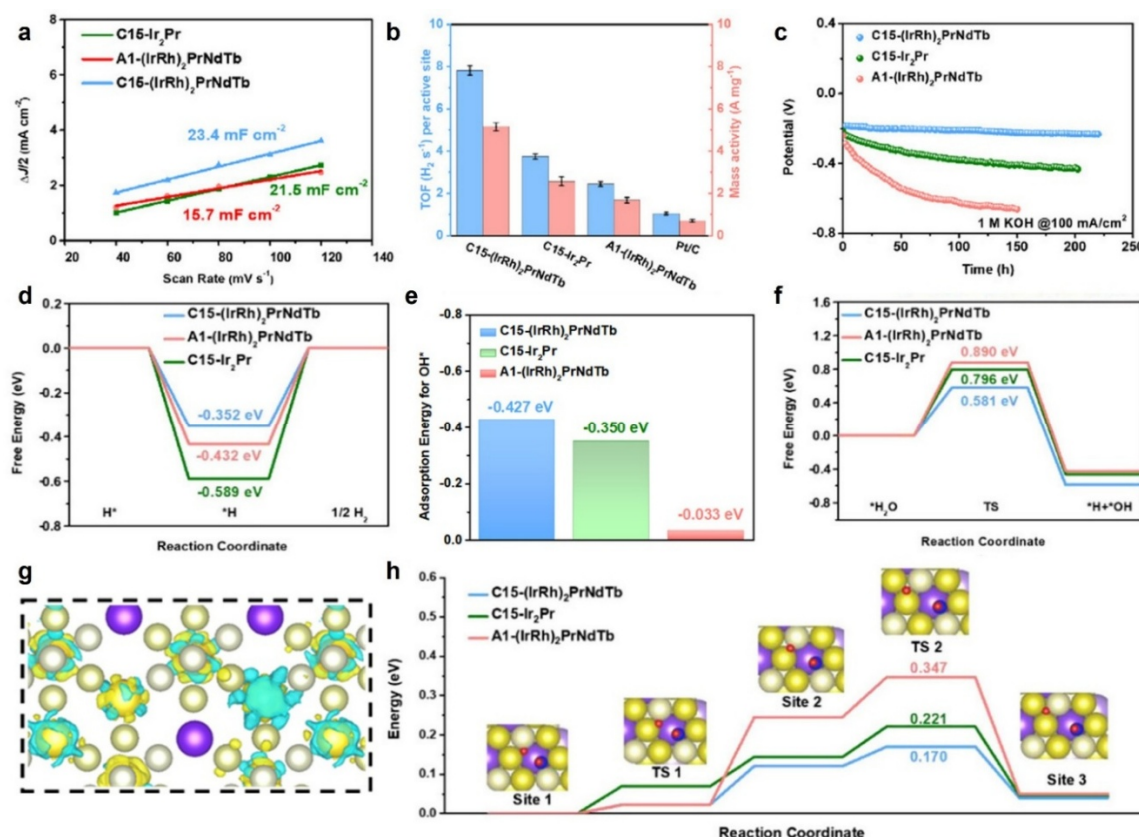


Figure 13. Electrocatalytic alkaline HER performance and theoretical analysis of $\text{C15-(IrRh)}_2\text{PrNdTb}$ HEI nanocrystals. (a) The double-layer capacitance (C_{dl}) curve. (b) The turnover frequency (TOF) values and mass activity. (c) Stability test recorded before and after 200h. (d) Calculated ΔG_{H^*} at the equilibrium potential. (e) Calculated ΔG_{OH^*} at the equilibrium potential. (f) The calculated energy barriers for water dissociation. (g) The difference charge density for $\text{C15-(IrRh)}_2\text{PrNdTb}$. (h) The kinetic energy barrier of the hydrogen spillover process. Reproduced with permission [61]. Copyright 2025, John Wiley and Sons.

Additionally, Huang et al. reported $\text{Pt}_4\text{FeCoNiCu}$ HEI nanocrystals with tunable degrees of chemical ordering [59]. In 1 M KOH, the highly ordered $\text{Pt}_4\text{FeCoNiCu}$ catalyst exhibited the best alkaline HER performance, requiring an overpotential of only 20 mV at 10 mA cm^{-2} and showing a Tafel slope of 31 mV dec^{-1} (Figure 14a). At a more practical potential of -0.2 V versus RHE, it delivered a mass activity of 71.9 $\text{A mg}_{\text{Pt}}^{-1}$, which is 2.1, 3.0, and 5.6 times higher than those of the partially ordered and disordered $\text{Pt}_4\text{FeCoNiCu}$ counterparts and commercial Pt/C, respectively (Figure 14b). These results clearly demonstrate that increasing the degree of ordering can substantially enhance alkaline HER kinetics. The improved activity is accompanied by markedly enhanced durability. Chronopotentiometry (CP) measurements showed that the highly ordered $\text{Pt}_4\text{FeCoNiCu}$ catalyst exhibited the smallest increase in potential during prolonged operation, indicating much slower deactivation than the partially

ordered, disordered, and Pt/C references (Figure 14c). This superior stability is further supported by the nearly overlapping HER polarization curves recorded before and after durability testing, confirming that the highly ordered catalyst retained its electrocatalytic performance with minimal degradation (Figure 14d). The ordering-dependent performance trend suggests that the chemically ordered intermetallic structure not only optimizes the electronic structure and adsorption behavior of reaction intermediates, but also enhances structural robustness under alkaline HER conditions. In this regard, stronger bonding within the ordered lattice and the higher thermodynamic stability of the intermetallic phase are likely key factors contributing to both the improved activity and long-term durability.

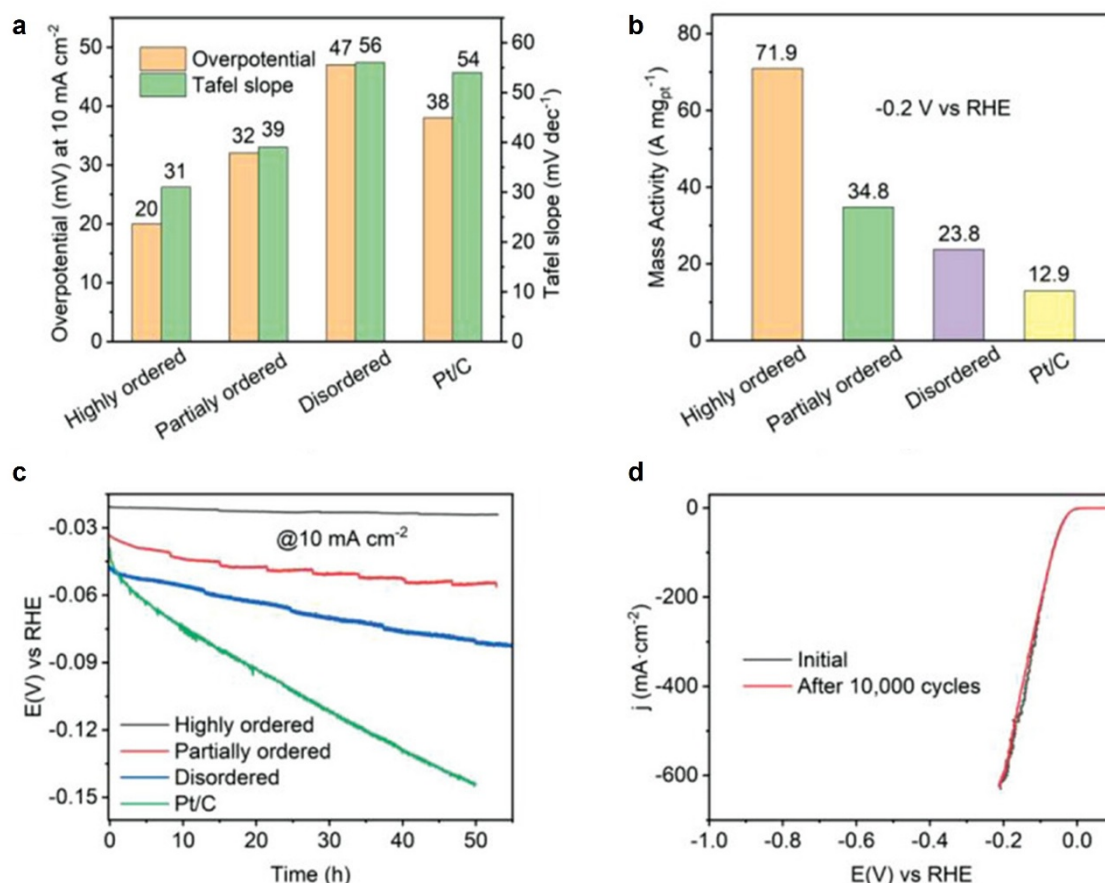


Figure 14. Electrocatalytic alkaline HER performance of Pt₄FeCoNiCu HEI nanocrystals in 1 M KOH. (a) Overpotentials at 10 mA cm⁻² and Tafel slopes of the catalysts. (b) HER mass activities of the catalysts. (c) CP curves of Pt₄FeCoNiCu catalysts. (d) HER polarization curves of the highly ordered HEI catalyst before and after durability testing. Reproduced with permission [59]. Copyright 2023, John Wiley and Sons.

4.2. Oxygen Reduction Reaction (ORR)

The ORR is the key cathodic half-reaction in fuel cells and metal-air batteries, but its practical application is limited by large overpotentials and intrinsically sluggish kinetics arising from complex proton-coupled electron-transfer processes [80]. ORR generally proceeds through either a direct four-electron pathway that reduces O₂ to H₂O or an indirect two-electron pathway involving H₂O₂ as an intermediate [81]. Although Pt-based catalysts remain the benchmark because of their high activity and selectivity, their widespread application is hindered by high cost, limited abundance, and insufficient long-term durability [39,82]. These challenges highlight the need for more efficient, durable, and economically viable alternatives to conventional Pt-based catalysts. One promising strategy to address these limitations is to introduce non-noble metals into Pt-based systems to create HEI structures. Such multicomponent design not only reduces noble-metal usage but also broadens the compositional space, strengthens interatomic interactions, and enables more flexible tuning of electronic structure and adsorption behavior.

In this context, Xia et al. reported well-ordered PtFeCoNiCuZn HEI nanocrystals with a face-centered tetragonal (FCT) intermetallic structure, which exhibited excellent ORR performance in acidic media [29]. The PtFeCoNiCuZn HEI delivered a mass activity of 2.403 A mg_{Pt}⁻¹ at 0.90 V versus RHE, which is approximately 19 times higher than that of commercial Pt/C (Figure 15a). Accelerated durability tests (ADTs) also showed that

the mass activity losses of PtFeCoNiCuZn HEI nanocrystals can be neglected after 10000 cycles of ADTs, indicating the high catalytic stability (Figure 15b). DFT calculations further revealed that the broadened d-band orbitals of the constituent elements enhance orbital overlap and interelement charge transfer, while also contributing to the generation of multiple catalytically active sites (Figure 15c–e). Furthermore, the charge density associated with the O–O bond in OOH at the Zn3 (Figure 15d) and Pt28 (Figure 15e) sites on the HEI (111) surface is significantly lower than that on Pt (111) (Figure 15c), indicating that the O–O bond can be more readily cleaved on the HEI surface. This suggests that the ordered multicomponent lattice creates a more favorable electronic environment for activating oxygenated intermediates and promoting the four-electron ORR pathway. This behavior is closely related to the modified electronic structure of the HEI catalyst. Because the electronic structure governs intermediate adsorption and reaction energetics, multielement incorporation and atomic ordering in PtFeCoNiCuZn broaden the d-band orbitals, enhance orbital overlap and charge transfer, and generate multiple active sites. As a result, both Pt and non-noble-metal sites can participate in ORR, leading to optimized adsorption behavior and improved catalytic kinetics. Building on this principle, Huang et al. demonstrated that highly ordered L1₀-Pt₄FeCoNiCu HEI nanocrystals have the best ORR mass activities than that of partially ordered HEAs, disordered HEAs, and Pt/C [59]. ADTs also proved that the highly ordered HEIs, characterized by increased configurational entropy, exhibit excellent ORR durability. These researches demonstrated that the ordered crystal structure with high entropy is more conducive to the selective exposure of active sites and the regulation of surface electronic structure, which significantly enhances the ORR catalytic activity.

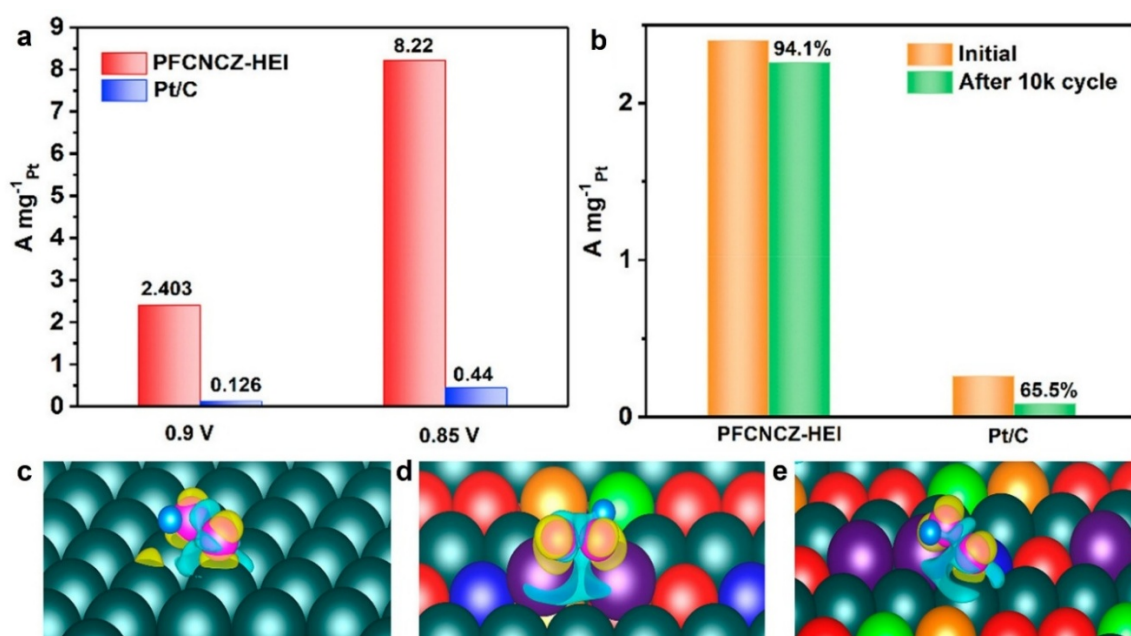


Figure 15. Electrocatalytic acidic ORR performance of PtFeCoNiCuZn HEI nanocrystals. (a) ORR mass activities of catalysts at 0.9 V vs RHE. (b) Mass activities before and after ADTs. Differential charge density of the OOH adsorbed system at (c) Pt (111), (d) the Zn3 site, and (e) the Pt28 site. Reproduced with permission [29]. Copyright 2023, American Chemical Society.

Furthermore, Luo et al. reported L1₂-ordered PdFeCoNiCu HEI nanocrystals, denoted as OHEA, which exhibited remarkable alkaline ORR activity with a high diffusion-limited current density (Figure 16a) [65]. At 0.9 V versus RHE, the OHEA catalyst delivered a mass activity of 2.037 $\text{mA } \mu\text{g}_{\text{Pd}}^{-1}$, which is substantially higher than those of disordered FCC PdFeCoNiCu HEA nanocrystals (DHEA) and commercial Pt/C (Figure 16b). In addition, OHEA showed a lower Tafel slope and superior durability, highlighting the beneficial effect of structural ordering on both ORR kinetics and stability. DFT calculations further revealed clear electronic-structure differences between OHEA and DHEA. In OHEA, the Co 3d orbitals are located closer to the Fermi level than in DHEA (Figure 16c,e), suggesting a more favorable electronic configuration for electrocatalytic electron transfer. Moreover, O₂ adsorption analysis showed evident orbital interaction between the catalyst and adsorbed oxygen on both materials, as reflected by the upshift of the O₂ s and p orbitals. Compared with DHEA, however, the Co 3d states in OHEA exhibit a more favorable shift near the Fermi level, which can facilitate electron transfer and stabilize adsorbed oxygen intermediates during ORR (Figure 16d, f). These results suggest that chemical ordering

not only modifies the electronic structure of HEI nanocrystals but also improves intermediate adsorption and charge-transfer behavior, thereby contributing to the enhanced alkaline ORR performance of OHEA.

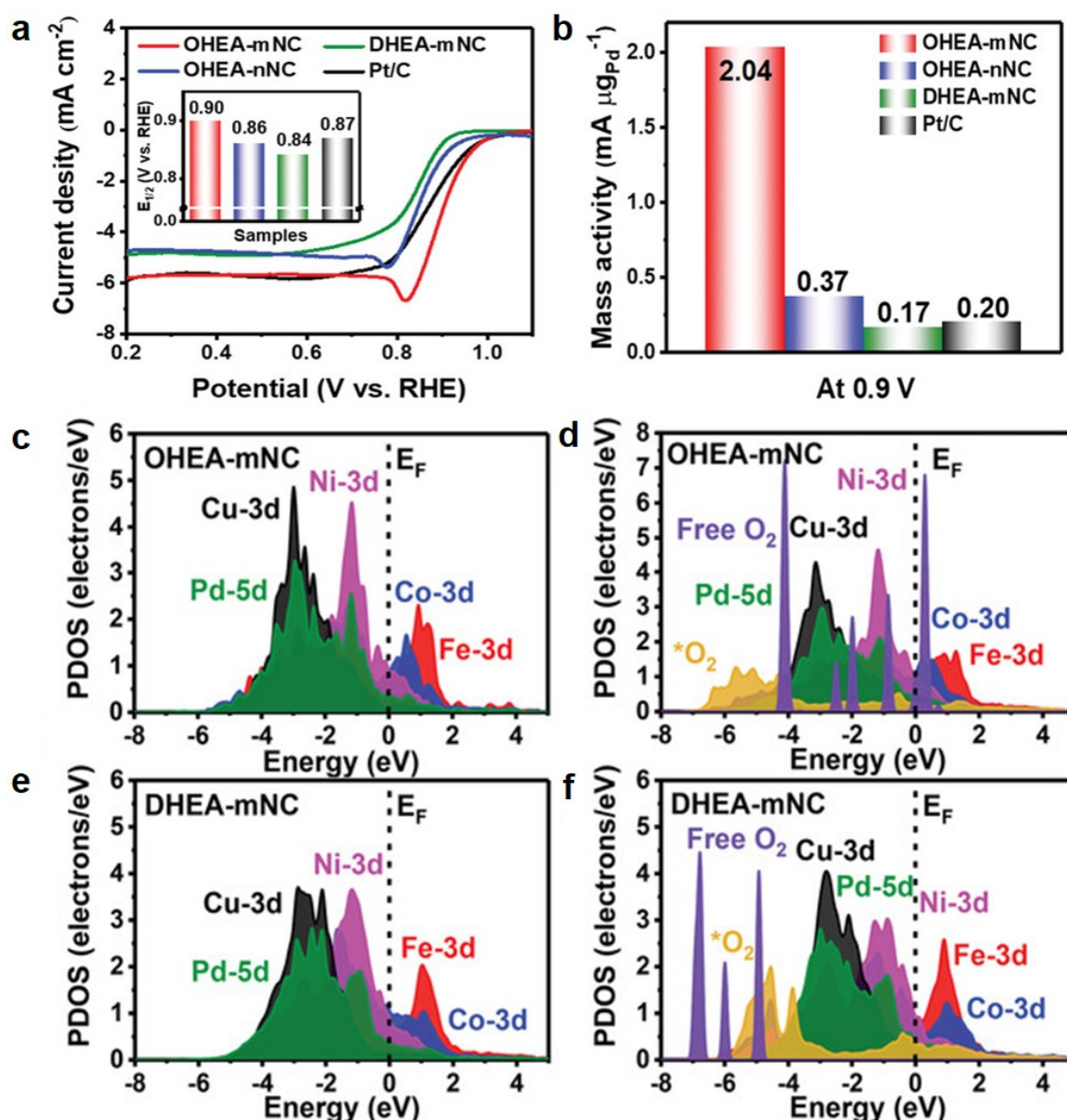


Figure 16. Electrocatalytic alkaline ORR performance of PdFeCoNiCu HEI nanocrystals. (a) Linear sweep voltammetry (LSV) curves. (b) Mass activities. (c,e) PDOSs of clean OHEA and DHEA, respectively. (d,f) PDOSs of O_2 -adsorbed OHEA and DHEA, respectively. Reproduced with permission [65]. Copyright 2022, John Wiley and Sons.

4.3. Ethanol Oxidation Reaction (EOR)

The EOR is a representative anodic reaction in fuel cells, and ethanol is considered a promising liquid fuel because of its high energy density and ease of storage and handling. EOR can proceed through two major pathways [83]. The C1 pathway corresponds to complete oxidation of ethanol to CO_2 , involving C-C bond cleavage and transfer of 12 electrons, and therefore enables maximal energy extraction. By contrast, the C2 pathway leads to incomplete oxidation products while preserving the C-C bond, which greatly limits the overall energy-conversion efficiency. In practice, most catalysts tend to favor the C2 pathway, and their performance is further compromised by sluggish C-C bond cleavage and poisoning from strongly adsorbed intermediates, especially CO. Therefore, a central challenge in EOR catalysis is to develop Pt-based catalysts with enhanced C-C bond-breaking ability, improved anti-poisoning capability, and higher overall activity [84].

In this context, Wang et al. reported structurally ordered FCC PtRhFeNiCu HEI nanocrystals that exhibited markedly improved EOR performance relative to the corresponding HEA nanocrystals and Pt/C [25]. The HEIs showed

higher mass activity, stronger CO tolerance, and superior durability, together with the lowest onset potential for CO removal among the compared catalysts. When used as the anode catalyst in a high-temperature polybenzimidazole-based direct ethanol fuel cell, the PtRhFeNiCu HEIs delivered a peak power density of 47.50 mW cm^{-2} , which is 2.97 times that of Pt/C (Figure 17a). More importantly, online gas chromatography revealed that the HEI nanocrystals generated the highest amount of C1 products, indicating a substantially enhanced ability to cleave the C-C bond and promote the complete oxidation pathway (Figure 17b). DFT calculations are further performed to clarify the origin of the superior EOR performance of the HEIs. The ordered HEI structure is thermodynamically more stable than the corresponding HEA structure (Figure 17c,d). In addition, the local density of states indicates that the d-band center of the HEIs is shifted farther away from the Fermi level than that of the HEAs, which helps weaken CO adsorption and thereby mitigate CO poisoning (Figure 17e). The calculated reaction-energy profiles further show that the HEIs exhibit a lower potential-limiting step for the electrochemical process and, more importantly, a substantially reduced energy barrier for C-C bond cleavage compared with the HEAs (Figure 17f,g). This result suggests that the ordered atomic arrangement of HEIs can facilitate both the electrochemical oxidation process and the key C-C bond-breaking step by altering the adsorption configuration of crucial intermediates.

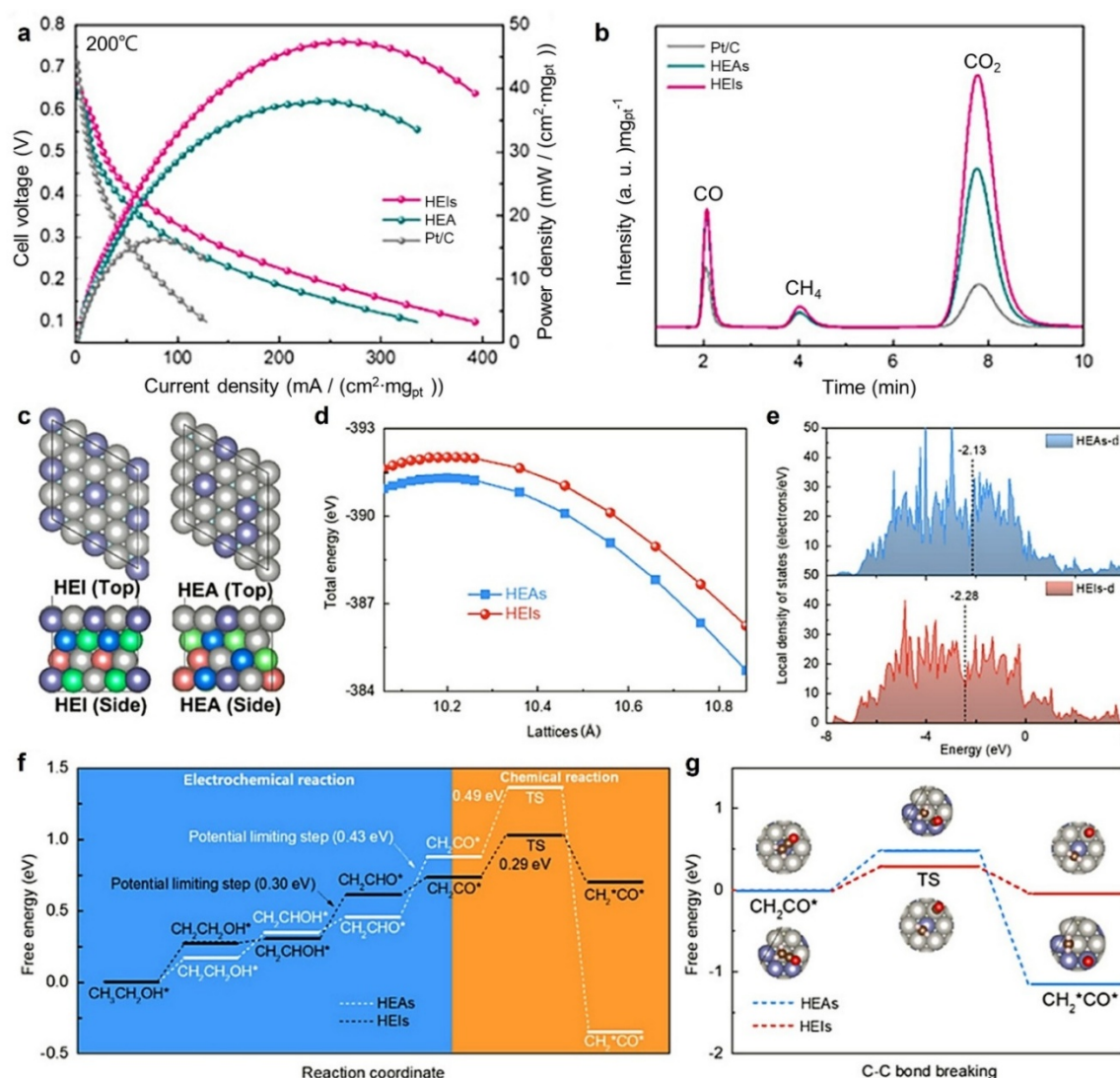


Figure 17. EOR performance and mechanistic analysis of PtRhFeNiCu HEI nanocrystals. (a) Polarization and power density curves obtained at 200 °C for the HEIs, HEAs, and Pt/C in a high-temperature polybenzimidazole-based direct ethanol fuel cell. (b) Gas-phase product distribution of ethanol electrooxidation measured by online gas chromatography. (c) Structural models of HEIs and HEAs viewed from the top and side. (d) Total energies of HEIs and HEAs with different lattice constants. (e) Local density of states of HEAs-d orbitals and HEIs-d orbitals. (f) Free-energy profiles of the EOR process on HEAs and HEIs, including electrochemical and chemical steps. (g) Energy barriers for C-C bond cleavage on HEAs and HEIs, together with the corresponding intermediate configuration. Reproduced with permission [25]. Copyright 2022, John Wiley and Sons.

5. Conclusions and Outlook

This review brings together recent progress in HEI nanocrystals, covering their formation principles, synthetic strategies, and electrocatalytic applications, and illustrates how atomic ordering and multicomponent complexity can be integrated within a single material platform. Compared with conventional disordered HEA nanocrystals and simpler intermetallic systems, HEI nanocrystals offer a distinctive opportunity to combine compositional diversity with structurally defined atomic arrangements, thereby creating new possibilities for regulating local coordination environments, heteroatomic interactions, and surface reactivity. In this Review, we have highlighted the thermodynamic and kinetic factors governing phase ordering in multicomponent nanoscale systems, summarized the key synthetic routes for accessing HEI nanocrystals with controlled structures, and discussed representative electrocatalytic applications in which structure, composition, and dynamic evolution jointly determine performance. We have also emphasized that the electrocatalytic advantages of HEI nanocrystals arise not only from their ordered frameworks, but also from the interplay between atomic occupation, surface structure, and reaction-dependent structural evolution. Taken together, these advances establish HEI nanocrystals as an emerging class of catalysts with considerable potential for achieving high activity, improved selectivity, and enhanced structural robustness.

Despite rapid progress in the development of HEI nanocrystals, the field remains at an early stage, and several key questions are still unresolved. In this regard, four critical directions are expected to shape future advances in the field.

5.1. Understanding the Growth Mechanisms of HEI Nanocrystals

A deeper understanding of the growth mechanisms of HEI nanocrystals is essential. Their formation often involves multiple precursors with distinct reduction potentials [22], which can lead to different reduction rates and thus increase the likelihood of phase separation, compositional heterogeneity, or kinetically trapped intermediates during synthesis. Beyond precursor conversion, the subsequent nucleation and growth processes may also involve extensive atomic diffusion and progressive structural transformation from disordered alloys to ordered intermetallic phases. A comprehensive understanding of these coupled processes is therefore required to reveal how composition, crystal phase, and atomic ordering evolve throughout synthesis. In particular, an important unresolved question is when and how atoms diffuse into specific crystallographic positions. It remains unclear whether preferred atomic occupation is established at the early nucleation stage, when the initial atomic framework is first created, or whether it mainly develops during later growth and post-growth rearrangement through thermally activated diffusion and ordering. Distinguishing between these possibilities is critical, because they imply fundamentally different synthetic control strategies. If atomic site occupation is largely determined during nucleation, then precursor conversion kinetics, local compositional fluctuations, and the earliest cluster structures may play a dominant role. By contrast, if ordering emerges primarily during growth or subsequent annealing, then diffusion barriers, interatomic interactions, and the balance between kinetic trapping and thermodynamic equilibration become more decisive.

This issue is further complicated by the fact that HEI nanocrystals contain multiple elements with different atomic sizes, bonding preferences, and mobilities. As a result, atomic diffusion is unlikely to occur uniformly across all elements, and some species may redistribute more readily than others during the transition from a disordered alloy to an ordered intermetallic structure. Understanding which elements migrate first, which atomic pairs preferentially form ordered motifs, and how these processes depend on temperature, particle size, and local chemical environment will be essential for clarifying the full growth pathway. Such insight would also help determine whether ordered HEI phases arise through direct nucleation of an ordered structure, through a disordered-to-ordered transformation during growth, or through post-synthetic atomic rearrangement after particle formation. In this regard, *in situ* characterization techniques will be particularly valuable. For example, *in situ* TEM [85] can track the evolution of particle size, morphology, composition, crystal phase, and structural ordering, while *in situ* spectroscopic methods such as XAS can provide complementary information on local coordination environments, oxidation states, and interatomic bonding [14,23]. When combined with time-resolved diffraction and theoretical modeling, these approaches can offer a more complete picture of the formation pathways of HEI nanocrystals. Ultimately, such mechanistic understanding will be essential for identifying how kinetic and thermodynamic parameters can be deliberately tuned to control precursor reduction, nucleation pathways, atomic diffusion, and final ordering behavior, thereby enabling the rational synthesis of HEI nanocrystals with targeted structures and properties.

5.2. Achieving Facet Control in HEI Nanocrystals

Facet control is critically important for catalysis because the exposed crystallographic planes determine the atomic arrangement, coordination environment, and electronic structure of active sites, thereby strongly influencing catalytic activity, selectivity, and durability. However, precise facet control in HEI nanocrystals remains highly challenging because of constraints arising from both thermodynamics and kinetics. Unlike simple alloys, HEIs require the simultaneous incorporation of five or more elements into a long-range ordered lattice, which greatly complicates surface and structural regulation. One major challenge is the thermodynamic instability of high-energy facets, which are prone to disappear during the high-temperature annealing typically required to drive the disorder-to-order transition. In addition, differences in the reduction potentials and reaction kinetics of multiple metal precursors often result in unbalanced deposition, phase separation, or loss of crystallographic uniformity, thereby hindering the formation of well-defined facets.

To address these challenges, several advanced synthetic strategies have been explored to regulate the surface structure of HEI nanocrystals. One effective approach is seed-mediated epitaxial growth combined with the dropwise addition of metal precursors, where preformed nanocrystal seeds with well-defined facets act as structural templates and the controlled precursor supply helps direct multicomponent deposition while preserving the desired symmetry [22,23,56,86–88]. Without such structural templates, facet control would need to rely more strongly on surface capping agents to direct crystal growth [89]. However, this remains particularly difficult in HEI systems because the chemically heterogeneous surface gives rise to highly complex ligand-surface interactions, and a single capping ligand rarely provides sufficiently selective and uniform adsorption across diverse atomic environments to stabilize a target crystallographic plane. In this regard, DFT calculations may serve as an important tool for screening and designing capping agents with appropriate facet-selective binding behavior for multicomponent HEI surfaces.

5.3. Probing Atomic-Scale Structures and Interatomic Interactions in HEI Nanocrystals

Atomic-scale characterization of HEI nanocrystals remains highly challenging because of their intrinsic structural and chemical complexity. Unlike conventional binary intermetallics, HEIs incorporate five or more principal elements into a long-range ordered lattice, generating highly heterogeneous local environments. Structural identification therefore requires not only verification of intermetallic ordering but also clarification of how multiple elements occupy specific atomic sites within individual nanocrystals. A major challenge is that conventional characterization techniques often lack sufficient sensitivity to resolve such local complexity. XRD mainly provides ensemble-averaged structural information and is therefore insufficient to distinguish local chemical ordering, site occupancy, or subtle compositional heterogeneity. Although HAADF-STEM offers much higher spatial resolution, direct identification of individual elements remains difficult when constituent atoms have similar atomic numbers. This limitation becomes even more severe in the presence of anti-site defects, vacancies, and local strain, making it difficult to determine whether the disorder-to-order transition occurs uniformly throughout the nanocrystal.

Elucidating interatomic interactions in HEIs is equally demanding. Their catalytic properties are governed by the coupled effects of local coordination, interatomic bonding, lattice distortion, and electronic structure modulation. In particular, the cocktail effect is often intertwined with local lattice distortion arising from atomic size mismatch and chemical heterogeneity, making it difficult to isolate the contribution of each factor. Future progress will require the integration of complementary methods that probe both local structure and electronic properties. XAS analysis, particularly EXAFS, can reveal local coordination environments and bond distances beyond the reach of diffraction methods. When combined with atomic-resolution electron microscopy, DFT calculations, and machine-learning-assisted simulations, these approaches can establish clearer correlations between local atomic configurations, electronic structure, and catalytic behavior, thereby guiding the rational design of HEI nanocrystals with optimized catalytic functionalities.

5.4. Elucidating Catalytic Mechanisms, Active Sites, and Interatomic Synergies in HEI Nanocrystals

Deciphering the catalytic mechanisms of HEI nanocrystals presents an unprecedented challenge due to the high configurational entropy and the vast diversity of potential adsorption sites on their surfaces. Unlike traditional catalysts with uniform active centers, the ordered yet multi-component nature of HEIs creates a complex “site-specific” landscape where the catalytic activity is governed by the precise atomic positions of five or more elements. A primary obstacle is the identification of the true active sites among countless possible atomic combinations (e.g., bridge, hollow, or top sites involving different metal neighbors). The structural ordering in HEIs imposes a sublattice constraint that creates unique coordination environments, yet the inherent chemical

randomness within these sublattices leads to a distribution of adsorption energies rather than a single value. This scaling relation break makes it difficult to pinpoint whether the observed activity stems from a specific high-symmetry site or from low-coordinated step sites and anti-site defects that are statistically prevalent in high-entropy systems.

Furthermore, probing the interatomic interactions that modulate these active sites requires sophisticated operando techniques to resolve how the electronic structure evolves during a reaction. The cocktail effect in HEIs implies that the adsorption strength of a reactant is not only determined by the atom it directly binds to but is also heavily tuned by the long-range electronic influence of neighboring atoms through orbital hybridization and charge redistribution. A major challenge is to decouple the geometric strain effect arising from atomic size mismatch from the ligand effect originating from electronic modulation at specific atomic positions. Future research must prioritize site-specific DFT simulations to resolve how local atomic configurations regulate catalytic behavior in HEI nanocrystals [90]. By mapping the d-band center shifts across different atomic coordinates, DFT calculations can help identify active site ensembles and clarify how synergistic interactions among diverse elements optimize the binding of reaction intermediates, ultimately providing a foundation for the rational design of HEI catalysts with superior atom-utilization efficiency.

Despite the rapid progress summarized in this Review, current HEI nanocrystals remain at an early stage with respect to practical and industrial implementation. Most reported synthetic methods still rely on precisely controlled rapid heating/quenching processes, thermally driven conversion, or solution-phase protocols, which are effective for mechanistic studies but remain difficult to translate into large-scale, reproducible production. In addition, although many HEI catalysts exhibit promising durability in laboratory-scale electrochemical tests, their long-term stability under realistic operating conditions, such as high current density, fluctuating potentials, and elevated temperatures, remains insufficiently evaluated. Another key consideration is the cost-performance balance, for which the intrinsic compositional diversity of HEI nanocrystals offers a distinct advantage by integrating noble metals with non-noble or earth-abundant elements in ordered multicomponent structures. Such noble-metal-diluted compositions can not only lower material cost but also create synergistic electronic, strain, and ensemble effects that contribute to enhanced activity and durability. Future studies should therefore place greater emphasis on quantitatively evaluating catalytic performance in relation to total catalyst cost, noble-metal content, mass loading, and device-level output, rather than relying only on activity normalized by geometric area or electrochemically active surface area. Addressing these challenges will be essential for moving HEI nanocrystals from proof-of-concept catalysts toward practically relevant catalytic materials.

Collectively, these four directions highlight the central challenges and opportunities that will define the next stage of HEI nanocrystal research. Future advances will rely on the tight integration of controllable synthesis, atomic-scale characterization, operando mechanistic analysis, and predictive theoretical modeling. By bridging these areas, researchers will be better positioned to uncover the fundamental principles governing atomic ordering, facet evolution, local electronic structure, and active site formation in HEIs. Such insight will provide a more rigorous foundation for the rational design of HEI nanocrystals with enhanced activity, selectivity, durability, and atom-utilization efficiency, ultimately broadening their impact in catalysis and sustainable energy technologies.

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Synergistic Effects of Topographical Guidance and Electrical Stimulation on Modulation of BMSC Behaviors Using Electrospun Nanofibers Decorated with Nanoscale Protrusions

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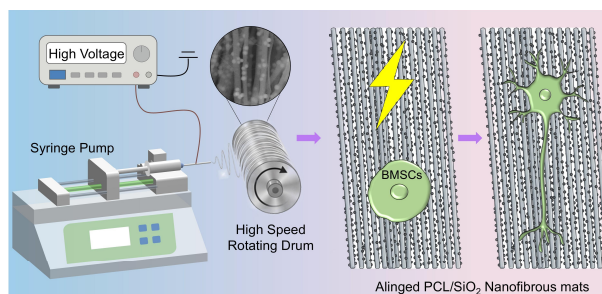
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Abstract: Peripheral nerve injury (PNI) often leads to disability and chronic pain, with limited options available to promote regeneration and functional recovery. Bone marrow mesenchymal stem cells (BMSCs) are considered promising candidates for cell therapy in PNI repair. However, the effective induction of BMSCs towards neurogenesis and the directed migration of cells remain challenging. Here, we constructed a biomimetic environment by combining uniaxially aligned polycaprolactone (PCL) nanofibers containing silica nanoparticles (SiO₂ NPs) with electrical stimulation (ES). A proper portion of SiO₂ NPs was uniformly integrated into uniaxially aligned PCL nanofibers to create nanoscale protrusions on fiber surfaces. Such fibrous mats showed uniaxially aligned morphology, uniform fiber diameter, and improved wettability. BMSCs were then cultured on both the nanofibers with smooth surfaces (PCL) or those decorated with nanoscale protrusions (PCL/SiO₂), followed by treatment with or without ES, with glass slides used as a control. All the fibrous mats showed good cell viability, and the uniaxially aligned fibers induced better cell extension and alignment in comparison to the control group. Both the contact guidance provided by the nanoscale protrusions on fiber surfaces and the biochemical signal from ES contributed to BMSC differentiation, with a combination of both promoting the greatest differentiation into neural-like cells. Immunofluorescence micrographs demonstrated significantly increased expression of NF200 and S100β, along with a higher proportion of NF200-positive cells compared with S100β, indicating the preferred differentiation of BMSCs to neuron-like cells under such conditions. Additionally, in migration assays the greatest number and longest migration distance of BMSCs were exhibited on the PCL/SiO₂ nanofibers with ES. These studies offer valuable strategies for the manipulation of stem cell behavior in PNI repair.



Keywords: bone marrow mesenchymal stem cells; nanofibers; nanoscale protrusions; electrical stimulation; neural differentiation

1. Introduction

Bone marrow mesenchymal stem cells (BMSCs) are regarded as highly promising cell sources for neural tissue engineering owing to their self-renewal capacity, multilineage differentiation potential, relative ease of harvest, and low immunogenicity [1,2]. Under appropriate microenvironmental cues, BMSCs can differentiate toward neural lineages, including neuron-like and Schwann cell-like phenotypes, secrete various neurotrophic factors, and modulate the local immune and inflammatory microenvironment via paracrine signaling [3–5].



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However, current strategies for inducing neurogenic differentiation of BMSCs mainly rely on exogenous supplementation of neurotrophic factors or pharmacological agents. These approaches are costly and often associated with suboptimal induction efficiency and limited stability of the differentiated phenotype. Thus, there is a need to develop alternative strategies such as using biophysical and biochemical cues to more effectively direct the fate of BMSCs. Increasing evidence demonstrates that cells can actively sense and respond to biophysical and mechanical factors [6,7]. Biophysical factors are considered one of the most important physical guidance cues that adjust cell behaviors including adhesion, morphology, migration, and differentiation [8–10]. Combined biophysical and biochemical factors have also been widely used to regulate cell behaviors [11–13].

After peripheral nerve injury (PNI), the directional migration of stem cells toward the injury site and their differentiation into neuron-like cells are critical for nerve regeneration and functional recovery [14]. In this regard, constructing a biomimetic microenvironment that integrates both biophysical and biochemical cues represents a key strategy for precisely regulating the adhesion, alignment, migration, and neurogenic differentiation of BMSCs. This approach facilitates the enrichment of both neuron-like and Schwann cell-like phenotypes at the injury site. Among the different types of biomaterials, nanofibrous scaffolds can closely mimic the architecture of native nerve fiber bundles and provide topographical guidance cues for neural cell growth [15]. By adjusting polymer composition and electrospinning parameters, it is possible to modulate fiber orientation, diameter, and surface secondary structures, as well as to introduce biochemical functionalization [16–18]. This enables the construction of engineered nerve regeneration microenvironments that provide multiple physical, chemical, and biological cues to guide neural cell behavior and stem cell differentiation [19,20]. Polycaprolactone (PCL) is a synthetic polymer approved by the U.S. Food and Drug Administration for *in-vivo* implantation. Electrospun PCL fibers often offer excellent biocompatibility, processability, and biodegradability, which have been widely employed in preparing tissue-engineered scaffolds [20,21]. By controlling the collector during the electrospinning process, uniaxially aligned PCL nanofibers can be obtained to mimic the linear arrangement of native nerve fascicles, thereby providing physical guidance for Schwann cell (SCs) migration and facilitating the formation of Büngner bands [22,23]. In addition, neural cells are highly sensitive to the nanoscale topography on fiber surfaces. Our previous study showed that silica nanoparticles (SiO₂ NPs), a commonly used inorganic material with controllable particle size and surface chemistry, can be readily incorporated into electrospun fibers to generate nanoscale protrusions on fiber surfaces and enable further functional modification [24,25]. The introduction of “cell climbing stone-like” structures provided by protrusions on the surfaces of aligned PCL fibers is expected to provide biomimetic cues more closely resembling axonal growth cones, thereby facilitating directed cell migration and axonal extension.

In addition to topographical guidance, external electrical stimulation (ES) usually serves as a clinically relevant and controllable physical modulation strategy, which exhibits clear potential in promoting peripheral nerve regeneration and attenuating muscle atrophy [26,27]. ES can modulate neuronal membrane potentials and ion channel activity, regulate intracellular Ca²⁺ signaling, cAMP levels, and related pathways, thereby promoting axonal sprouting and extension [28–32]. ES also influences SCs proliferation, migration, and the expression of myelin-associated genes, together with promoting a pro-regenerative microenvironment by upregulating neurotrophic factors such as brain-derived neurotrophic factor, nerve growth factor, and glial cell line-derived neurotrophic factor [32–34]. In terms of the modulation of stem cell behaviors, ES shows advantages in regulating stem cell proliferation, migration, and lineage commitment. Several studies have demonstrated the positive effects of ES on lineage-specific differentiation of BMSCs to neural, osteogenic, and chondrogenic lineages [35–38]. Compared with the induction of BMSC differentiation solely based on structural cues, the integration of ES offers temporal controllability, adjustable parameters, and compatibility with nerve conduits, providing an important complement for realizing “structure–function integrated” nerve repair [39]. The combined effects of structural guidance, provided by the aligned nanofibers with nanoscale protrusions, together with the ES treatment on BMSCs neurogenic differentiation and migration, are worth fully elucidating.

In this study, we designed and fabricated SiO₂ NPs-doped PCL (PCL/SiO₂) nanofibrous mats by electrospinning. After systematically characterizing their morphology, wettability, and chemical structure, BMSCs were cultured on the nanofibrous mats, with or without ES, to evaluate cytocompatibility, cell morphology, neurogenic differentiation, and migratory behavior. Neurofilament 200 (NF200) and anti-S100 Beta (S100β) were selected as representative markers of neuronal axons and SCs, respectively, to assess lineage-specific differentiation. By integrating topographical guidance from nanoscale protrusions with ES, this work aims to elucidate the synergistic effects of combined biophysical cues on directing BMSC fate, and to provide a rational design strategy based on cell therapy for PNI repair.

2. Experimental Section

2.1. Materials

Ammonium hydroxide aqueous solution and ethanol absolute were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tetraethyl orthosilicate (TEOS) and Hexafluoroisopropanol (HFIP) were obtained from Macklin (Shanghai, China). Polycaprolactone (PCL, $M_w = 8 \times 10^4$) and poly-D-lysine (PDL) were supplied by Sigma-Aldrich (Shanghai, China). Electrospinning equipment was bought from Beijing Ucalery Technology and Development Co., Ltd. (Beijing, China). Fetal bovine serum (FBS), low glucose dulbecco's modified eagle medium and phosphate-buffered saline (PBS) were procured from Wuhan Procell Biotechnology Co., Ltd. (Wuhan, China). Specialized growth media design for mesenchymal stem cells was procured by Xirui Infinity Biotechnology Co., Ltd. (Hangzhou, China). Penicillin-Streptomycin Solution was obtained from Biosharp (Beijing, China). 4% paraformaldehyde, mounting medium, antifade (with DAPI), Triton X-100, and bovine serum albumin (BSA) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Phalloidin-iFluor 488 was bought from Abcam (Cambridge, UK). Osteogenic, adipogenic, and chondrogenic differentiation kits were purchased from Haixing Biosciences (Jiangsu, China). NF200 Polyclonal antibody (NF200) and anti-S100 Beta Monoclonal antibody (S100 β) were procured from Proteintech (Wuhan, China).

2.2. Preparation of PCL/SiO₂ Nanofibrous Mats

The Stöber process was used to synthesize SiO₂ NPs [40,41]. Briefly, 5 mL of ammonium hydroxide aqueous solution and 12 mL of deionized water were added to a beaker and stirred using a magnetic stirrer for 10 min to obtain stock solution A. Stock solution B was prepared by mixing 3 mL TEOS and 47 mL absolute ethanol. Stock solutions A and B were then mixed uniformly, and the reaction was allowed to proceed at room temperature for 2 h. Subsequently, the SiO₂ NPs were obtained by centrifugation, washing, and subsequent drying. The morphology of the particles was analyzed using a scanning electron microscope (Regulus8100, Hitachi, Tokyo, Japan). The SiO₂ NPs were then investigated by dynamic light scattering (DLS) in triplicate (90Plus PALS, Brookhaven Instruments Corporation, New York, USA).

According to our previous study, a dispersion of SiO₂ NPs with concentrations of 6% (w/v) was prepared by ultrasonication in HFIP for 2 h uniformly [24]. PCL was dissolved in suspension to formulate an electrospinning solution with a final concentration of 10 wt%. The prepared PCL/SiO₂ aligned nanofibers were fabricated by electrospinning, with a high-speed rotating drum employed as the collector. The electrospinning parameters used included an applied voltage of 15 kV, collection distance of 18 cm, and rotating speed of the drum at 3000 rpm. The morphology of the fibers was analyzed by the scanning electron microscope. The distribution of SiO₂ NPs within the fibers was observed using a transmission electron microscope (TEM) (HT7700, Hitachi, Tokyo, Japan). Fourier transform infrared spectroscopy (FTIR) (Nicolet iS50, Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate whether SiO₂ NPs were successfully encapsulated. The hydrophilicity and hydrophobicity of the nanofibrous mats were measured at room temperature using a contact angle analyzer (Theta Lite, Biolin Scientific, Gothenburg, Sweden).

2.3. In Vitro Experiments

Different samples were placed in 24-well or 6-well plates and subjected to plasma sputtering for 30 s, followed by sterilization with 75% ethanol for 4 h and subsequent UV irradiation for 30 min. The samples were then coated with PDL overnight. After removing the PDL, the samples in each well were rinsed with PBS three times. Direct current ES was used in this study at 0.14 mA for 15 min, once daily. The designated experimental groups were as follows: Control (C), Control with ES (CE), PCL (P), PCL with ES (PE), PCL/SiO₂ (S), and PCL/SiO₂ with ES (SE).

2.3.1. Characterization of BMSCs

Morphological Observation: BMSCs at passage 3 (P3) were cultured in specialized growth media. When the cells reached approximately 80–90% confluence, their morphology was examined and photographed under an inverted light microscope.

Osteogenic differentiation: BMSCs at P3 were seeded at 4×10^4 cells/well in 24-well plates. When the cells reached approximately 90–100% confluence, the medium was replaced with osteogenic induction medium and cultured for 21 days, with the medium changed every 3 days. After fixation with 4% paraformaldehyde, mineralized nodules were stained with Alizarin Red S solution for 5 min at room temperature and observed under a light microscope.

Adipogenic differentiation: BMSCs at P3 were seeded at 4×10^4 cells/well in 24-well plates. When the cells reached approximately 80–90% confluence, adipogenic induction medium was added. After 3 days, the medium was changed to adipogenic maintenance medium for 1 day, followed by another 3-day induction cycle. This cycle was repeated for 14 days. Cells were fixed with 4% paraformaldehyde for 30 min and stained with Oil Red O working solution (stock:saline = 3:2) for 30 min.

Chondrogenic differentiation: BMSCs at P3 were seeded at 4×10^5 cells/well in 24-well plates. After 3 h of attachment, 200 μ L of medium was added. The cells were cultured for 14 days with medium changes every 3 days. Cells were fixed with 4% paraformaldehyde for 30 min, then stained with Alcian Blue solution for 30 min in the dark. All staining results were observed under an inverted light microscope.

2.3.2. Cell Viability

The CCK-8 assay was used to assess the biocompatibility of various nanofibrous mats with or without ES after 3 and 7 days. Low glucose Dulbecco's modified eagle medium supplemented with 1% (v/v) penicillin-streptomycin solution and 10% (v/v) FBS was used to culture BMSCs. BMSCs were co-cultured with the different material groups in 24-well plates with 2×10^4 cells/well. After cell attachment, ES was applied, and the absorbance of the supernatant was measured at 450 nm by a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany).

2.3.3. Cell Morphology

BMSCs were seeded into 24-well plates with 1×10^4 cells/well in 400 μ L of specialized growth media. After cell adhesion, ES was applied. The media was changed every other day, and ES was continuously applied for 3 days. After the media was removed, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 1% BSA for 1 h. The cells were then stained with Phalloidin-iFluor 488 followed by antifade (with DAPI) and imaged on an inverted fluorescence microscope.

2.3.4. Cell Differentiation

BMSCs were seeded into the 24-well plates at a density of 5×10^3 cells/well in 400 μ L of specialized growth media. After cell adhesion, ES was applied continuously for 7 days, and the media was changed every other day. At 7 days post seeding, the samples were fixed, permeabilized, and blocked following the same protocol as described in Section 2.3.2. Cells were subsequently stained with NF200, S100 β , and antifade (with DAPI), respectively, for observation. ImageJ software was used to quantify the fluorescence intensity of NF200 and S100 β . Briefly, fluorescence images were imported into ImageJ, and identical thresholding and analysis settings were applied to all groups. Regions of interest (ROIs) were defined for each image, the mean fluorescence intensity was measured after background subtraction, and the values of experimental groups were normalized to the average intensity of the control group. $I_{sample,i}$ is the mean fluorescence intensity of an individual experimental group sample, $I_{control,mean}$ is the average intensity of all control samples, and n is the number of experimental samples. This calculation gives the mean relative intensity normalized to the control group. The relative fluorescence intensity was calculated by normalizing the mean fluorescence intensity of each sample to the average fluorescence intensity of the control group, according to the following formula, as well as the proportion of differentiated cells. The proportion of differentiated cells (NF200-positive or S100 β -positive cells) was calculated.

$$\text{Relative intensity} = \frac{1}{n} \sum_{i=1}^n \frac{I_{sample,i}}{I_{control,mean}}$$

2.3.5. Cell Migration

PCL and PCL/SiO₂ fibrous mats were placed in 6-well plates, respectively, with glass slides as a control. BMSCs were seeded at a density of 1×10^5 cells/well on the left side of each sample with a 0.3-mm wide area produced using a polydimethylsiloxane (PDMS) block. After the cells had adhered to the substrate, the PDMS block was removed to allow the migration of cells into a region of 1 cm, and the media was replaced with specialized growth media to low-glucose DMEM containing 1% FBS. At 3 days post seeding and daily ES, the cells were fixed, permeabilized, blocked, and stained with Phalloidin-iFluor 488 and antifade (with DAPI) following the same procedure as described in Section 2.3.2. The micrographs were captured under the inverted fluorescence microscope, and the total number of migrated cells as well as the cell counts in each migration region were quantified using the ImageJ software.

2.4. Statistical Analysis

All experiments in this study were conducted with at least three replicates. All data were processed and analyzed using GraphPad Prism 10.1.2 (GraphPad Software, San Diego, CA, USA) and ImageJ (National Institutes of Health, Bethesda, MD, USA), and results were presented as mean $\pm$ standard deviation (SD). Student's *t*-test was used for comparisons between two groups only. For experiments involving the six groups C, CE, P, PE, S, and SE and two independent variables, a two-way ANOVA was applied. Statistical significance is presented as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results and Discussion

3.1. Characterization of SiO₂ NPs and PCL/SiO₂ Nanofibrous Mats

SiO₂ NPs were synthesized via the Stöber method. The scanning electron microscopy (SEM) images in Figure 1A show that the SiO₂ NPs have smooth surfaces, good dispersibility, and a uniform size distribution around 200 nm. DLS analysis in Figure 1E shows that the hydrodynamic diameter of the SiO₂ NPs was 241.29 ± 21.87 nm. This discrepancy arises because DLS measures the hydrodynamic diameter of particles in solution, including the hydration layer surrounding the nanoparticles, whereas SEM images show the physical diameter of dry particles under vacuum. Such differences are commonly observed in nanoparticle characterization. Our previous study demonstrated that PCL microfibers engraved with nanoscale grooves around 200 nm showed the best performance in inducing neurite extension and SCs migration [42]. Additionally, we integrated SiO₂ NPs with biocompatible PCL to fabricate hybrid nanofibers featuring surface protrusions as secondary structures with distinct sizes and SiO₂ concentrations [24]. The results also confirmed that the SiO₂ NPs with a diameter of around 200 nm demonstrated the most obvious influence on the migration of SCs and neural stem cells and contributed to the axonal extension of neurons. These results are mainly attributed to the modulation of surface roughness and hydrophilicity of SiO₂ NPs, which may enhance cell adhesion and cytoskeletal remodeling. Meanwhile, 200 nm protrusions match the dimension of growth cones, thereby reinforcing cell–material interactions and amplifying guidance effects.

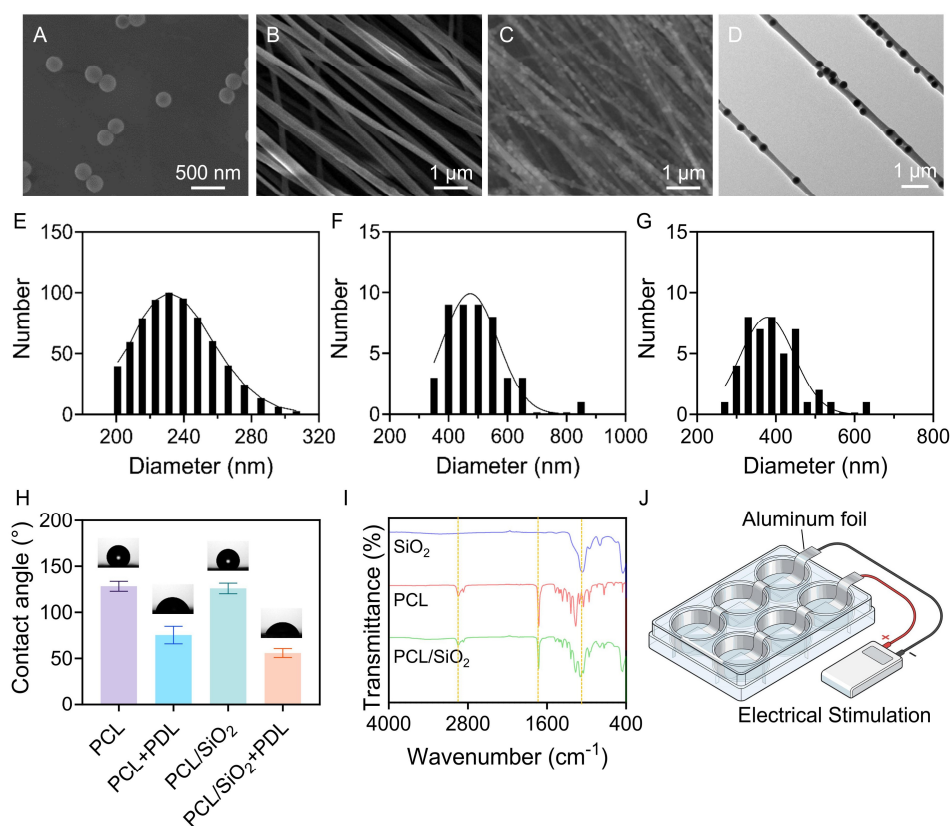


Figure 1. SEM images of (A) SiO₂ NPs, (B) PCL nanofibers, and (C) PCL/SiO₂ nanofibers. (D) TEM image of PCL/SiO₂ nanofibers. (E) Size distribution of SiO₂ NPs measured by DLS. Diameter distribution of (F) PCL and (G) PCL/SiO₂ nanofibers. (H) Water contact angle analysis. (I) FTIR spectra of SiO₂ NPs, PCL and PCL/SiO₂ nanofibers. (J) Schematic illustration of the electrical stimulation system used for in vitro cell stimulation.

Aligned PCL and PCL/SiO₂ nanofiber mats were successfully fabricated by electrospinning. SEM observations in Figures 1B and 1C confirm the morphologies of PCL and PCL/SiO₂ nanofibers, which were uniformly distributed and exhibited a predominantly aligned morphology, as observed by SEM. Such fibers are known to provide contact guidance cues for cell alignment and migration [24]. TEM analysis in Figure 1D presents that SiO₂ NPs are distributed along the PCL nanofibers, showing both surface-decorated and embedded morphologies. Although the present study did not quantitatively determine the density, height, or spatial distribution of the nanoscale protrusions by AFM or profilometry, SEM and TEM observations confirmed the aligned fibrous morphology and the presence of SiO₂ nanoparticle-derived surface features. The particle dispersion and aggregation state are expected to strongly influence the surface properties of composite fibers. Statistical analysis in Figures 1F and 1G showed that the average fiber diameter of PCL mats was 496.33 ± 96.1 nm, whereas that of PCL/SiO₂ mats decreased to 394.58 ± 71.1 nm. The incorporation of SiO₂ NPs affected the electrospinning process and resulted in a narrower diameter distribution. The decrease in fibrous diameter can be explained by the typical parameters for electrospinning and the polymer composition [43,44]. When SiO₂ NPs are added to the solution for electrospinning, the NPs significantly improve the conductivity and charge density, resulting in stronger stretching forces and enhanced whipping instability of the jet in the electric field, thereby enabling the fibers to be drawn thinner before solidification. Moreover, the SiO₂ NPs could influence the surface tension or increase the electrostatic interaction between the solution and the collector.

Wettability plays a critical role in regulating cell adhesion and spreading [45]. The water contact angles of PCL and PCL/SiO₂ nanofiber mats were $128.34 \pm 4.93^\circ$ and $126.02 \pm 5.2^\circ$, respectively (Figure 1H). Plasma was then applied to treat the nanofiber mats, followed by coating with PDL. The contact angle was markedly decreased to $75.39 \pm 8.66^\circ$ and $55.92 \pm 4.42^\circ$, respectively. The improved wettability is likely related to the surface activation effect of plasma treatment. Plasma exposure may cause mild surface etching and generate nanoscale irregularities, potentially increasing the apparent surface roughness. Meanwhile, plasma treatment introduces oxygen-containing polar groups, such as hydroxyl (-OH), carbonyl (C=O), and carboxyl (-COOH), onto polymer surfaces. These changes are expected to enhance surface energy and facilitate subsequent PDL deposition, thereby promoting cell adhesion and migration. The chemical structures of SiO₂ NPs, PCL and PCL/SiO₂ nanofibers were characterized by FTIR, as shown in Figure 1I. For the PCL/SiO₂ nanofibers, a characteristic absorption band at 1710 cm^{-1} was observed, corresponding to the C=O stretching vibration of PCL. Moreover, the peaks at approximately 2940 cm^{-1} ascribed to C–H stretching were enhanced after the incorporation of SiO₂ NPs [46]. The appearance of bands around 765 cm^{-1} in the PCL/SiO₂ spectrum was assigned to the Si–O stretching vibration of SiO₂ [47], demonstrating the successful incorporation of SiO₂ NPs into the PCL fibers.

3.2. Characterization of BMSCs

The isolated and cultured cells exhibited typical mesenchymal stem cell morphology, as shown in Figure S1, with adherent growth and a spindle-shaped or fibroblast-like appearance under an inverted microscope. To further identify their stem cell characteristics, trilineage differentiation assays were performed. After osteogenic induction, Alizarin Red S staining showed distinct red-stained mineralized nodules, indicating calcium deposition and osteogenic differentiation potential (Figure S2). Following chondrogenic induction, positive Alcian blue staining was observed, demonstrating the accumulation of acidic glycosaminoglycans in the extracellular matrix and confirming chondrogenic differentiation ability (Figure S3). After adipogenic induction, Oil Red O staining revealed abundant intracellular lipid droplets, and Figure S4 indicates adipogenic differentiation potential. Together, these results confirmed that the cells used in this study possessed the typical morphological features and multilineage differentiation capacity of BMSCs.

3.3. In Vitro Biocompatibility Evaluation

As shown in Figure 1J, an in vitro electrical stimulation system was constructed for subsequent cell experiments. Aluminum foil was used as the electrode material, integrated into a culture plate, and connected to an external ES. This simple setup allowed electrical cues to be applied during cell culture and served as the basis for evaluating the synergistic effects of topography and electrical stimulation on BMSC behaviors.

To evaluate the biocompatibility of the topographical nanofibrous mats combined with ES, BMSCs cultured on the different substrates were evaluated using the CCK-8 assay at days 3 and 7. As shown in Figure 2, there are no statistically significant differences in overall BMSCs viability, indicating that the aligned PCL/SiO₂ nanofibers, with or without ES, exhibit good cytocompatibility and support BMSCs growth. Fluorescent micrographs in Figure 3A present the morphologies of BMSCs after culture in the different groups for 3 days. In the control group (C), BMSCs displayed a typical fibroblast-like morphology with a relatively spread and polygonal shape. With the

introduction of aligned fibers and ES, cells gradually became more elongated and aligned along the fiber direction. As shown in Figure 3B, the orientation angle of BMSCs was widely distributed in the C group, indicating no obvious directional preference. In contrast, the aligned fibrous mat groups exhibited a narrow angular distribution, with a larger proportion of cells oriented close to the fiber direction. These data demonstrate that aligned fibers play a major role in guiding BMSCs' orientation, while ES can further improve the degree of ordered cell arrangement. This morphological transition implies that the combined physical guidance of fiber orientation and ES may induce a neurogenic-like response in BMSCs. Such morphological changes are often associated with cytoskeletal rearrangement and may represent an early indicator of lineage commitment toward neural phenotypes. Therefore, the synergistic effects of topographical guidance and electrical cues appear to modulate BMSCs' morphology and potentially prime cells for neurogenic differentiation.

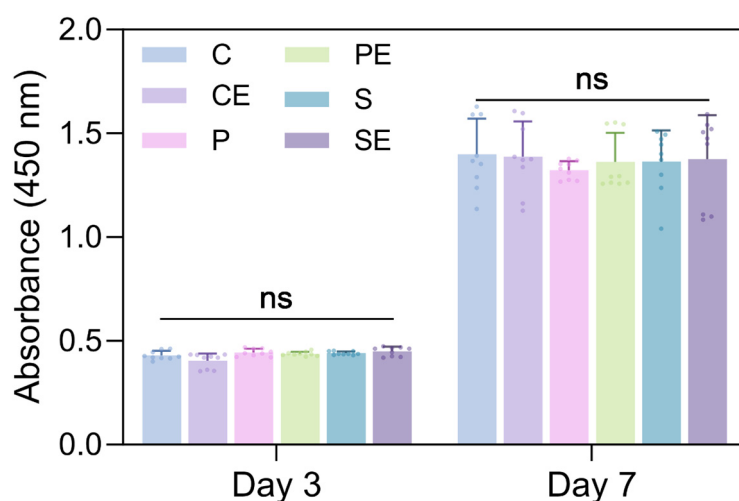


Figure 2. Viability of BMSCs after culture in the different groups for 3 and 7 days. The experimental groups were designed as follows: control (C), control with ES (CE), PCL (P), PCL with ES (PE), PCL/SiO₂ (S), and PCL/SiO₂ with ES (SE). ns indicates no significant differences between the compared groups.

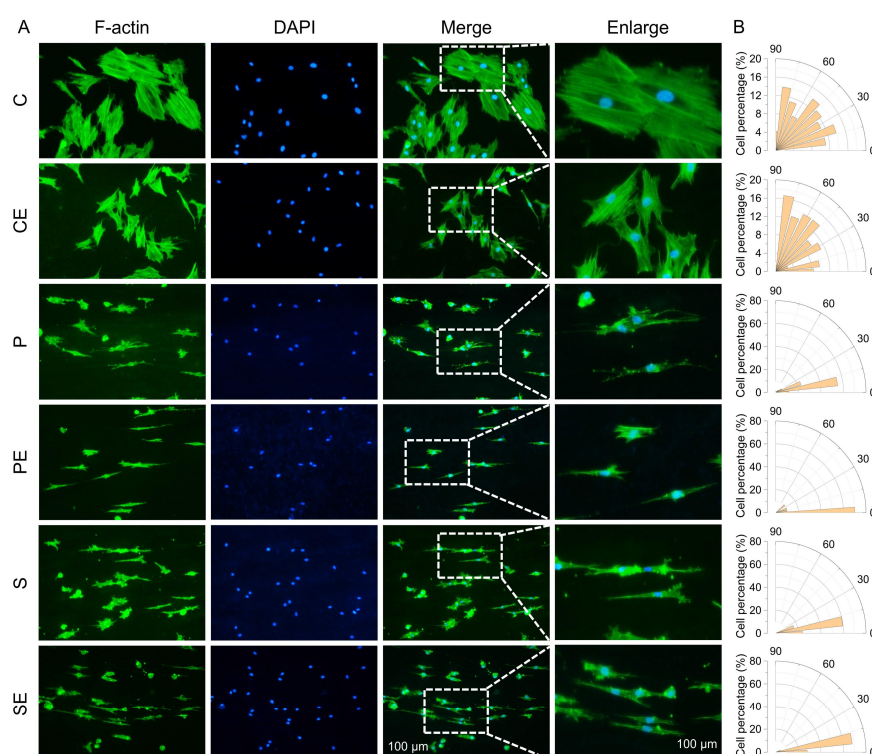


Figure 3. (A) The morphology of BMSCs after culture on the different groups for 3 days; (B) Orientation angles of BMSCs on the different groups. F-actin (green) represents the cytoskeleton and DAPI (blue) is a nuclei stain. The experimental groups were designed as follows: control (C), control with ES (CE), PCL (P), PCL with ES (PE), PCL/SiO₂ (S), and PCL/SiO₂ with ES (SE).

3.4. Differentiation of BMSCs

Following PNI, remyelination and axonal extension are two critical biological steps for functional regeneration [48]. The myelin sheath is an insulating structure formed by SCs that wraps around the axons [49]. It not only enables saltatory conduction of action potentials but also provides neurotrophic support to the axon [50,51]. Axons, as the extending part of the neuron, are responsible for conducting nerve impulses and serve as the foundation for establishing functional neural connections [52]. Therefore, simultaneously promoting axonal regeneration and remyelination is the goal of neural tissue engineering. NF200, as a structural protein in neurons, implies the growth of nerve fibers, while S100 β serves as a cytoplasmic protein marker for SCs. To investigate the regulatory effects of topographical cues combined with ES on neural-like differentiation of BMSCs, the expression of NF200 and S100 β was assessed by immunofluorescence. With the introduction of aligned fibers and ES, consistent with the morphological observations, BMSCs demonstrated elongated cell morphology and aligned growth and expressed both NF200 and S100 β (Figure 4).

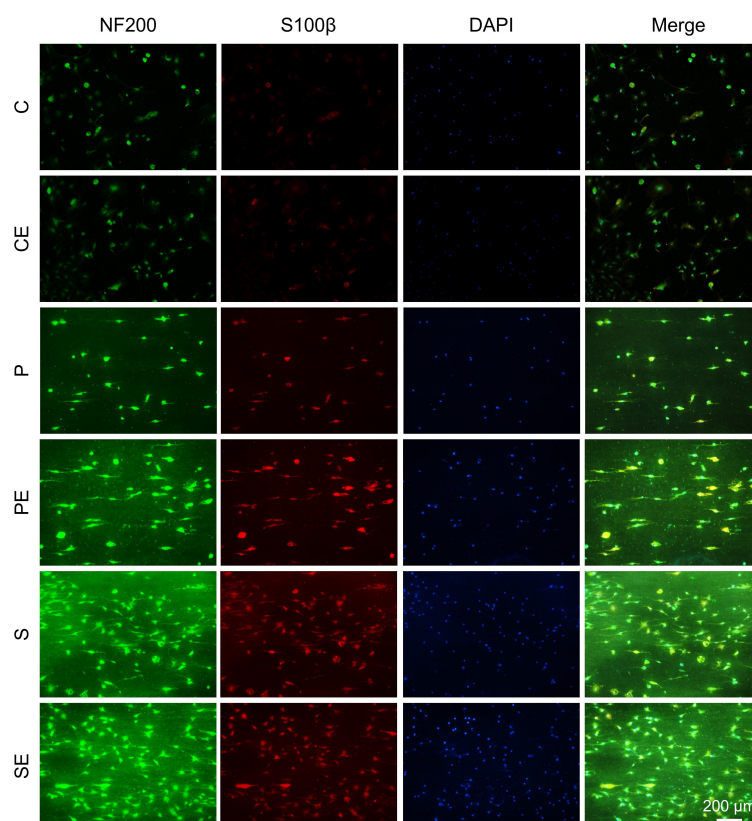


Figure 4. Immunofluorescence micrographs showing NF200 and S100 β expression after BMSCs were cultured on the different groups for 7 days. Green: NF200; Red: S100 β ; Blue: DAPI nuclei stain. The experimental groups were designed as follows: control (C), control with ES (CE), PCL (P), PCL with ES (PE), PCL/SiO₂ (S), and PCL/SiO₂ with ES (SE).

Quantitative analysis of fluorescence intensity in Figures 5A and 5B revealed that both the nanoscale protrusions on fiber surfaces and the ES contributed to the induction of BMSCs differentiation, with the highest NF200 and S100 β expression seen in the SE group. Compared to the C group, aligned PCL nanofibers (P group) also induced a significant upregulation of NF200 expression. Incorporation of SiO₂ NPs in aligned PCL nanofibers (S group) further enhanced NF200 expression compared with the C group, indicating that nanoscale protrusions augment the physical guidance effect of fiber alignment. The expression of NF200 was significantly higher in the groups exposed to ES compared to those without, particularly in the SE group. On the other hand, BMSCs exposed to both nanoscale protrusions and ES showed the highest Schwann cell-like differentiation in comparison to the other groups, suggesting beneficial synergistic effects of physical and biochemical signals. These results suggest that the integration of nanoscale topography with ES effectively enhances the neural-like differentiation of BMSCs. Notably, compared with nanoscale protrusions, ES more effectively induced differentiation into Schwann cell-like cells. Overall, aligned fibers with nanoscale protrusions on fiber surfaces offered a structure similar to Büngrer bands, which provided guidance for cell elongation. Additionally, ES could regulate the level of Ca²⁺ and activate downstream pathways, which played a significant role in regulating axonal extension. The synergistic

effects may stem from the elongation of cell morphology introduced by nanoscale protrusions on aligned fiber surfaces, which enhances the sensitivity to ES, thereby enlarging neuronal signaling.

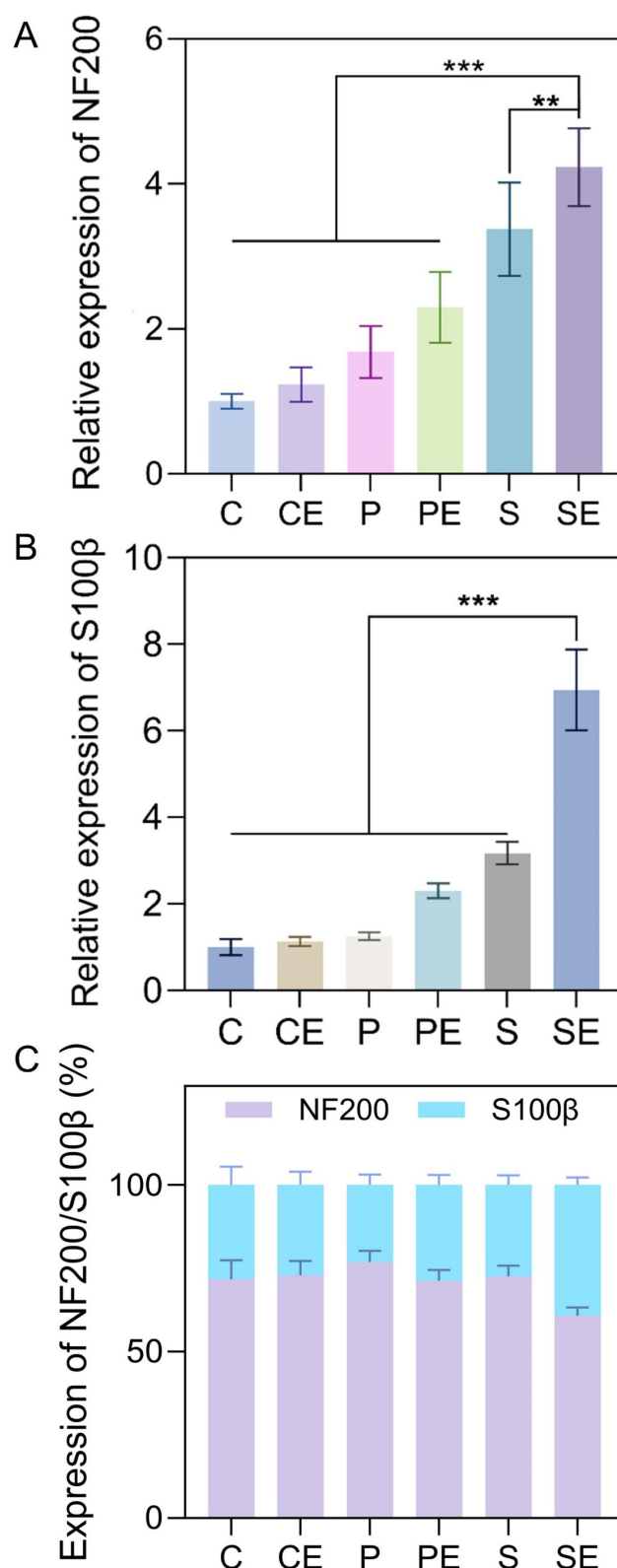


Figure 5. Relative expression of NF200 and S100β proteins according to the results shown in Figure 4: (A) NF200; (B) S100β; (C) Comparison of NF200/S100β expression. The experimental groups were designed as follows: control (C), control with ES (CE), PCL (P), PCL with ES (PE), PCL/SiO₂ (S), and PCL/SiO₂ with ES (SE). ** $p < 0.01$ and *** $p < 0.001$ indicate significant differences between the compared groups.

Moreover, data showed that the proportion of cells expressing NF200 was higher than that expressing S100β in each group, as presented in Figure 5C. Aligned PCL nanofibers induced a significant increase in NF200 relative

to the C group and altered the NF200/S100 β ratio in a direction consistent with a more neuron-like phenotype. Following the application of ES, the expression level of NF200 exhibited a slight decrease. Nevertheless, it remained significantly elevated compared with that of S100 β . These results indicate a preference for neuronal-like differentiation rather than Schwann cell-like differentiation under these conditions. The synergy between the nanoscale topography of SiO₂-containing aligned fibers and ES can effectively promote the neurogenic differentiation of BMSCs, with a bias toward a neuron-like phenotype.

3.5. Migration of BMSCs

Cell migration is a critical process during nerve regeneration, as stem cells must migrate toward injury sites and participate in tissue repair [53]. We further evaluated the influence of PCL/SiO₂ fibers combined with ES on the migration of BMSCs. Fluorescence micrographs in Figure 6A showed that BMSCs in all groups exhibited migration behaviors on the underlying substrates. Compared to the C group, the migration of BMSCs on uniaxially aligned fibers (P, PE, S, and SE) displayed substantially enhanced migration, characterized by a longer migrated distance. This result indicates that the aligned fiber architecture provided effective contact-guidance cues to facilitate directional cell movement. Furthermore, the BMSCs cultured on fiber surfaces with nanoscale protrusions (S group) exhibited a more significant response to directional migration, compared to the smooth nanofibers (P group). When combined with ES, the BMSCs migrated the farthest distance in the SE group. Quantitative statistical analysis in Figure 6B further revealed that the total number of migrated cells in the SE group was significantly greater than that in the other groups ($*** p < 0.001$), suggesting a synergistic contribution of biomimetic nano-topography and ES in enhancing BMSCs migration. To further evaluate migration distribution, the migration zone was evenly divided into three regions: the proximal (region I), middle (region II), and distal area (region III) in Figure 6C. In regions I and II, the number of migrated BMSCs in the SE group was significantly greater ($*** p < 0.001$) than in the C and CE groups. Even in region III, the SE group exhibited a significantly higher number of migrated cells ($* p < 0.05$) than the C and CE groups. These results revealed that the combination of biophysical and biochemical cues enabled BMSCs to migrate over longer distances.

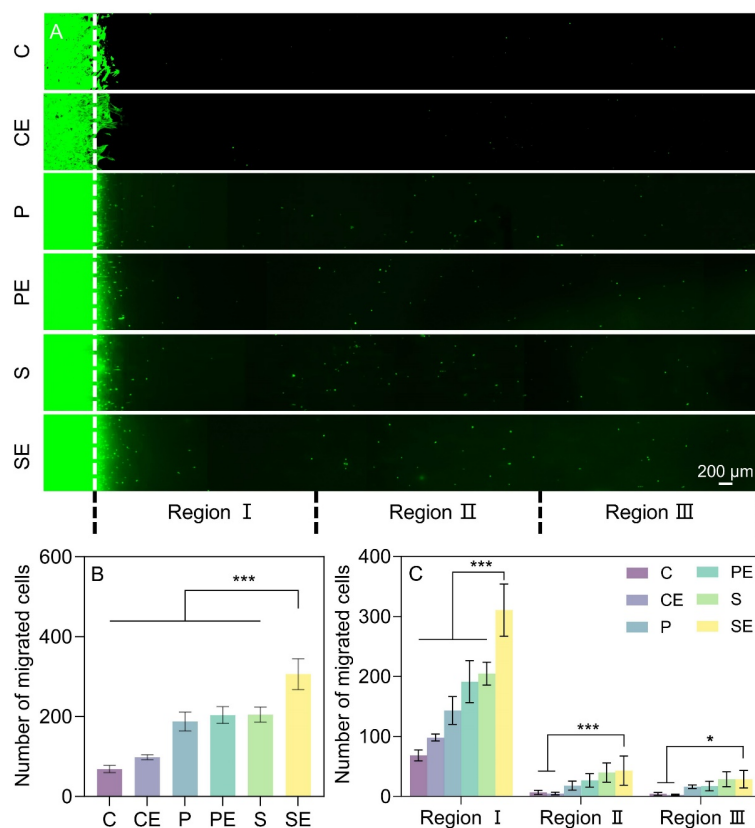


Figure 6. (A) Fluorescence micrographs showing the migration of BMSCs after culture on the different groups for 3 days. (B) Statistics of the total number of migrated BMSCs. (C) The number of BMSCs in the different migration zones. The experimental groups were designed as follows: control (C), control with ES (CE), PCL (P), PCL with ES (PE), PCL/SiO₂ (S), and PCL/SiO₂ with ES (SE). $* p < 0.05$ and $*** p < 0.001$ indicate significant differences between the compared groups.

Uniaxially aligned nanofibers with nanoscale protrusions offer more anchoring sites to reduce the randomness of cell movement and improve migration efficiency. Such nano-topographical features may also enhance signaling related to cell migration, such as the reaction of actin mediated by Rho, Rac, and Cdc42 GTPases, and actin remodeling [54]. Meanwhile, ES served as an external bioelectrical cue that further enhanced directional migration, possibly by modulating membrane potential, ion channel activity, and intracellular calcium dynamics, thereby accelerating cell movement and improving directional persistence. More importantly, the simultaneous presence of topographical and electrical cues may create a more biomimetic microenvironment, enabling BMSCs to integrate multiple external signals and respond more sensitively to guidance stimulation [55]. This cooperative effect likely underlies the superior migration performance observed in the SE group and may be particularly beneficial for promoting endogenous stem cell recruitment and tissue regeneration in peripheral nerve repair.

4. Conclusions

By utilizing electrospinning, SiO₂ NPs were integrated with biocompatible PCL to fabricate hybrid nanofibers featuring surface protrusions as secondary structures. We explored the regulatory influence of PCL/SiO₂ nanofibrous mats combined with ES on modulating BMSCs' behavior. Our results demonstrated that the fabricated scaffolds possessed favorable physicochemical properties and cytocompatibility, effectively promoting cell adhesion, migration, and viability. The synergistic effect of the uniaxially aligned topographical structure and applied ES induced a neural-like morphological transition of BMSCs. In particular, this combined approach significantly upregulated the neuronal marker NF200 and the Schwann cell marker S100 β , with a bias toward neuronal-like differentiation. The uniaxially aligned PCL/SiO₂ nanofibers combined with ES also markedly enhanced the long-distance migration of BMSCs. Collectively, these findings indicate that the combination of aligned PCL/SiO₂ nanofibrous scaffolds with ES creates a biomimetic microenvironment that resembles native neural tissue architecture. Furthermore, it is indicated that fibrous mats seeded with stem cells to create nerve guidance conduits represent a significant potential for future applications in PNI repair.

Supplementary Materials: The following supporting information can be downloaded at: <https://media.scilit.com/articles/others/2606081418394895/MI-26040102-SI.pdf>, Figure S1: Representative light microscopy image showing the typical spindle-shaped and fibroblast-like morphology of cultured BMSCs; Figure S2: Alizarin Red S staining showing BMSCs formed mineralized nodes under osteogenic induction, showing the formation of mineralized nodules; Figure S3: Alcian blue staining after chondrogenic induction, showing the deposition of cartilage-associated extracellular matrix; Figure S4: Oil Red O staining after adipogenic induction, showing the formation of intracellular lipid droplets.

Author Contributions: Y.Y.: Investigation, Methodology, Visualization, Writing—original draft; B.S.: Methodology; M.L.R.: Writing—review & editing; Y.W.: Supervision, Resources; T.W.: Conceptualization, Supervision, Resources, Funding acquisition, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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SERS Studies on the Dynamic Response of Membrane Interface Lipids to Proteins: Concentration Effect and Redox State Conversion

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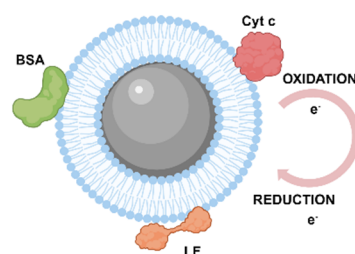
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Abstract: The dynamic behavior of lipids at membrane interfaces is essential for maintaining the structural stability and functional specificity of biomembranes, with its regulation closely linked to protein interactions and the redox microenvironment. Understanding the interaction between proteins and phospholipid membranes, as well as the impact of redox processes on membrane structures, is therefore crucial for clarifying membrane functions and mechanisms. This study developed a phospholipid bilayer-on-plasmonic nanoparticle (BOM) configuration using an extruder. Based on this configuration, we conducted a systematic investigation of the dynamic interactions and concentration-dependent effects of bovine serum albumin (BSA), cytochrome c (Cyt c), and lactoferrin (LF) on zwitterionic dioleoylphosphatidylcholine (DOPC) bilayers coated silver nanoparticles (DOPC@Ag) *via* surface-enhanced Raman spectroscopy (SERS). Furthermore, by leveraging a non-physiological artificial electron-transport chain model, we elucidated the redox-dependent regulatory mechanisms of Cyt c on both synthetic phospholipid membranes and natural cellular membranes. Our observations provide insight into the lipid-protein-redox interaction network, offering suggestions for membrane biosensor design, targeted drug delivery systems, and the study of membrane-related physiological and pathological processes.



Keywords: surface-enhanced Raman spectroscopy; phospholipid membrane; lipid-protein interaction; redox; cytochrome c

1. Introduction

Biomembranes are fundamental components of cells and their internal organelles. Their inherent fluidity drives continuous processes of synthesis, remodeling, fission, and fusion [1,2]. This dynamic nature renders the biomembrane a highly adaptable compartment, providing an indispensable structural foundation for all bio units. Crucially, core cellular functions, including receptor signaling, intercellular communication, and membrane transport, rely on the precise modulation of membrane properties by key proteins (such as cytochrome c and iron transporters) and lipid components (such as cholesterol and cardiolipin) [3–5]. These molecular players exert spatiotemporal control over biomembrane function by regulating membrane fluidity, phase distribution, and curvature stress [6–8].

The analysis of lipid-protein interactions faces significant challenges with conventional techniques such as nuclear magnetic resonance (NMR) [9], infrared spectroscopy (IR) [10,11], and quartz crystal microbalance with



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dissipation monitoring (QCM-D) [12]. NMR demands exceptionally high sample purity and quantity, poses challenges in establishing membrane-mimetic environments, and entails complex data interpretation. IR spectroscopy primarily yields low-resolution information on protein secondary structure and suffers from severe interferences from water vibrational bands. While QCM-D enables real-time monitoring of adsorbed mass changes and viscoelastic properties, it lacks chemical specificity and pre-identification of interacting molecular species; furthermore, its measurement of “hydrated mass” complicates quantitative analysis [13–16].

Raman spectroscopy offers distinct advantages for probing lipid-protein interactions [17]. Surface-enhanced Raman spectroscopy (SERS) combines ultra-high sensitivity with exceptional interfacial specificity, enabling not only single-molecule detection but also precise interrogation of nanoscale dynamic processes at membrane interfaces [18]. Critically, it concurrently provides real-time molecular fingerprint information, establishing a powerful tool for investigating biomembrane interfacial processes [16,19,20].

Although significant progress has been made in the studies of lipid-protein interactions, the mechanism by which the redox microenvironment orchestrates hierarchical protein-phospholipid interactions across spatiotemporal scales remains elusive [21–23]. To address this fundamental question, we constructed a phospholipid bilayer-on-metal particle (BOM) structure using an extruder and investigated its interactions with diverse proteins *via* SERS. In this study, we took dioleoylphosphatidylcholine (DOPC) to mimic the cell membrane and coated silver nanoparticles (Ag NPs) with DOPC to achieve a phospholipid bilayer-on-plasmonic nanoparticle (BOM) configuration, DOPC@Ag. Owing to the plasmonic enhancement effect of Ag NPs, the SERS spectra of DOPC bilayers can be significantly improved and analyzed in detail. Our observations revealed that the SERS behaviors of lipids are concentration-dependent in the presence of spiked proteins. Furthermore, we systematically compared the redox responses of this biomimetic DOPC@Ag system with the red blood cell membrane-on-Ag NP system (RCM@Ag). A direct causal relationship between the redox state of membrane-bound cytochrome c (Cyt c) and its regulatory function on the structure and dynamics of lipid bilayers was built. This work delineates the concentration-dependent interaction modes between three representative proteins and zwitterionic phospholipid membranes. It also elucidates the molecular mechanism by which redox-state transitions modulate dynamic processes at the lipid-protein interface, providing a valuable theoretical basis for the field of membrane redox biology. Additionally, our study demonstrates that this BOM configuration can be a powerful tool for investigating lipid-protein interactions.

2. Experimental Section

2.1. Preparation of DOPC@Ag

The 200-nm pore-size filter membrane was installed in a micro liposome extruder (as shown in Figure 1a). Subsequently, 450 μ L of the DOPC vesicle solution was subjected to 11 consecutive extrusion cycles through the membrane. Following this, 500 μ L of AgNP solution (~50 nm in diameter, see Supplementary Materials for more details) was passed through the same membrane. The filter membrane was then replaced with a 100-nm-pore-size membrane, and the resulting mixture was further extruded 11 times. The final product, DOPC@Ag, was collected and stored at 4 °C for future use.

2.2. Protein Assembly on DOPC@Ag

To prepare protein-conjugated DOPC@Ag complexes, DOPC@Ag was mixed with BSA in equal volumes and incubated at 37 °C for 40 min to facilitate complex assembly, then centrifuged at 5300 rpm for 10 min to remove unbound proteins. The resulting BSA-DOPC@Ag complex was resuspended in PBS for further characterization. The same procedure was repeated to prepare Cyt c-DOPC@Ag and LF-DOPC@Ag complexes.

2.3. Quartz Crystal Microbalance with Dissipation (QCM-D) Measurements

We employed QCM-D to trace the protein assembly on the DOPC membrane. First, we need a supported lipid bilayer (SLB) film on the surface of a QCM chip. To fabricate the SLB film, we prepared a bicelle solution (Stage I, Figure 2). More details for the protocols of bicelles and SLB films can be found in our previous publication [24]. Briefly, a DOPC chloroform solution was thoroughly mixed at the predetermined molar ratio. The solvent was then slowly evaporated using nitrogen blowing until the chloroform had completely evaporated, resulting in a uniform lipid film on the inner wall of the container. The lipid film was subsequently transferred to a vacuum desiccator and dried continuously for 6–8 h to remove residual organic solvents completely. The dried lipid film was hydrated with PBS buffer (pH 7.4) and vigorously shaken to form a turbid vesicle suspension. Next, short-chain lipids were added at a molar ratio of long-chain to short-chain lipids of 3:1 to construct a binary

phospholipid particle system. The mixed suspension underwent 5–10 freeze-thaw cycles (rapid freezing in liquid nitrogen for 1 min, thawing in a 60 °C water bath for 5 min, followed by vortex mixing for 30 s each time) until the solution transitioned from turbid to clear, indicating the formation of homogeneous nanostructures.

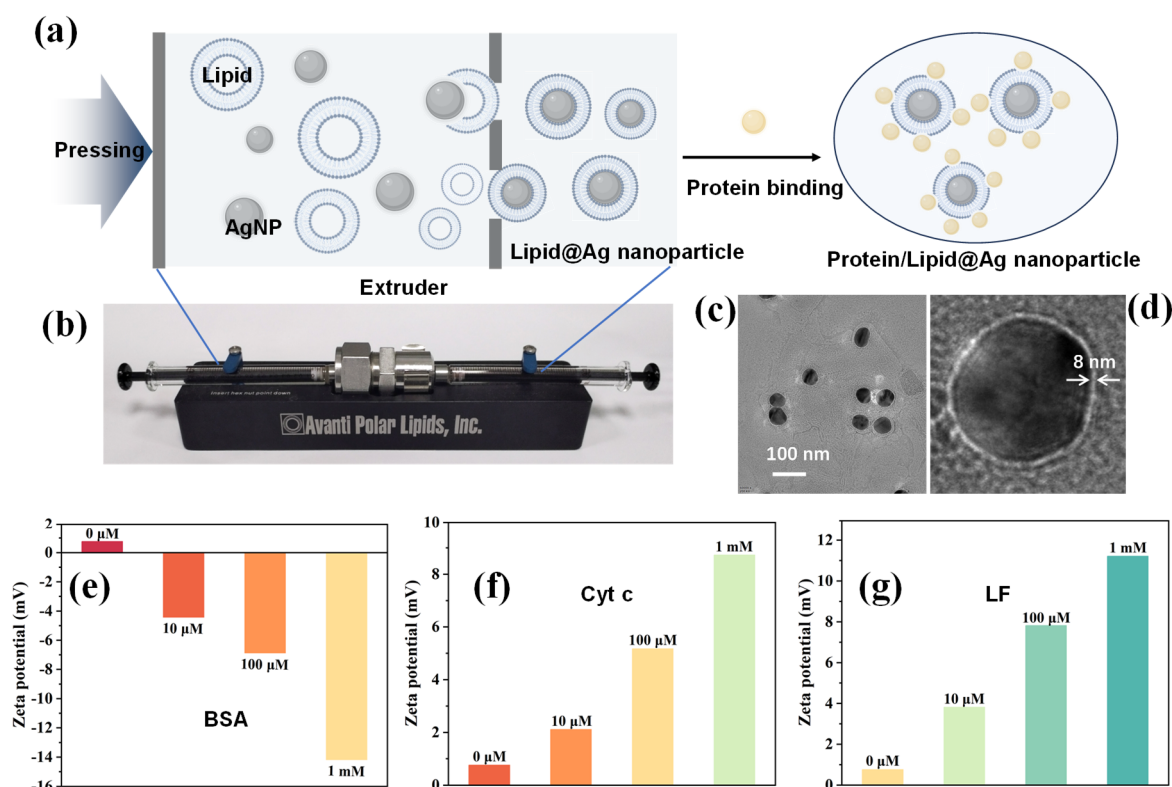


Figure 1. (a) Schematic diagram of phospholipid-encapsulated nanoparticles. (b) Liposome extruder schematic diagram. (c) TEM images of DOPC@Ag. (d) A high-magnification TEM image of a DOPC@Ag. (e–g) Zeta potential measurements of the DOPC@Ag before and after proteins (BSA, Cyt c, and LF) were loaded.

The final bicelles were injected into the QCM-D detection cell at a constant flow rate of 50 μL/min to monitor real-time changes in frequency and dissipation on the surface of the quartz crystal oscillator chip (QSX303, 5 MHz). Once the short-chain lipids were completely dissociated and washed away (Stage II, Figure 2 top panel), an intact supported DOPC bilayer was left on the chip surface (Stage III, Figure 2 top panel). The system was immediately switched to continuous perfusion with protein solutions at the same flow rate (maintaining the same flow rate). This enabled real-time monitoring of the dynamic interactions between the proteins and the lipid bilayer.

This experiment uses a modular fluid control system integrated with QCM-D technology. For fluid control, a high-precision, multi-channel peristaltic pump from the Swiss company Ismatec is used to maintain a constant sample flow rate in the flow cell. The core detection device is a Qense E4 four-channel QCM-D system from Finland Biolin Scientific AB. This system integrates a constant-temperature control unit and simultaneously performs real-time monitoring of four independent detection channels at 25.0 ± 0.1 °C. The test module uses the standard flow cell component of the system, and the liquid volume between the flow cell and the sensing chip is approximately 40 μL. Data collection was accomplished using the QSoft₄₀₁ software. The experimental data were parsed using the QTools module.

2.4. SERS Measurements

The experimental procedure involved mixing DOPC@Ag and Ag@RCM solutions with the protein solution at a 1:1 volume ratio, followed by incubation at 25 °C for 40 min to allow the complexes to self-assemble completely. A series of concentration gradient systems was systematically prepared for subsequent use. After incubation, the reaction mixtures were centrifuged (5300 rpm, 10 min) to remove unbound proteins, with the resulting precipitates being retained for further characterization.

SERS measurements were performed a confocal Raman spectrometer (T64000, HORIBA JOBIN YVON, Villeneuve d'Ascq, France) using a 532 nm excitation laser. The laser power was carefully maintained at 1 mW by a precision optical power meter. All spectral acquisitions were conducted under identical experimental conditions,

employing a single accumulation with an integration time of 20 s. Throughout the experimental process, a constant-temperature environment was rigorously maintained to ensure system stability and measurement reproducibility.

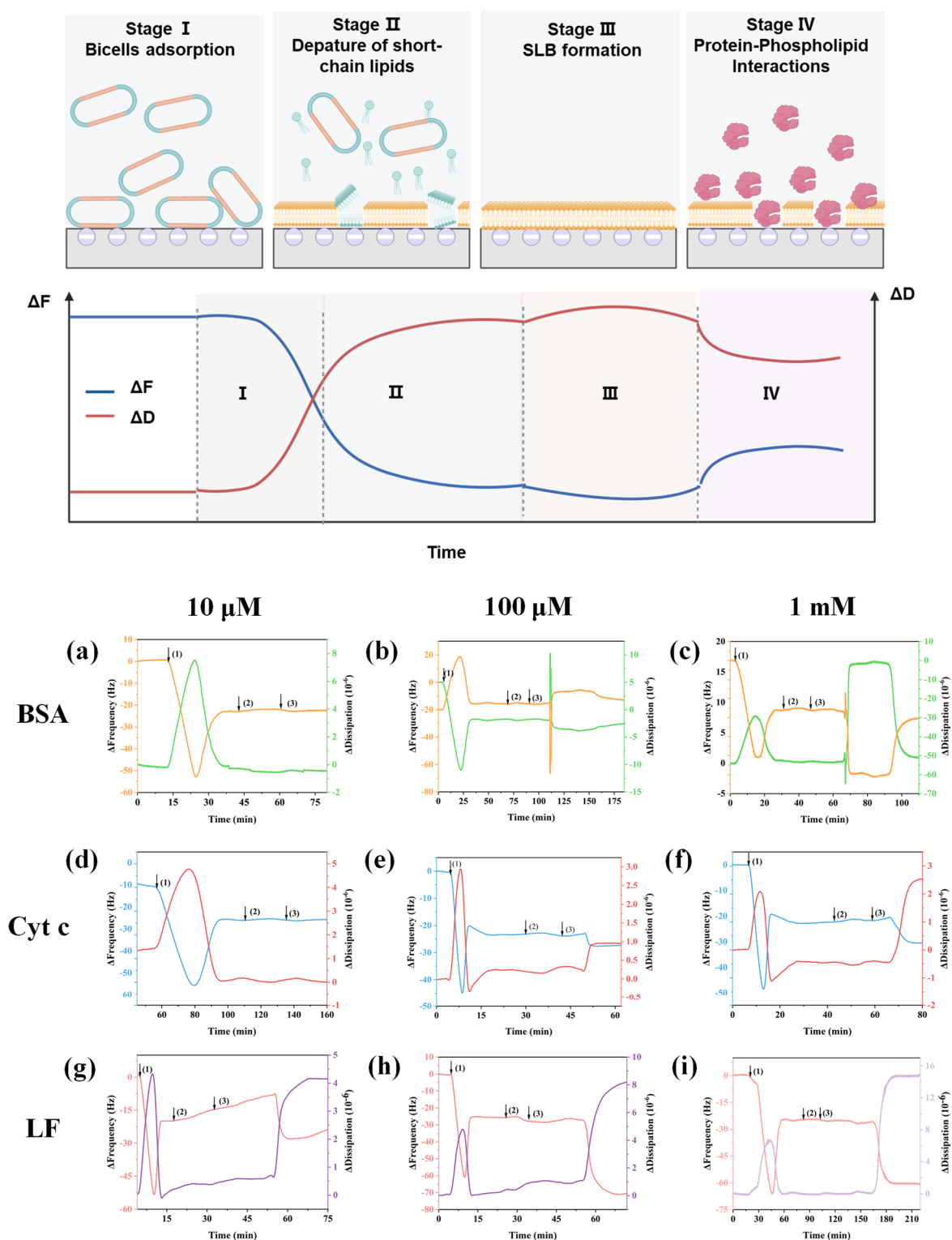


Figure 2. Schematic diagram of QCM-D analysis for protein adsorption kinetics (Top panel). QCM-D analysis of protein adsorption kinetics on DOPC (ΔF denotes the frequency variation; ΔD denotes the dissipation variation). Arrows indicate injection time points. BSA (a–c), Cyt c (d–f), and LF (g–i), respectively, with the concentration of 10 μM , 100 μM , and 1.0 M.

2.5. Spectral Processing

To quantitatively analyze the lateral packing order of lipid acyl chains, peak deconvolution fitting was performed on the C-H stretching vibration region (2800–3000 cm^{-1}). The position of the third peak, along with

the full width at half maximum (FWHM) and height of all peaks, was treated as a free parameter and iteratively optimized using the nonlinear least squares method until convergence. The spectra in this region were fitted with a Voigt profile. The peaks near 2850 and 2890 cm^{-1} correspond to the symmetric stretching vibration and asymmetric stretching vibration of the methylene group, respectively. The peak near 2927 cm^{-1} is assigned to the primary symmetric stretching vibration of methylene groups [17].

To ensure the physical significance of the fitting results and avoid overfitting, the peak positions of the three methylene vibration bands at 2848, 2890, and 2927 cm^{-1} were fixed during the fitting process, using values derived from the spectral characteristics of highly ordered pure DOPC control samples. The peak position of the third peak, as well as the full width at half maximum (FWHM) and peak height of all fitted peaks, were set as free variables. The fitting was optimized iteratively *via* nonlinear least squares until convergence. The goodness of fit was verified by a coefficient of determination ($R^2 = 0.97 > 0.95$) and the random distribution of residual plots [25,26].

3. Result and Discussion

3.1. Characterizations of Protein Assembly on DOPC@Ag

DOPC liposomes, prepared *via* the thin film hydration method [27], were mixed with the as-prepared Ag NPs. The mixture was then extruded through a polycarbonate membrane with a 100-nm pore size using a liposome extruder. Figure 1a shows schematic diagrams of phospholipid-encapsulated nanoparticles. Figure 1b is a photo of the liposome extruder used in this study. During extrusion, phospholipid molecules undergo dynamic reorganization under shear forces and membrane-pore constraints, thereby enabling the passive encapsulation of nanoparticles from the aqueous phase into the liposome bilayer and yielding the DOPC@Ag composite.

To mimic cell membranes, we decorated the DOPC@Ag with various proteins: bovine serum albumin (BSA), cytochrome c (Cyt c), and lactoferrin (LF). To verify protein interaction with electrically neutral DOPC@Ag, TEM and Zeta potential characterization were performed. Figure 1c shows TEM images of DOPC@Ag. It can be observed that DOPC was successfully coated onto the surface of Ag NPs. Figure 1d is a high-magnification TEM image. The thickness of the DOPC bilayers is 4.8 nm, indicating the successful formation of an intact phospholipid bilayer on the surface of Ag NPs. Decorating proteins on DOPC@Ag induces shifts in zeta potential upon interaction. It can be observed that negatively charged BSA decreased the potential from an initial value of -0.1 mV to -4.4 mV, -6.8 mV, and -14.2 mV at BSA concentrations of 10 μM , 10 μM , and 1.0 mM, respectively. Conversely, incubation with positively charged Cyt c and LF increased the potential to $+2.1$, $+5.2$, and $+8.7$ mV for Cyt c, and $+3.8$, $+7.8$, and $+11.2$ mV for LF as the concentration increased from 10 μM to 1.0 mM. These potential changes provide direct evidence of the protein's adsorption onto the DOPC@Ag.

To further investigate the lipid-protein interaction, QCM-D was used to monitor protein adsorption kinetics on DOPC bilayers. We first fabricated DOPC bilayers on the QCM chip using the bicelle method [24]. Next, protein solutions at different concentrations (BSA, Cyt c, and LF) were injected. The protein adsorption kinetics on DOPC bilayers were recorded, as shown in Figure 2. The measured frequency shift (ΔF) and dissipation shift (ΔD) reveal pronounced protein-specific and concentration-dependent behaviors in interfacial adsorption, membrane embedding, and structural remodeling of DOPC bilayers, with each protein exhibiting a distinct mode of action at each tested concentration (Figure 2j). At the lowest protein concentration of 10 μM , overall interactions between the proteins and DOPC membranes were negligible, with only LF yielding measurable interfacial binding. Specifically, BSA shows only trace adsorption and fails to form a stable interfacial structure (Figure 2a). For Cyt c, only a minimal decrease in ΔF and limited fluctuations in ΔD are detected, further confirming the near-absence of adsorption under this condition (Figure 2d). In contrast, LF forms a loose, stable interfacial structure *via* surface adsorption onto the phospholipid membrane, representing the only protein capable of effective interfacial binding to DOPC at 10 μM (Figure 2g).

Upon increasing the protein concentration to 100 μM , all three proteins exhibited significant interfacial interactions with DOPC membranes, albeit with fundamentally distinct interfacial structural features and molecular mechanisms. The BSA system shows a marked decrease in ΔF accompanied by a continuous increase in ΔD , a profile indicative of BSA predominantly embedding in the phospholipid bilayer *via* hydrophobic interactions and concentration-driven conformational rearrangement, establishing a dynamic adsorption equilibrium at the interface (Figure 2b). By comparison, increasing the Cyt c concentration to 100 μM results in a significant reduction in ΔF while ΔD stabilized after the initial change, suggesting the formation of a more compact interfacial layer (Figure 2e). This enhanced structural integrity is attributed to the directional insertion of the heme domain of Cyt c into the phospholipid bilayer in a vertical orientation, enabling tight association with DOPC molecules. At the same 100 μM concentration, the LF system exhibits initial dynamic profiles similar to those of BSA, namely a pronounced decrease in ΔF and a continuous increase in ΔD . However, unlike BSA, the increase

in ΔD for LF is substantially larger and sustained without reaching a plateau, indicating that LF did not achieve a stable embedded equilibrium state. Consequently, the adsorption of LF leads to a much looser, more dynamic phospholipid interface (Figure 2h).

At the highest protein concentration (1.0 mM), all three proteins induced substantial structural remodeling of the DOPC membrane, ultimately forming flexible protein-phospholipid composite interfaces, yet with marked differences in interaction strength, interfacial structural characteristics, and the underlying molecular mechanisms. BSA exhibits a distinct adsorption mode at this concentration. QCM-D monitoring reveals a continuous decrease in ΔF alongside a synchronous increase in ΔD , with both parameters eventually intersecting at a new equilibrium. These trends demonstrate that BSA is deeply embedded into the phospholipid bilayer *via* the synergistic effects of hydrophobic and electrostatic interactions, transforming the native DOPC membrane into a water-rich, soft BSA-DOPC composite interface (Figure 2c). This transition is mediated by concentration-triggered conformational rearrangement of BSA, which exposes specific functional domains and thereby markedly enhances its binding affinity to the DOPC bilayer. In contrast, Cyt c at 1.0 mM also induced a membrane softening effect, characterized by a decrease in ΔF and an increase in ΔD (Figure 2f). This phenomenon arises from a concentration-dependent transition in adsorption behavior, indicating that Cyt c formed a relatively compact, flexible interface with DOPC in dynamic equilibrium. More notably, LF at 1.0 mM triggers the most pronounced ΔF and ΔD responses among the three proteins, revealing that LF molecules induced a deeper level of structural perturbation to the phospholipid bilayer, significantly altering the assembly structure of the lipid membrane without compromising its integrity (Figure 2i). This effect stems from the high cationic charge density of LF. The dense positive charges electrostatically shield the negatively charged phospholipid headgroups, while a fraction of LF molecules can even penetrate the headgroup region into the hydrophobic core of the bilayer, inducing disordered lipid packing. Meanwhile, the formation of protein-phospholipid complexes further drives membrane curvature remodeling and redistribution of interfacial tension.

Taken together, although BSA, Cyt c, and LF all form flexible composite interfaces with DOPC at 1.0 mM, their interaction strengths and molecular mechanisms are fundamentally distinct. In short, BSA acts primarily *via* conformational rearrangement-mediated membrane embedding, Cyt c relies on directional insertion of its heme domain as the core binding mode, and LF induces the most significant interfacial softening through strong electrostatic interactions, hydrophobic insertion, and global structural perturbation of the bilayer. These distinct molecular interaction modes—weak adsorption, conformationally regulated membrane embedding, charge screening, and hydrophobic insertion—exhibit characteristic interfacial stability profiles across the protein concentration gradient. Elucidating these molecular mechanisms provides a critical theoretical foundation for the precise regulation of protein-phospholipid interactions.

These distinct concentration-dependent and protein-specific interaction behaviors observed by QCM-D are fully consistent and quantitatively correlated with the SERS results as described in Section 3.2. The mass adsorption, membrane embedding, and structural remodeling processes, as revealed by changes in ΔF and ΔD , directly induce lipid chain disordering, packing variation, and fluidity modulation, as quantified by the SERS lateral order parameter (S_{lat}). Specifically, QCM-D provides the dynamic kinetic evidence of how proteins interact with the membrane over time, while SERS offers the molecular structural evidence of how lipids respond to protein binding at the atomic level. Together, they establish a complete and multi-scale mechanistic description of lipid-protein interactions.

3.2. SERS Characterization of protein assembly on DOPC bilayer

By analyzing the characteristic spectral peaks of phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, we can accurately assess membrane structural stability [28,29]. Spectral analysis combined with multidimensional data models can reveal associations between abnormal phospholipid metabolism and cardiovascular and neurodegenerative diseases, facilitating biomarker discovery and early diagnosis [30]. Thus, studies on the spectral nature of phospholipids using Raman analysis have always attracted considerable attention [31]. However, normal Raman usually provides low signals of analytes. To amplify the Raman signal of lipids, we developed a unique phospholipid bilayer-on-metal particle (BOM) configuration using an extruder. Owing to the phospholipid being close to the plasmonic nanoparticle, the short-range and long-range enhancement effects are both involved, which significantly amplify the Raman scattering signals of the phospholipid. Thus, we can further explore protein-induced phospholipid disturbances using high-quality SERS spectra of phospholipids obtained with the BOM configuration.

To directly verify and mechanistically explain the protein-induced membrane structural changes observed by QCM-D at the molecular level, SERS was employed to monitor the lipid acyl chain ordering and packing density.

The SERS results are in excellent agreement with QCM-D observations. The adsorption, insertion, and interfacial remodeling behaviors identified by frequency and dissipation shifts directly correspond to the changes in lipid chain conformation and membrane fluidity revealed by SERS. Accordingly, we correlated the QCM-D kinetic trends with the SERS spectral features and the quantitative S_{lat} values for each protein across all tested concentrations.

To investigate the effects of proteins at distinct binding sites on phospholipids, the lateral interaction order parameter [S_{lat} ; see Formula (1)] was calculated from the ratio defined in Formula (2). S_{lat} quantifies both the strength of lateral interactions between phospholipid chains and the degree of chain ordering [17,28].

$$S_{lat} = \frac{I_{CH_2} - 0.7}{1.5} \quad (1)$$

$$I_{CH_2} = \frac{I_{2890}}{I_{2850}} \quad (2)$$

S_{lat} was calculated from the Raman peak intensity ratio I_{2890}/I_{2850} . All data represent the mean $\pm$ standard deviation of three independent experiments. The mean and variance of the I_{2890}/I_{2850} ratio are shown in Supplementary Figure S1.

Comparative analyses of the data in Figure 3g–i and Table 1 indicate that LF exhibits unique membrane interactions, fully supported by QCM-D results. Here, QCM-D provides real-time, quantitative information on the kinetics and thermodynamics of protein adsorption, as well as the viscoelastic properties of the resulting interfacial layer. SERS provides molecular-level insight into the structural changes in lipid molecules induced by protein binding, directly linking macroscopic interfacial properties to microscopic molecular conformations. At the trial of 10 μ M LF, QCM-D shows that LF was the only protein capable of effective interfacial binding to DOPC (Figure 2g), corresponding to a remarkable 380% increase in S_{lat} in SERS—far higher than BSA (50%) and Cyt c (205%) at the same concentration. This confirms that LF has superior membrane-binding affinity even at low concentrations. In the case of 100 μ M LF, ΔD for LF increased substantially and sustained without reaching a plateau (Figure 2h), indicating a much looser, more dynamic phospholipid interface. This corresponds to a further increase in S_{lat} to 400%, consistent with the continuous structural perturbation observed by QCM-D. As for the result of 1.0 mM LF, it can be observed that LF triggers the most pronounced ΔF and ΔD responses among the three proteins (Figure 2i), corresponding to an extraordinary 995% increase in S_{lat} . This demonstrates that LF molecules induce the deepest structural perturbation of the phospholipid bilayer, significantly altering lipid membrane assembly without compromising its integrity. This effect stems from the high cationic charge density of LF. The dense positive charges electrostatically shield the negatively charged phospholipid headgroups, while a fraction of LF molecules penetrate the headgroup region into the hydrophobic core of the bilayer, inducing disordered lipid packing. This is corroborated by the QCM-D signal, which indicates an increase in intact membrane mass. This observed diversity in protein-membrane interactions and their specific concentration dependencies provides multi-dimensional experimental insights into the mechanisms of biomacromolecular transmembrane transport. Taken together, the QCM-D and SERS results are fully consistent and mutually reinforcing.

The correlation between $\Delta F/\Delta D$ trends and S_{lat} values across all three proteins and all concentrations confirms that both techniques are measuring the same underlying protein-membrane interaction processes. This cross-validation significantly strengthens the reliability of our conclusions and provides a comprehensive understanding of how different proteins interact with phospholipid membranes.

Table 1. S_{lat} variation rates in protein/DOPC@Ag assembly systems at varying concentrations.

Specimen	I_{2890}/I_{2850}	S_{lat}	S_{lat} Change Rate/%
DOPC@Ag	0.90 $\pm$ 0.02	0.13 $\pm$ 0.01	
10 μ M BSA/DOPC@Ag	1.00 $\pm$ 0.04	0.20 $\pm$ 0.03	50.0
100 μ M BSA/DOPC@Ag	1.66 $\pm$ 0.09	0.64 $\pm$ 0.06	380.0
1.0 mM BSA/DOPC@Ag	1.26 $\pm$ 0.06	0.37 $\pm$ 0.04	180.0
10 μ M Cyt c/DOPC@Ag	1.31 $\pm$ 0.03	0.41 $\pm$ 0.02	205.0
100 μ M Cyt c/DOPC@Ag	1.30 $\pm$ 0.04	0.40 $\pm$ 0.03	200.0
10 μ M LF/DOPC@Ag	1.66 $\pm$ 0.04	0.64 $\pm$ 0.03	380.0
100 μ M LF/DOPC@Ag	1.7 $\pm$ 0.03	0.67 $\pm$ 0.02	400.0
1.0 mM LF/DOPC@Ag	2.89 $\pm$ 0.03	1.46 $\pm$ 0.02	995.0

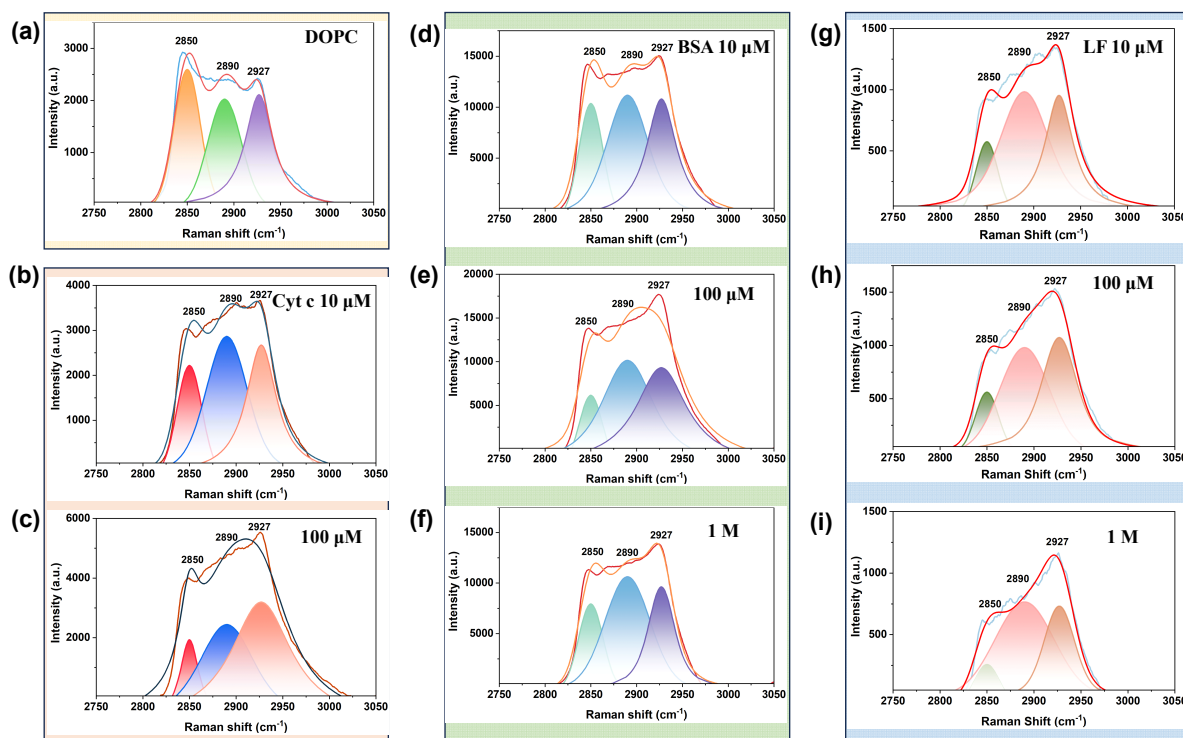


Figure 3. SERS spectra of DOPC@Ag (a) upon exposure to different proteins corresponding to BSA (b,c), Cyt c (d–f), and LF (g–i), with the concentration of 10, 100 μM , and 1.0 mM, respectively.

3.3. SERS Characterizations of Redox State Transitions of Cyt c on DOPC@Ag and RCM@Ag

We further investigated lipid behavior at the membrane interface during redox-state transitions. The significant attenuation of the characteristic peak at 1635 cm^{-1} serves as a key indicator of Cyt c reduction [32,33]. Figure 4a demonstrates that Cyt c immobilized on the DOPC@Ag nanocomposite surface remains fully reduced across the NADPH concentration range of 25–200 μM . Notably, at an NADPH concentration of 50 μM , Figure 4b–d shows a significant peak in the S_{lat} parameter for the phospholipid (Table 2), corresponding to an approximately 2.4-fold increase relative to the control group. This phenomenon elucidates the regulatory mechanism by which Cyt c-mediated redox reactions influence lipid-protein interfacial dynamics. During electron transfer, conformational changes in Cyt c likely attenuate van der Waals interactions between adjacent lipid acyl chains. This attenuation promotes an increase in the proportion of gauche conformers relative to all-trans conformers within the acyl chains, thereby reducing the lateral packing order. This molecular-level structural reorganization decreases the lateral packing density of the phospholipid membrane, inducing a localized relaxation effect within the bilayer microstructure. This dynamically modulated membrane environment significantly enhances fluidity and, crucially, establishes a low-energy-barrier interface conducive to conformational transitions of membrane-associated proteins and transmembrane substrate transport. Collectively, these structural adaptations likely underpin the system's superior performance in biosensing and targeted delivery applications. The S_{lat} parameter was calculated using the intensity ratio (I_{2890}/I_{2850}) of the characteristic vibrational bands at 2890 cm^{-1} (CH_2 antisymmetric stretching mode) and 2850 cm^{-1} (CH_2 symmetric stretching mode). The mean and variance of the I_{2890}/I_{2850} ratio are shown in Supplementary Figure S2.

Table 2. Percentage change in S_{lat} of DOPC@Ag following Cyt c reduction at varying NADPH concentrations.

NADPH (μM)	I_{2890}/I_{2850}	S_{lat}	S_{lat} Change Rate/%
0	1.26 ± 0.04	0.37 ± 0.03	
25	1.36 ± 0.02	0.44 ± 0.01	17.9
50	2.03 ± 0.09	0.89 ± 0.06	137.5
100	1.64 ± 0.05	0.63 ± 0.03	67.9
200	1.46 ± 0.04	0.51 ± 0.03	35.7

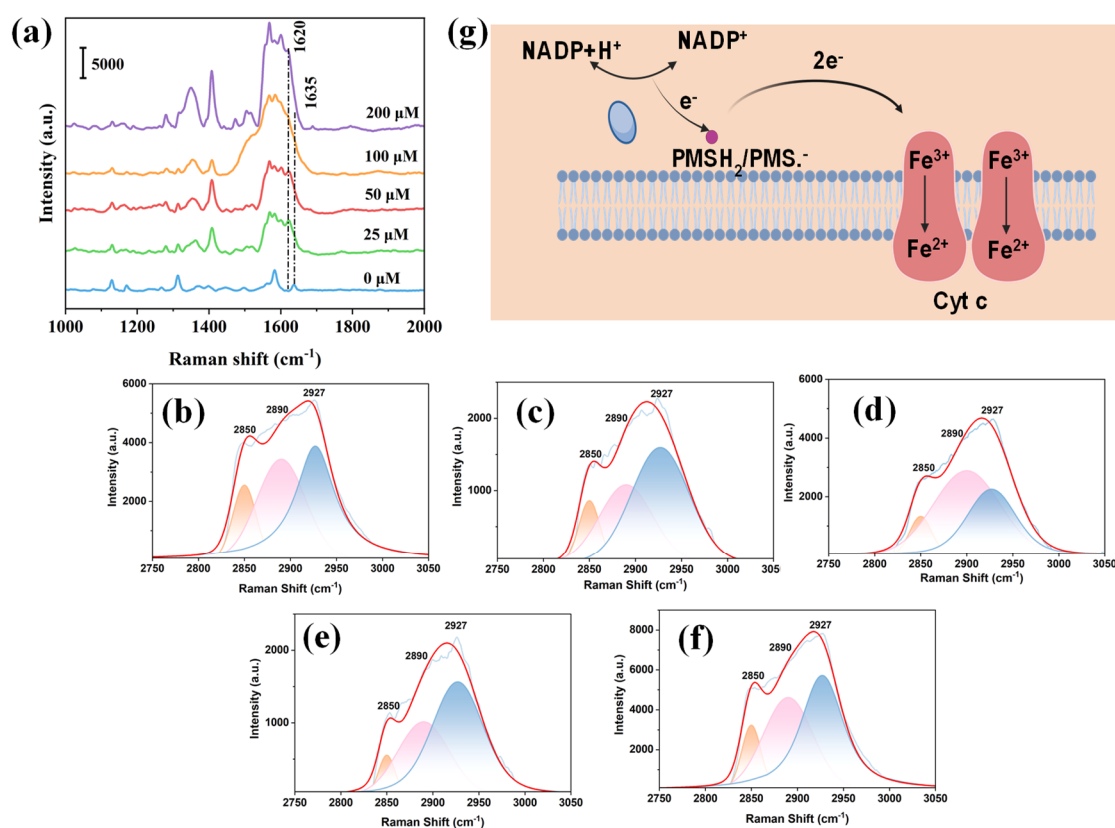


Figure 4. In situ monitoring of Cyt c redox state transformation *via* SERS. (a) SERS of lipids in the reduction process of cytochrome c adsorbed on DOPC@Ag. (b–f) respectively, with the concentration of 0, 25, 50, 100, and 200 μM NADPH. (g) The redox state transformation diagram of Cyt c.

To further verify the aforementioned conclusion regarding Cyt c redox states, we fabricated RCM@Ag from red blood cells and investigated its Cyt c redox states using SERS. RCM@Ag with an identical structure was prepared using readily available natural red blood cell membranes (RCM). As evidenced by the TEM results (Figure S3), the erythrocyte membrane prepared by the extrusion method successfully formed a homogeneous coating layer on the surface of Ag NPs. DLS characterization of the nanocomposite system (Figure S4a,b) reveals that upon RCM coating, the hydrodynamic diameter of the Ag NPs increased from 44 nm to 50 nm, while the surface zeta potential decreased significantly from −18 mV to −3.3 mV. This concurrent increase in size and reduction in surface charge indicates the formation of a stable erythrocyte membrane coating on the nanoparticle surface, mediated by electrostatic interactions. Notably, following the electrostatic assembly of Cyt c, the hydrodynamic diameter further increased to 58 nm, and the zeta potential rebounded to +2.9 mV. This charge strongly demonstrates the successful electrostatic anchoring of Cyt c onto the negatively charged cell membrane surface. The successful assembly of Cyt c on the erythrocyte membrane surface is confirmed, in good agreement with the above spectroscopic analysis, particle size, and zeta potential characterization (Figure S4).

The same spectral processing was applied to RCM@Ag, and the mean and variance of the I_{2890}/I_{2850} ratio are shown in Figure S5. Figure 5c–g demonstrate that surface-loaded Cyt c within a nanocomposite system undergoes a reduction reaction under a gradient NADPH concentration of 0–200 μM. Notably, at an NADPH concentration of 50 μM, the S_{lat} exhibited a significant response, with its relative change rate approximately 4.6-fold higher than that of the blank system (Table 3). This response quantitatively aligns with observations in the DOPC@Ag biomimetic membrane system (where the S_{lat} change rate increased by 2.4-fold), further confirming a universal regulatory mechanism whereby Cyt c-mediated redox reactions modulate phospholipid interfacial dynamics. System evidence suggests that Cyt c conformational changes during electron transfer influence membrane structure through two synergistic pathways: (1) charge fluctuations at its redox center directly weaken van der Waals interactions between acyl chains, and (2) increased rotational freedom of the heme prosthetic group induces local hydrodynamic perturbations. These effects systematically elevate the proportion of gauche conformers within the acyl chains. This molecular-scale dynamic restructuring reduces the lateral packing density of the phospholipid bilayer, forming a metastable, relaxed interfacial domain. Crucially, the higher S_{lat} responsivity observed in the native membrane system (3.1-fold vs. 2.4-fold) is likely attributable to cholesterol modulation. The rigid steroid

ring of cholesterol generates enhanced steric hindrance relaxation, thereby significantly amplifying the change in the order parameter [34–36]. This consistency in behavior between biomimetic and native systems provides a key theoretical basis for designing nanobiodevices that precisely regulate membrane environments.

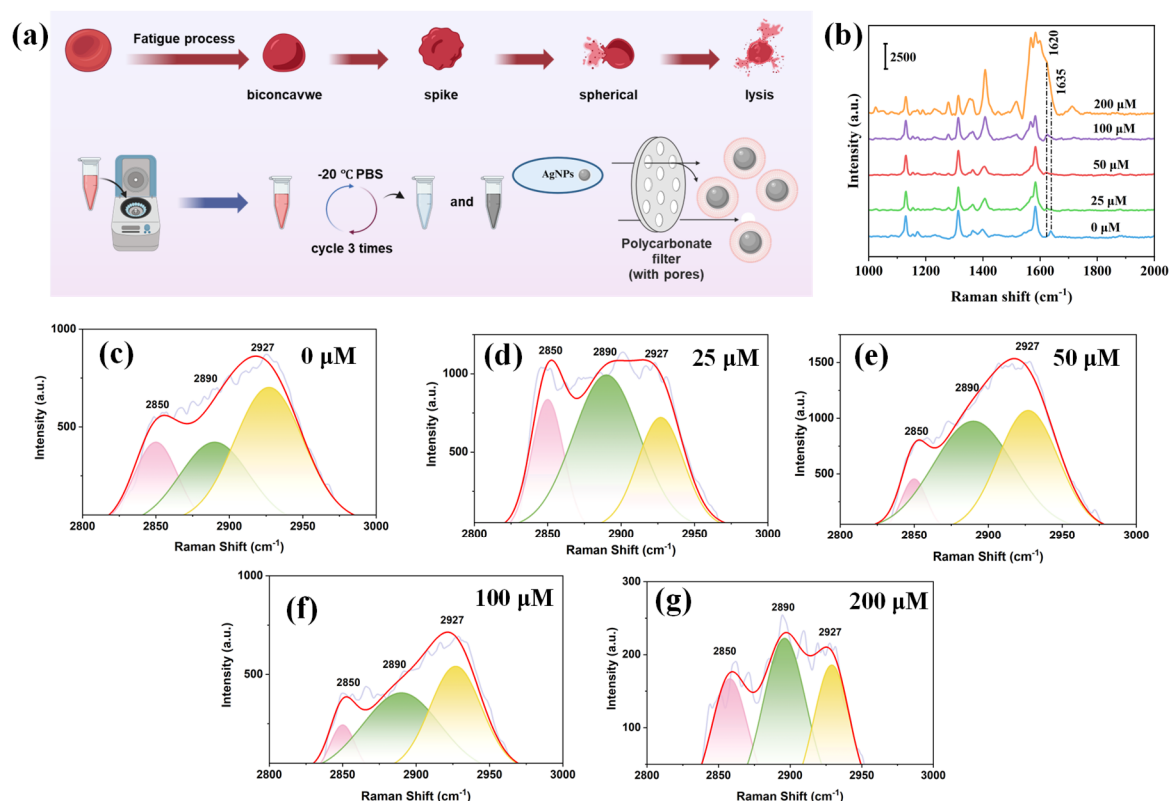


Figure 5. (a) Preparation flow chart of RCM@Ag. (b) SERS of lipids in the reduction process of Cyt c adsorbed on RCM@Ag. (c–g) respectively, with the NADPH concentrations of 0, 25, 50, 100, and 200 μM.

Table 3. Percentage changes in S_{lat} of RCM@Ag following Cyt c reduction at varying NADPH concentrations.

NADPH (μM)	I_{2890}/I_{2850}	S_{lat}	S_{lat} Change Rate/%
0	1.04 ± 0.03	0.23 ± 0.02	
25	1.20 ± 0.03	0.33 ± 0.02	47.0
50	2.09 ± 0.11	0.93 ± 0.07	308.8
100	1.58 ± 0.04	0.59 ± 0.03	158.8
200	1.49 ± 0.02	0.53 ± 0.01	132.3

4. Conclusions

We systematically elucidated the dynamic interaction mechanisms and concentration-dependent behaviors of BSA, Cyt c, and LF with DOPC phospholipid bilayers. Key findings reveal distinct protein-specific binding modes: BSA embeds in the hydrophobic membrane core *via* hydrophobic interactions, exhibiting weak adsorption at low concentrations and triggering deep insertion, acyl chain loosening, and the formation of flexible interfacial layers at high concentrations. Cyt c demonstrates orientation-dependent binding anchoring to polar headgroups *via* its heme moiety at low concentrations, while undergoing hydrophobic insertion that drives membrane softening and formation of a relatively compact yet flexible interface at elevated concentrations. LF exhibits concentration-dependent binding through strong electrostatic interactions and hydrophobic insertion, significantly enhancing lipid chain fluidity without compromising membrane integrity. Furthermore, the order parameter for lateral interactions of phospholipids S_{lat} (I_{2890}/I_{2850}) is established as a sensitive probe for quantifying phospholipid bilayer order, providing a novel basis for analyzing protein-membrane dynamic equilibria. Notably, the study reveals the redox-responsive behavior of Cyt c in both biomimetic (DOPC@Ag) and real cell membrane (RCM@Ag) systems, detailing its regulatory mechanism at the membrane interface. Redox reactions weaken van der Waals forces between acyl chains, increase gauche conformer populations, and reduce membrane lateral packing density. Comparative analysis highlights that cholesterol-mediated synergistic steric hindrance in real cell membranes significantly amplifies this interfacial remodeling. Collectively, this work elucidates the intrinsic molecular link between redox-

state transitions and membrane dynamic remodeling, providing a crucial theoretical foundation for designing redox-responsive biosensors and developing targeted delivery systems that leverage enhanced membrane fluidity.

Supplementary Materials: The following supporting information can be downloaded at: <https://media.scilit.com/articles/others/2606161457297769/MI-26030236-SM.pdf>. Figure S1. Normalized I_{2890}/I_{2850} ratio of DOPC@Ag treated with BSA, Cyt c, and LF at varying concentrations. Data are normalized to pure DOPC@Ag and presented as mean $\pm$ SD ($n = 3$). The ratio refers to the Raman peak intensity ratio I_{2890}/I_{2850} . Figure S2. Normalized I_{2890}/I_{2850} lipid ratio of DOPC@Ag during the reduction of adsorbed Cyt c. Data are normalized to pure DOPC@Ag and presented as mean $\pm$ SD ($n = 3$). The ratio refers to the Raman peak intensity ratio of lipid acyl chains at 2890 cm^{-1} and 2850 cm^{-1} . Figure S3. TEM images of RCM@AgNPs. Figure S4. (a) Zeta potential of Cyt c assembled RCM@AgNPs; (b) Size distribution of Cyt c assembled RCM@AgNPs. Figure S5. Normalized I_{2890}/I_{2850} lipid ratio of RCM@Ag during the reduction of adsorbed Cyt c. Data are normalized to pure RCM@Ag and presented as mean $\pm$ SD ($n = 3$). The ratio refers to the Raman peak intensity ratio of lipid acyl chains at 2890 cm^{-1} and 2850 cm^{-1} . Reference [37] are cited in supplementary materials.

Author Contributions: J.S. and S.X.: conceived the idea; J.S. and Y.C.: conducted material preparation and characterizations; X.W. and Y.Z.: performed TEM image and zeta potentials; J.Z.: designed the QCM experiment; J.S. and L.C.: performed the SERS experiment; J.S.: wrote the paper; J.C. and S.X.: supported the research with funding and supervision; S.X.: revised the draft. All authors have read and agreed to the published version of the manuscript.

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Breath-Based SERS Detection of Propofol Metabolites during Anesthesia Using Mesoporous Au Foam Chips

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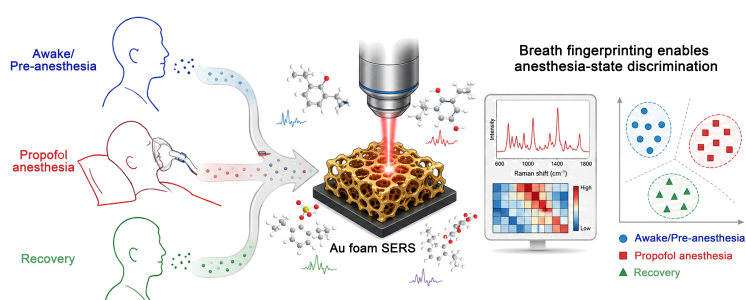
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Abstract: Propofol anesthesia is commonly guided by hemodynamic signs, infusion parameters, and electroencephalography-derived indices, yet noninvasive molecular approaches for distinguishing anesthesia-related states remain limited. Here, we developed a mesoporous Au foam-based surface-enhanced Raman spectroscopy



(SERS) chip for breath analysis of propofol metabolites signatures and anesthesia-state discrimination. The Au foam was fabricated by selective dealloying of Au-Ag alloy films, producing an interconnected ligament-pore architecture with a pore size of 55 nm and ligament width of 24 nm, which showed broad optical extinction near 785 nm, and strong SERS activity with an enhancement factor of approximately 6.84×10^5 , as well as spatially uniform mapping over $30 \times 30 \mu\text{m}^2$, batch-to-batch reproducibility, humidity tolerance, 30-day storage stability, and resistance to breath-like gas flow. Using propofol, 4-hydroxypropofol, propofol glucuronide, and propofol sulfate as representative targets, molecule-specific SERS fingerprints and marker peaks were identified, enabling excellent discrimination of these structurally related molecules with classification accuracy above 85% with multivariate analysis. Quantitatively, all of these selected marker peaks showed linear responses over 2–75 ppb, with detection limits down to 0.663 ppb and spike recoveries above 90% in artificial breath matrix. Finally, breath SERS fingerprints collected from awake/pre-anesthesia, propofol anesthesia, and recovery states were analyzed by spectral scoring, PCA, supervised classification, and anesthesia-probability output, which achieved an overall state-discrimination accuracy of 86.7%, demonstrating the potential of mesoporous Au foam SERS chips for noninvasive molecular assessment of propofol anesthesia-related breath states. This work offered a compact optical strategy for molecularly informed assessment of propofol anesthesia-related breath signatures.

Keywords: mesoporous Au foam; surface-enhanced Raman scattering; exhaled breath analysis; propofol metabolites; anesthesia-state discrimination

1. Introduction

Propofol is one of the most widely used intravenous anesthetics for the induction and maintenance of general anesthesia because of its rapid onset, controllable hypnotic effect, and short recovery profile [1,2]. In clinical practice, propofol administration is commonly guided by infusion models, hemodynamic responses, and electroencephalography-derived indices [1–3]. However, these indirect parameters do not provide direct molecular information on the dynamic distribution, metabolism, and elimination of propofol during anesthesia. Blood-based drug monitoring can offer more accurate pharmacokinetic information, but repeated blood sampling is invasive, discontinuous, and incompatible with real-time perioperative decision-making. Therefore, developing a



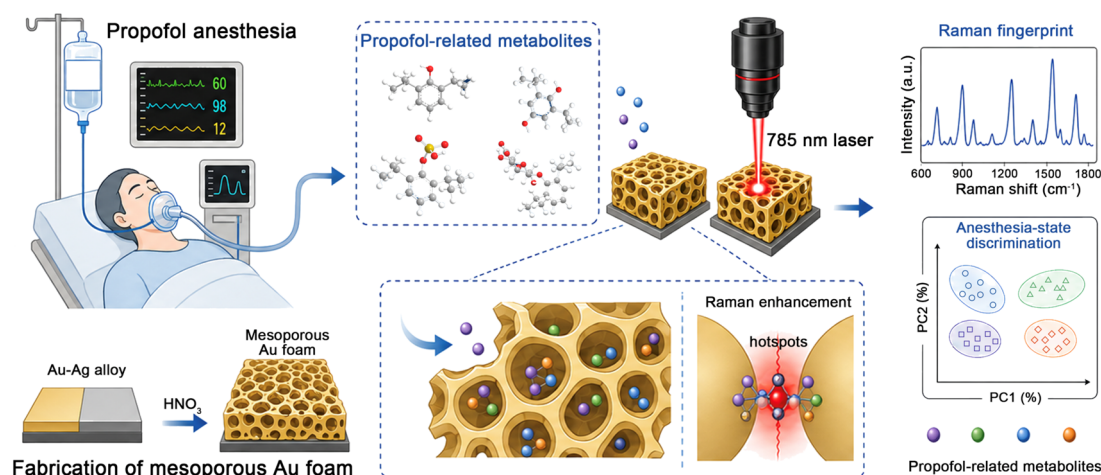
noninvasive analytical strategy capable of rapidly tracking propofol metabolites signatures would provide a valuable complement to conventional anesthesia monitoring.

Exhaled breath has emerged as an attractive matrix for noninvasive drug and metabolic monitoring because volatile and semi-volatile molecules can be continuously exchanged through the blood-air interface [4,5]. Previous studies have shown that propofol and volatile propofol-related species can be detected in exhaled breath during surgery, supporting the feasibility of breath-based monitoring of intravenous anesthesia [6–10]. Early real-time breath studies reported the detection of propofol and volatile metabolites, including 2,6-diisopropyl quinone and 2,6-diisopropyl phenol, in expired gases from patients undergoing surgery. More recent studies and reviews have further highlighted the potential of exhaled propofol monitoring using advanced analytical platforms such as proton-transfer-reaction mass spectrometry, selected-ion-flow-tube mass spectrometry, ion mobility spectrometry, secondary electrospray ionization high-resolution mass spectrometry, and gas chromatography-based techniques [6–12]. However, most breath-analysis technologies still rely on bulky instrumentation, complex ionization or separation modules, and specialized operating conditions, which limit their translation into compact, rapid, and real-time sensing formats.

Surface-enhanced Raman spectroscopy (SERS) provides a promising alternative for breath-based molecular detection because its molecular fingerprint specificity, high sensitivity, rapid optical readout, and compatibility with miniaturized instrumentation [13–16]. Unlike mass spectrometric methods that identify analytes by ion mass-to-charge ratios, SERS directly reports vibrational fingerprints of target molecules and their interfacial binding states, which is particularly attractive for detecting aromatic phenolic and quinone-like propofol metabolites. However, breath samples are highly humid, chemically complex, and contain target molecules at low concentrations [4,13,17]. Conventional planar noble-metal substrates often suffer from insufficient molecule capture, limited hotspot density, and poor reproducibility under gas-phase or high-humidity conditions. Therefore, a SERS interface specifically engineered for volatile molecule enrichment and stable signal amplification is needed.

Mesoporous gold structures offer a powerful materials platform for addressing limitations in breath samples analysis [18–22]. A well-established strategy of continuous mesoporous gold provided high surface area, abundant plasmonic nanogaps, tunable ligament/pore dimensions, and excellent chemical stability, which has been widely explored as a robust interface for molecular adsorption and a SERS substrate with electromagnetic enhancement, providing an attractive platform simultaneously as a molecular enrichment layer and a plasmonic hotspot scaffold [22–24].

Herein, we report a dealloyed mesoporous Au foam SERS chip for noninvasive breath analysis of propofol metabolites signatures and anesthesia-state discrimination (Scheme 1). The chip was prepared by selective dealloying of Au-Ag alloy films, producing a three-dimensional interconnected Au ligament–pore network with tunable structural parameters and broad optical extinction near the 785 nm Raman excitation wavelength, which provided a robust breath-compatible SERS interface, combining strong 4-mercaptobenzonitrile (4-MBN) enhancement, homogeneous signal distribution, batch reproducibility, humidity tolerance, storage stability, and resistance to breath-like gas flow. The Au foam-enhanced fingerprints enabled reliable multivariate discrimination of structurally related propofol species, with supervised classification achieving an accuracy above 85%. In artificial breath matrix, the selected marker peaks exhibited linear ppb-level responses over 2–75 ppb with low detection limits of 0.663–1.259 ppb, and spike recoveries above 90%, confirming the analytical reliability of the platform under breath-relevant conditions. Finally, breath SERS fingerprints from awake/pre-anesthesia, propofol anesthesia, and recovery states were analyzed through composite spectral scoring, PCA, supervised classification, and anesthesia-state probability output, achieving an overall state-discrimination accuracy of 86.7%, which can identify awake/pre-anesthesia and propofol anesthesia states with accuracies of 93.3% and 90.0%, respectively. This work demonstrated a mesoporous Au foam SERS interface that integrated breath-compatible molecular enrichment, ppb-level detection, and state discrimination, offering a compact optical strategy for molecularly informed assessment of propofol anesthesia-related breath signatures.



Scheme 1. Mesoporous Au foam-based SERS platform for propofol-related metabolites in exhaled breath detection.

2. Experimental Section

2.1. Fabrication of Mesoporous Au Foam Chips

Mesoporous Au foam chips were fabricated by selective dealloying of Au-Ag alloy films. Briefly, Si/SiO₂ substrates were sequentially cleaned by sonication in acetone, ethanol, and ultrapure water, followed by drying under a nitrogen stream. Au-Ag alloy films were then deposited onto the cleaned substrates by magnetron sputtering. The obtained Au-Ag alloy films were immersed in nitric acid solution to selectively remove Ag and generate an interconnected porous Au network. The dealloying time was varied to tune the porous structure, giving Au foam samples dealloyed for 5, 15, and 30 min. After dealloying, the chips were thoroughly rinsed with ultrapure water and ethanol to remove residual acid and dissolved metal ions, and then dried under nitrogen.

2.2. Structural and Compositional Characterization

The morphology of the Au-Ag alloy films and dealloyed Au foam chips was characterized by field-emission scanning electron microscopy (Zeiss GeminiSEM 300, Carl Zeiss Microscopy GmbH, Jena, Germany) operated at an accelerating voltage of 3–5 kV. Transmission electron microscopy (TEM), high-resolution TEM (HRTEM), high-angle annular dark-field scanning TEM (HAADF-STEM), and energy-dispersive X-ray spectroscopy (EDS) mapping were performed on a JEM-2100F transmission electron microscope (JEOL, Tokyo, Japan). Atomic force microscopy (AFM) was performed using a Dimension Icon AFM system (Bruker, Billerica, MA, USA) in tapping mode under ambient conditions. Nitrogen adsorption-desorption measurements were conducted on a Micromeritics ASAP 2460 surface area and porosity analyzer (Micromeritics, Norcross, GA, USA) at 77 K. X-ray diffraction (XRD) patterns were recorded using an X-ray diffractometer (SmartLab, Rigaku, Tokyo, Japan) equipped with a Cu K α radiation source ($\lambda = 1.5406 \text{ \AA}$). The diffraction data were collected over a 2θ range of 30–90° at a scanning rate of 2–5° min⁻¹. X-ray photoelectron spectroscopy (XPS) was performed on an ESCALAB 250Xi spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) using a monochromatic Al K α X-ray source ($h\nu = 1486.6 \text{ eV}$). UV-vis-NIR extinction spectra of the Au-Ag alloy and Au foam substrates were recorded using a UV-vis-NIR spectrophotometer (UV-3600, Shimadzu, Kyoto, Japan) over the wavelength range of 400–1000 nm. The optical extinction around the 785 nm Raman excitation wavelength was specifically analyzed to evaluate the plasmonic compatibility of the Au foam substrate for SERS measurements. Raman and SERS spectra were collected using a confocal Raman microscope (Renishaw inVia, Renishaw plc, Wotton-under-Edge, Gloucestershire, UK) equipped with a 785 nm diode laser. The laser was focused onto the sample surface through a 50 $\times$ objective lens. The laser power at the sample surface was controlled at 1 mW. The spectral acquisition time was 10 s, and each spectrum was accumulated 3 times.

2.3. 4-MBN Modification and SERS Performance Evaluation

4-MBN was used as a standard Raman reporter to evaluate the SERS activity of the Au foam substrates. Before modification, the Au foam chips were rinsed sequentially with ethanol and ultrapure water, dried under a nitrogen stream, and treated by UV-ozone for 10 min to remove possible organic contaminants. A 4-MBN stock solution was prepared in ethanol and diluted to a working concentration of 1 mM. The cleaned Au foam chips were immersed in the 4-MBN ethanol solution at room temperature for 12 h. After incubation, the chips were

thoroughly rinsed with ethanol three times to remove physically adsorbed or unbound 4-MBN molecules, followed by drying under nitrogen. The obtained 4-MBN-modified Au foam chips were used immediately for SERS measurements or stored in a dry container before testing.

2.4. SERS Detection of Propofol Metabolites

Propofol, 4-hydroxypropofol, propofol glucuronide, and propofol sulfate were selected as representative propofol metabolites for SERS fingerprint analysis. Stock solutions of each compound were prepared in ethanol or ethanol/water mixed solvent depending on molecular solubility. For propofol and 4-hydroxypropofol, ethanol was used as the primary solvent. For propofol glucuronide and propofol sulfate, ethanol/water mixed solvent was used to improve dissolution. Before SERS measurement, the stock solutions were diluted to the desired working concentrations. For molecular fingerprint acquisition, the optimized Au foam chips were first rinsed with ethanol and ultrapure water, dried under nitrogen, and then exposed to the target molecular solution. Typically, 20–50 μL of the target solution was dropped onto the Au foam surface and incubated at room temperature for 10–30 min to allow molecular adsorption into the porous Au network. After incubation, the chip was gently rinsed with the corresponding solvent to remove excessive unbound molecules and dried under nitrogen. Blank Au foam chips treated with solvent alone were measured as background controls.

2.5. Artificial Breath Preparation and Gas-Phase SERS Calibration

Artificial breath matrix was prepared to simulate the humid gas environment of exhaled breath. Briefly, carrier gas was passed through a humidification chamber containing ultrapure water to generate humidified gas. The relative humidity was adjusted by controlling the humidification temperature and gas-flow ratio. For matrix-matched calibration experiments, known amounts of propofol metabolites were introduced into the artificial breath stream to generate ppb-level vapor or aerosol-associated standards. The concentration range was set as 0, 2, 5, 10, 20, 35, 50, and 75 ppb, covering the breath-relevant low-concentration range.

For each measurement, the artificial breath containing the target molecule was directed onto the optimized Au foam SERS chip for 5 min, to allow molecular adsorption/enrichment within the porous Au foam. After exposure, the chip was immediately transferred to the Raman microscope for SERS measurement. The same Raman acquisition parameters were used throughout the calibration experiments.

2.6. Spike-Recovery Experiments in Artificial Breath Matrix

Spike-recovery experiments were performed to evaluate the quantitative accuracy of the Au foam SERS platform in artificial breath matrix. Propofol, 4-hydroxypropofol, propofol glucuronide, and propofol sulfate were individually spiked into artificial breath matrix at 10 ppb. The spiked artificial breath was introduced onto the Au foam chip under the same gas-exposure conditions used for calibration. After exposure, SERS spectra were collected, and the corresponding marker peak intensities were extracted. The measured concentration of each target molecule was calculated from its matrix-matched calibration curve. The recovery rate was calculated according to:

$$\text{Recovery (\%)} = C_{\text{found}}/C_{\text{spiked}} \times 100\%$$

where C_{found} is the calculated concentration from the calibration curve and C_{spiked} is the spiked concentration.

2.7. Anti-Interference Evaluation under Breath-Relevant Conditions

To evaluate the selectivity and anti-interference capability of the Au foam SERS platform, common breath-related volatile compounds were introduced into the artificial breath matrix. The tested interferents included acetone, ethanol, isoprene, ammonia, acetaldehyde, and a mixture of these interferents. High-humidity mixed-interferent conditions were also tested by introducing the interferent mixture into artificial breath at 95% relative humidity. For each anti-interference experiment, the target molecule was maintained at a fixed concentration, while the interferent was introduced into the artificial breath stream. The signal retention of each marker peak was calculated as:

$$\text{Signal retention (\%)} = I_{\text{interferent}}/I_{\text{control}} \times 100\%$$

where $I_{\text{interferent}}$ is the marker peak intensity measured in the presence of interferents, and I_{control} is the marker peak intensity measured in artificial breath without interferents.

2.8. Breath Sample Collection and Au Foam SERS Analysis

This study was approved by the Ethics Committee of Shanghai First People's Hospital, with the approval number 2025-192. Basic demographic and clinical information of the enrolled patients, including age, gender, ASA physical status, surgical type, and anesthesia protocol. Breath samples were collected from three anesthesia-related states: awake/pre-anesthesia, propofol anesthesia, and recovery. Breath samples were collected using a breath sampling device. The exhaled breath was transferred and directed onto the Au foam chip for molecular adsorption and enrichment for 30 min. After breath exposure, the Au foam chip was immediately subjected to Raman measurement. SERS spectra were collected in the 600–1800 cm^{-1} fingerprint region.

It should be noted that awake/pre-anesthesia samples were collected before propofol administration, anesthesia-state samples were collected after a stable propofol anesthesia condition was reached, and recovery samples were collected after discontinuation of propofol during the recovery period. Patients with severe respiratory disease, severe hepatic or renal dysfunction, active infection, metabolic disorders, recent alcohol intake, or other conditions that could markedly influence breath VOC profiles were excluded.

2.9. Breath SERS Score Calculation

For breath-state analysis, the integrated areas of four propofol-related spectral regions were extracted from each breath spectrum: 842 cm^{-1} region, 1048–1088 cm^{-1} region, 1452 cm^{-1} region, and 1600 cm^{-1} region. Each regional area was normalized across all breath samples. A composite breath SERS score was then calculated by averaging the normalized integrated areas:

$$\text{Breath SERS score} = (I_{842} + I_{1048-1088} + I_{1452} + I_{1600})/4$$

where I represents the normalized integrated area of each selected spectral region.

2.10. PCA and Classification Analysis

Principal component analysis was performed to visualize the unsupervised separation of breath SERS fingerprints from different anesthesia-related states. Input features included the integrated areas and peak intensities of the selected propofol-related spectral regions. Before PCA, all features were standardized to zero mean and unit variance. The first two principal components were used to construct the PCA score plot. Meanwhile, supervised classification was performed using a random forest (RF) classifier. The input features were manually selected based on the assigned Raman marker peaks and propofol-related spectral regions. For molecular-standard classification, representative marker-peak intensities were used as input features. For breath-state classification, the integrated areas and peak intensities of selected spectral regions were extracted. The dataset was divided into training and testing sets using a stratified strategy to maintain class balance. Five-fold cross-validation was performed within the training set for model optimization, and the independent testing set was used for final evaluation.

3. Results and Discussion

3.1. Fabrication and Structural Characterization of Dealloyed Mesoporous Au Foam Chips

To construct a plasmonically active interface for breath-based SERS detection, mesoporous Au foam chips were fabricated through a dealloying strategy using Au-Ag alloy films as sacrificial precursors. As schematically illustrated in Figure 1A, the morphological evolution of the Au foam was first examined by scanning electron microscopy. The pristine Au-Ag alloy film exhibited a relatively compact and continuous surface, without obvious nanoscale porosity. After dealloying, a distinct porous morphology was clearly observed, confirming the successful transformation from a dense alloy film into an interconnected Au foam structure (Figure 1B). The dealloyed Au foam displayed a continuous ligament-pore network over a large area, indicating that selective Ag removal occurred uniformly across the film. By adjusting the dealloying time, the pore structure could be systematically regulated. As displayed in Figure S1, the Au foam sample dealloyed for 5 min showed small and densely distributed pores with an average pore size of approximately 24 ± 6 nm and a ligament width of approximately 13 ± 3 nm, suggesting an early-stage dealloying structure with fine Au ligaments but partially developed pore channels. With the dealloying time extended to 15 min, Au foam exhibited a more open and well-connected porous network, with an average pore size of approximately 55 ± 9 nm and a ligament width of approximately 24 ± 4 nm. This structure provided a favorable balance between accessible pores for molecule diffusion and nanoscale Au ligaments for plasmonic hotspot generation. Further prolonging the dealloying time to 30 min led to obvious coarsening of the porous framework, giving enlarged pores of approximately 105 ± 18 nm and thicker ligaments of approximately 42 ± 6 nm. Such ligament coarsening reduced the density of nanoscale

junctions and narrow interligament gaps, which are critical for electromagnetic field localization. Thus, the Au foam with dealloying time 15 min sample was selected as the optimized substrate because it preserved a continuous Au ligament network with moderate ligament width while providing sufficiently open pores for volatile-molecule access. Moreover, as shown in Figure 1C, the porous Au layer was continuously attached to the Si/SiO₂ substrate with a depth of 330 nm, suggesting good structural integrity after dealloying. Transmission electron microscopy (TEM) image revealed that the Au foam consisted of interconnected nanoscale ligaments and open pores, which was consistent with the SEM results (Figure 1D). The high-resolution TEM image displayed clear lattice fringes with an interplanar spacing of approximately 0.235 nm, which can be assigned to the Au (111) plane, confirming the crystalline nature of the dealloyed Au ligaments. In addition, The High-angle annular dark-field (HAADF) image and corresponding energy dispersive spectrometer (EDS) mapping showed that Au was continuously distributed throughout the porous ligament network, whereas the Ag signal was greatly weakened after dealloying, indicating efficient removal of Ag from the precursor alloy film (Figure 1E). In addition to electron microscopy, atomic force microscopy (AFM) analysis further quantified the roughness evolution associated with dealloying (Figure S2). The Au-Ag alloy precursor showed a relatively smooth surface with low arithmetic average roughness (Ra) and root-mean-square roughness (Rq) <5 nm. In contrast, the Au foam displayed a significant increase in roughness with Ra and Rq values reaching approximately 17.5 nm and 22.0 nm, respectively, indicating the presence of nanoscale protrusions and recessed pore structures across the dealloyed surface, which was consistent with the SEM observation.

To further support the porous nature of the Au foam, Brunauer-Emmett-Teller (BET) nitrogen adsorption-desorption analysis was performed. As shown in Figure S3, The Au foam showed a type-IV-like adsorption-desorption isotherm with an apparent hysteresis loop, indicating the presence of accessible mesoporous/macroporous channels. The BET surface area was approximately 21–22 m² g^{−1}, and the total pore volume was approximately 0.292 cm³ g^{−1}. The BJH pore-size distribution showed a dominant pore-size peak centered at approximately 55 nm, together with a smaller shoulder around 23–26 nm, which was consistent with the SEM-derived average pore diameter of Au foam. The smaller shoulder may originate from pore throats and nanoscale channels within the interconnected Au foam network.

Furthermore, as shown in Figure 1F, the dealloyed samples exhibited characteristic X-ray diffraction (XRD) pattern corresponding to face-centered cubic Au, including Au (111), Au (200), Au (220), and Au (311). Compared with the Au-Ag alloy precursor, the diffraction peaks of the dealloyed Au foam shifted toward the standard Au positions, further supporting the removal of Ag and the formation of an Au-rich porous framework. The high-resolution X-ray photoelectron spectroscopy (XPS) survey spectra provided additional evidence for the compositional change. As displayed in Figure 1G, in the Au-Ag alloy precursor, the dominant Au-related signals were observed at approximately 84.0 and 87.7 eV, corresponding to Au 4f_{7/2} and Au 4f_{5/2}, respectively, confirming the metallic Au state in the alloy film. The alloy precursor also exhibited characteristic Ag peaks, including Ag 3d_{5/2} and Ag 3d_{3/2} at approximately 368.2 and 374.2 eV. After dealloying, the Au 4f doublet remained clearly visible, no obvious positively shifted Au oxide peak was observed in the survey spectra, supporting the chemical stability of the dealloyed Au foam. While the Ag 3d signals were markedly weakened, indicating the selective removal of Ag from the alloy matrix and the formation of an Au-rich porous framework.

The optical response of the Au foam chips was then evaluated by UV-vis-NIR spectroscopy (Figure 1H). Compared with the relatively smooth Au-Ag alloy precursor, the dealloyed Au foam exhibited a broad extinction response across the visible to near-infrared region centered at ~700 nm, which can be attributed to the interconnected Au ligament network, nanoscale curvature, and heterogeneous pore geometry. Importantly, the Au foam maintained strong optical extinction around the commonly used Raman excitation wavelength of 785 nm, enabling sufficient SERS activity towards standard Raman reporter 4-MBN which can be stably anchored on Au through Au-S bonding. As shown in Figure S4, 4-MBN exhibited characteristic Raman bands of ring vibration modes at approximately 1589 cm^{−1}, together with a distinct nitrile stretching band near 2222 cm^{−1} [25,26]. Compared with the Si/SiO₂, the dealloyed Au foam chips showed dramatically enhanced 4-MBN signals with an enhancement factor about 6.84×10^5 , confirming the formation of abundant SERS-active sites after dealloying. Collectively, the dealloyed Au foam offers an optimized SERS-active interface for converting weak surface-bound molecular vibrations into clearly distinguishable Raman fingerprints, providing the analytical foundation for subsequent breath detection of propofol-related species.

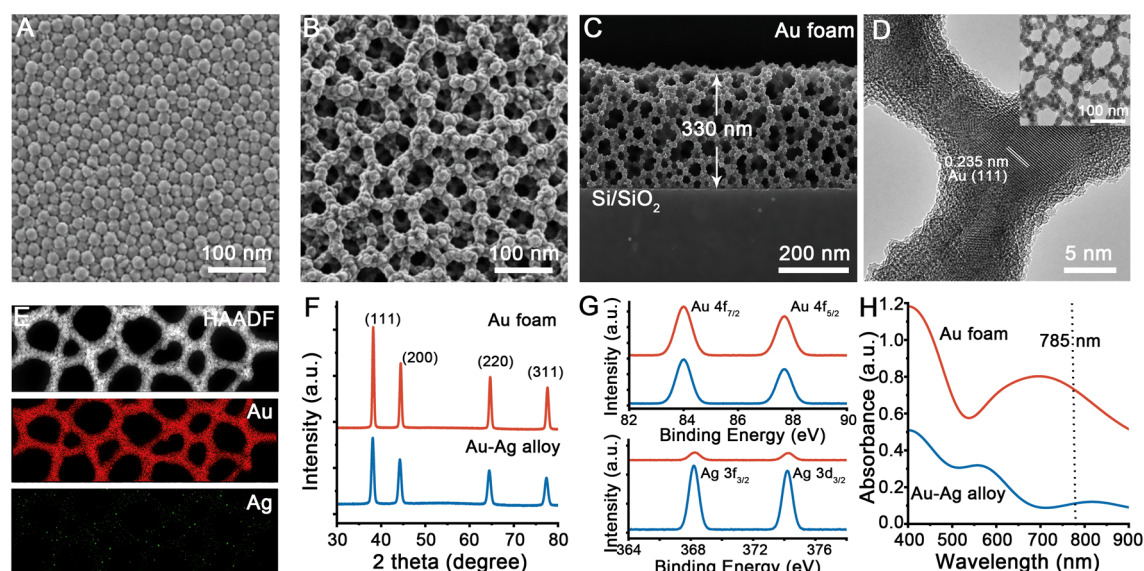


Figure 1. (A,B) SEM images of (A) Au-Ag alloy and (B) Au foam. (C) SEM side view of Au foam platform, (D) High resolution TEM image of Au foam, inset: TEM image of Au foam. (E) EDS mapping of Au foam. (F) XRD pattern of Au-Ag alloy and Au foam. (G) XPS spectra of Au 4f and Ag 3d on Au foam. (H) UV-Vis spectra of Au-Ag alloy and Au foam.

3.2. SERS Performance and Stability of Optimized Au Foam Substrates

Further SERS performance comparison was performed Figure 2A. All 4-MBN-modified substrates displayed two characteristic Raman bands, which were similar with Au foam. Significantly, compared with the flat Au substrate and the undealloyed Au-Ag alloy film, the dealloyed Au foam substrates generated much stronger SERS signals, confirming that the porous Au architecture formed during dealloying effectively enhanced the Raman scattering of 4-MBN. Moreover, as quantified in Figure 2B, the Au foam dealloyed for 15 min showed the highest 2226 cm^{-1} intensity among all tested substrates, whereas the Au foam dealloyed for 5 min and 30 min produced weaker responses, which was consistent with the structural evolution described above. Specifically, the 5 min dealloyed Au foam contained relatively small pores, which may limit molecular accessibility to the inner porous network. In contrast, prolonged dealloying for 30 min resulted in enlarged pores and coarsened ligaments, reducing the density of nanoscale junctions and plasmonic hotspots. Moreover, the Au foam dealloyed for 15 min exhibited the highest enhancement factor among the representative Au foam substrates, further confirming that the intermediate dealloying condition produced the most effective SERS-active structure (Figure 2C).

Furthermore, SERS mapping was performed on the Au foam substrate using the 2226 cm^{-1} peak intensity as the readout signal to evaluate the spatial signal uniformity. As shown in Figure 2D, the mapping image collected over a $30 \times 30\text{ }\mu\text{m}^2$ area displayed a relatively homogeneous signal distribution without large inactive regions. The corresponding intensity distribution histogram further showed that the measured intensities were concentrated within a narrow range (RSD $\approx$ 9%), which can be attributed to the continuous three-dimensional Au ligament network, providing distributed SERS-active sites rather than isolated hotspots. In addition, the signal reproducibility of the optimized Au foam substrate was further evaluated under different breath-relevant atmospheres. As shown in Figure 2E, the 2226 cm^{-1} signal remained relatively stable under dry N₂, different relative humidity conditions, and artificial breath. Although a slight decrease in signal intensity was observed under high-humidity and artificial breath conditions, the overall SERS response was well preserved, suggesting that the Au foam substrate can maintain stable molecular readout in humid environments, which is essential for subsequent breath analysis. Similarly, the 2226 cm^{-1} intensity showed only moderate attenuation during high-humidity exposure to 95% RH for different durations, indicating that the 4-MBN-modified Au foam retained its SERS activity under prolonged humid conditions, which is likely associated with the chemical inertness of the Au-dominant framework and the open porous structure (Figure 2F).

As shown in Figure 2G, the 2226 cm^{-1} intensities obtained from different batches remained comparable, demonstrating good batch-to-batch reproducibility of the dealloying-based fabrication strategy (RSD < 2.5%). Meanwhile, the long-term storage stability of the optimized Au foam substrate was also investigated in Figure 2H, the 2226 cm^{-1} signal was largely retained over 30 days of storage with only a gradual decrease in intensity. Finally,

as shown in Figure 2I, the SERS signal showed only a moderate decrease after repeated flow exposure, indicating that the Au foam substrate can withstand gas flushing without severe signal loss.

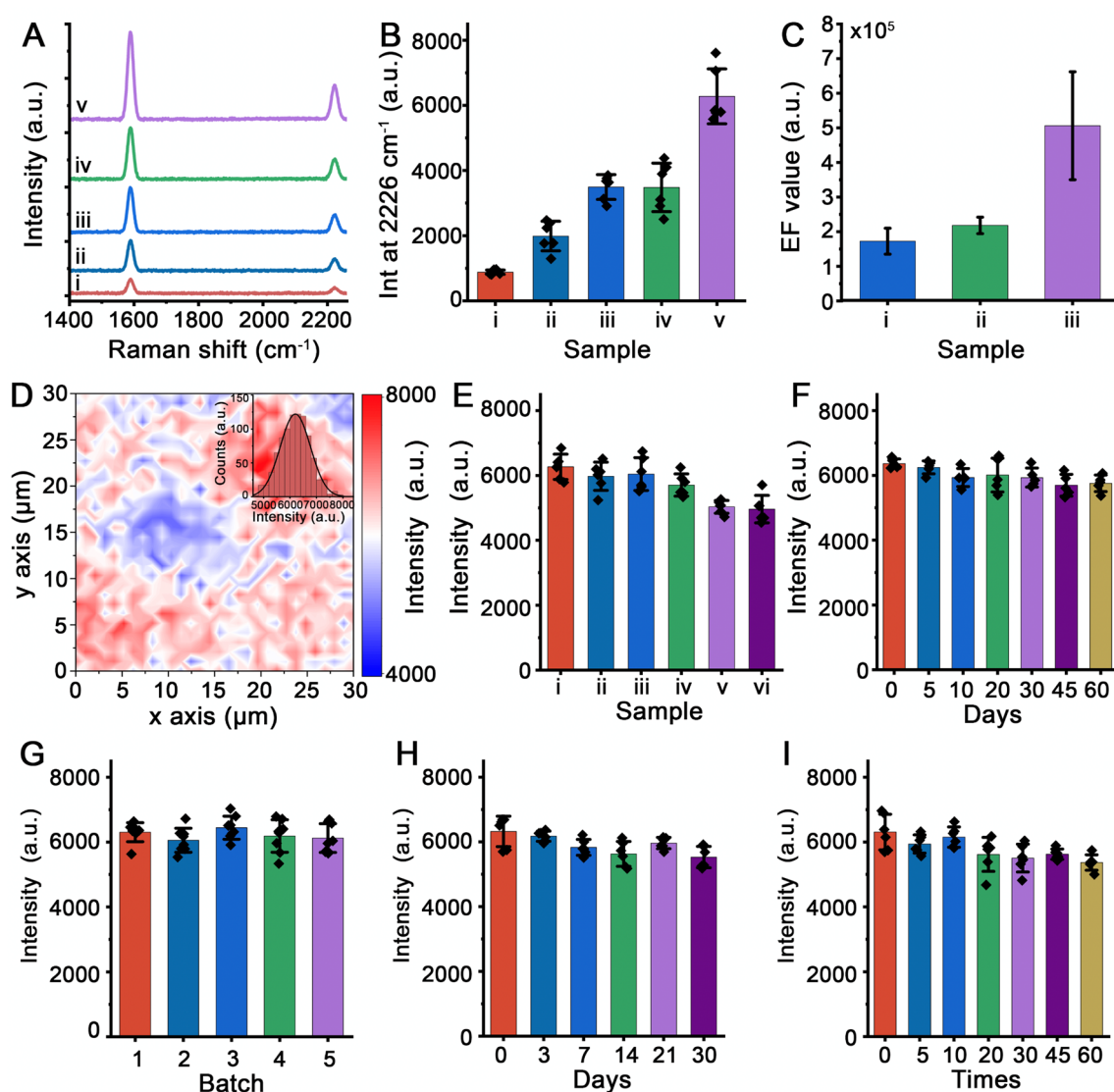


Figure 2. (A) Representative SERS spectra of 4-MBN collected from different substrates (i) flat Au substrate, (ii) Au-Ag alloy; (iii) Au foam dealloyed for 5 min; (iv) Au foam dealloyed for 30 min; (v) Au foam dealloyed for 15 min. (B) Quantitative comparison of the 2226 cm^{-1} peak intensity among samples in Figure 2A. (C) Calculated SERS enhancement factor values based on the 2226 cm^{-1} C≡N stretching band of 4-MBN for representative samples, Au foam dealloyed for (i) 5 min; (ii) 30 min; (iii) 15 min. (D) SERS mapping image of the optimized Au foam substrate based on the 2226 cm^{-1} peak intensity over a $30 \times 30 \mu\text{m}^2$ area. Inset: the corresponding intensity distribution histogram. (E) Signal reproducibility of the Au foam substrate measured under different breath atmospheres (i) dry N_2 , (ii) 40% RH, (iii) 60% RH, (iv) 80% RH, (v) 95% RH, (vi) artificial breath. (F) SERS signal stability under 95% RH conditions storage for different days. (G) Batch-to-batch reproducibility of five independently prepared Au foam substrates. (H) Storage stability of the Au foam substrate over 30 days. (I) Signal retention after repeated breath-like gas flow exposure for different times. Error bars represent standard deviations from repeated measurements.

3.3. SERS Fingerprint Discrimination of Propofol Metabolites

To further investigate the resolve molecular fingerprints ability associated with propofol metabolism, a representative propofol metabolites library was established, including propofol, 4-hydroxypropofol, propofol glucuronide, and propofol sulfate, which were selected to represent the parent anesthetic molecule together with structurally related oxidative and conjugated products [27,28]. As shown in Figure 3A, propofol, 4-hydroxypropofol, propofol glucuronide, and propofol sulfate were selected as representative propofol-related model analytes, which covered the major structural categories involved in propofol biotransformation, including

the parent drug, oxidative aromatic hydroxylation product, O-glucuronide conjugate, and O-sulfate conjugate. Figure S5 indicated that the SERS signal increased rapidly during the initial exposure stage and reached a near-plateau after approximately 5 min, indicating that the adsorption/enrichment of propofol metabolites on the mesoporous Au foam substrate had approached a stable state. As shown in Figure 3B, the blank Au foam substrate showed only weak background signals, whereas propofol, 4-hydroxypropofol, propofol glucuronide, and propofol sulfate all produced clear and distinguishable SERS spectra. For propofol, the band at 755 cm^{-1} was tentatively assigned to aromatic ring deformation coupled with isopropyl-related vibration, while the peak at 1008 cm^{-1} was attributed to aromatic ring breathing. The bands at 1178 and 1266 cm^{-1} were assigned to phenolic C-O stretching and C-H in-plane bending vibrations. The strong peak at 1452 cm^{-1} was associated with C-H bending from the isopropyl groups, and the band at 1602 cm^{-1} was attributed to aromatic C=C stretching.

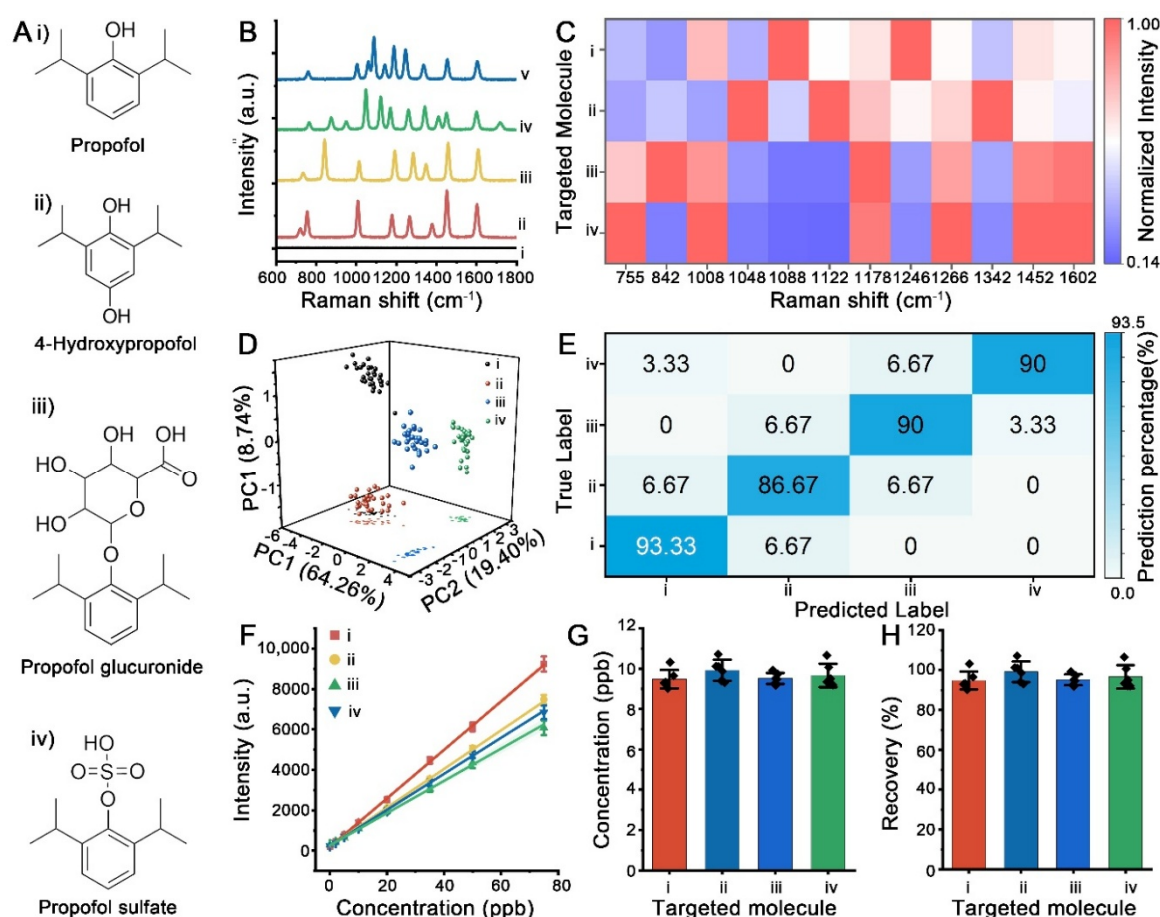


Figure 3. SERS fingerprint discrimination and ppb-level artificial-breath validation of propofol metabolites on the optimized Au foam substrate. (A) Chemical structures of the selected propofol metabolites library: (i) propofol, (ii) 4-hydroxypropofol, (iii) propofol glucuronide, and (iv) propofol sulfate. (B) Representative SERS spectra collected on the optimized Au foam substrate, including (i) blank Au foam and the four target molecules (ii) propofol, (iii) 4-hydroxypropofol, (iv) propofol glucuronide, and (v) propofol sulfate. (C) Heatmap of selected Raman features extracted from the SERS spectra. (D) PCA score plot based on selected SERS features ($n = 30$). (E) Row-normalized confusion matrix for supervised classification of the target molecules using selected Raman features ($n = 30$). (F) Matrix-matched calibration curves of the four target molecules in artificial breath matrix at ppb-level concentrations ($n = 5$). (G) Calculated concentrations of 10 ppb spiked target molecules in artificial breath matrix ($n = 5$). (H) Corresponding spike recovery rates of the four target molecules in artificial breath matrix ($n = 5$). Unless otherwise indicated, labels i–iv correspond to propofol, 4-hydroxypropofol, propofol glucuronide, and propofol sulfate, respectively.

Compared with propofol, 4-hydroxypropofol exhibited altered relative intensities and additional hydroxylation-sensitive features. In particular, the band around 842 cm^{-1} became more prominent, which may be associated with hydroxylation-induced changes in aromatic ring deformation and C-O-related vibrations. The enhanced features near 1192 , 1284 , 1458 , and 1608 cm^{-1} further indicate that the introduction of an additional hydroxyl group changes the local vibrational environment of the aromatic ring. Additionally, propofol glucuronide

displayed a different spectral pattern with characteristic bands around 1048, 1122, 1170, 1260, and 1342 cm^{-1} , which can be attributed mainly to C-O, C-O-C, and glycosidic-bond-related vibrations from the glucuronide moiety, and coupled aromatic vibrations from the propofol skeleton. Meanwhile, propofol sulfate showed pronounced features around 1088, 1188, and 1246 cm^{-1} , consistent with sulfate-associated vibrations and substituent-sensitive aromatic modes. These peak assignments suggest that although the four molecules share a related aromatic framework, their peripheral metabolic modifications introduce distinct vibrational signatures that can be captured by the Au foam SERS substrate (Table S1).

Furthermore, a set of representative Raman features was extracted to construct a feature-intensity heatmap (Figure 3C). The intensity of each Raman feature was normalized to the maximum value among the target molecules, allowing direct comparison of the relative contribution of each feature to molecular discrimination, clearly showing that propofol was characterized by strong aromatic and phenolic features, 4-hydroxypropofol by hydroxylation-sensitive vibrations, propofol glucuronide by C-O/C-O-C-dominated features, and propofol sulfate by sulfate-associated vibrations. Typically, several molecule-specific bands with high intensity and low spectral overlap were identified as candidate quantitative marker peaks, including 1452 cm^{-1} for propofol, 842 cm^{-1} for 4-hydroxypropofol, 1048 cm^{-1} for propofol glucuronide, and 1088 cm^{-1} for propofol sulfate. PCA was then performed using the selected SERS features to evaluate the discrimination ability towards these propofol metabolites in an unsupervised manner. As shown in Figure 3D, the four target molecules formed distinguishable clusters in the PCA score space. The PC1, PC2 and PC3 were explained 64.3%, 19.4% and 8.74% of the total variance, respectively, accounting for 100% of the spectral variance in combination, suggesting that differences in the Au foam-enhanced SERS fingerprints are sufficient to differentiate propofol and its related molecular derivatives. To further assess the classification capability of the SERS fingerprints, a supervised classification model was trained using the selected Raman features [29,30]. The row-normalized confusion matrix shown in Figure 3E demonstrated accurate classification of the four propofol metabolites using a balanced test set containing 30 spectra per class. The overall classification accuracy reached over 85%, indicating that the spectral differences observed in the SERS fingerprints could be translated into reliable molecular recognition.

Moreover, as shown in Figures S6 and S7, common breath-related interferents, including acetone, ethanol, isoprene, ammonia, acetaldehyde, mixed interferents, and high-humidity mixed interferents, caused only limited signal variation in the molecule-specific marker peaks. The propofol-related marker signals were largely retained under single-interferent conditions, and remained detectable over 80%, even in the presence of mixed interferents and 95% RH background, indicating the suitability for subsequent artificial-breath and real-breath analysis.

Furthermore, matrix-matched calibration curves were established at ppb-level concentrations. As shown in Figure 3F, the SERS intensities of these marker peaks increased linearly with concentration over the tested range of 2–75 ppb with an excellent limit of detection (LOD) of 0.847 ppb for propofol, 0.663 ppb for 4-hydroxypropofol, 1.259 ppb for propofol glucuronide and 1.035 ppb for propofol sulfate, respectively, demonstrating that the optimized Au foam substrate enabled sensitive ppb-level quantification of propofol metabolites, which can be reliably used for concentration estimation in breath-relevant environments (Table S2). Then, spike-recovery experiments were further performed at 10 ppb to assess quantitative accuracy. As shown in Figure 3G,H, the calculated concentrations were close to the spiked values, and the recovery rates remained >90%, which was also observed across different concentration levels (Figure S8), confirming the reliability of the Au foam SERS platform for ppb-level detection in artificial breath, which showed better performance than reported works before (Table S3).

3.4. Breath SERS Fingerprinting for Discrimination of Propofol Anesthesia-Related States

As illustrated in Figure 4A, exhaled breath samples were collected from three representative physiological states, including awake/pre-anesthesia, propofol anesthesia, and recovery (Table S4). The collected breath samples were introduced onto the optimized Au foam SERS chip, followed by Raman spectral acquisition, feature extraction, and state discrimination. Representative breath SERS spectra from the three states are shown in Figure 4B. Unlike the standard-molecule spectra, the breath spectra displayed several broader bands, shoulder peaks, reflecting the complex molecular composition of exhaled breath. Compared with the relatively weak and broad spectral profile observed in the awake/pre-anesthesia state, the propofol anesthesia state exhibited enhanced SERS features in several spectral regions around 842, 1048–1088, 1452, and 1600 cm^{-1} , which exhibited high correlation towards the Raman features previously identified from propofol-related standards. Moreover, the recovery-state spectrum showed an intermediate pattern, in which the propofol-related spectral features were still observable but attenuated compared with the anesthesia state, suggesting that the Au foam substrate can capture anesthesia-associated molecular changes from complex breath backgrounds.

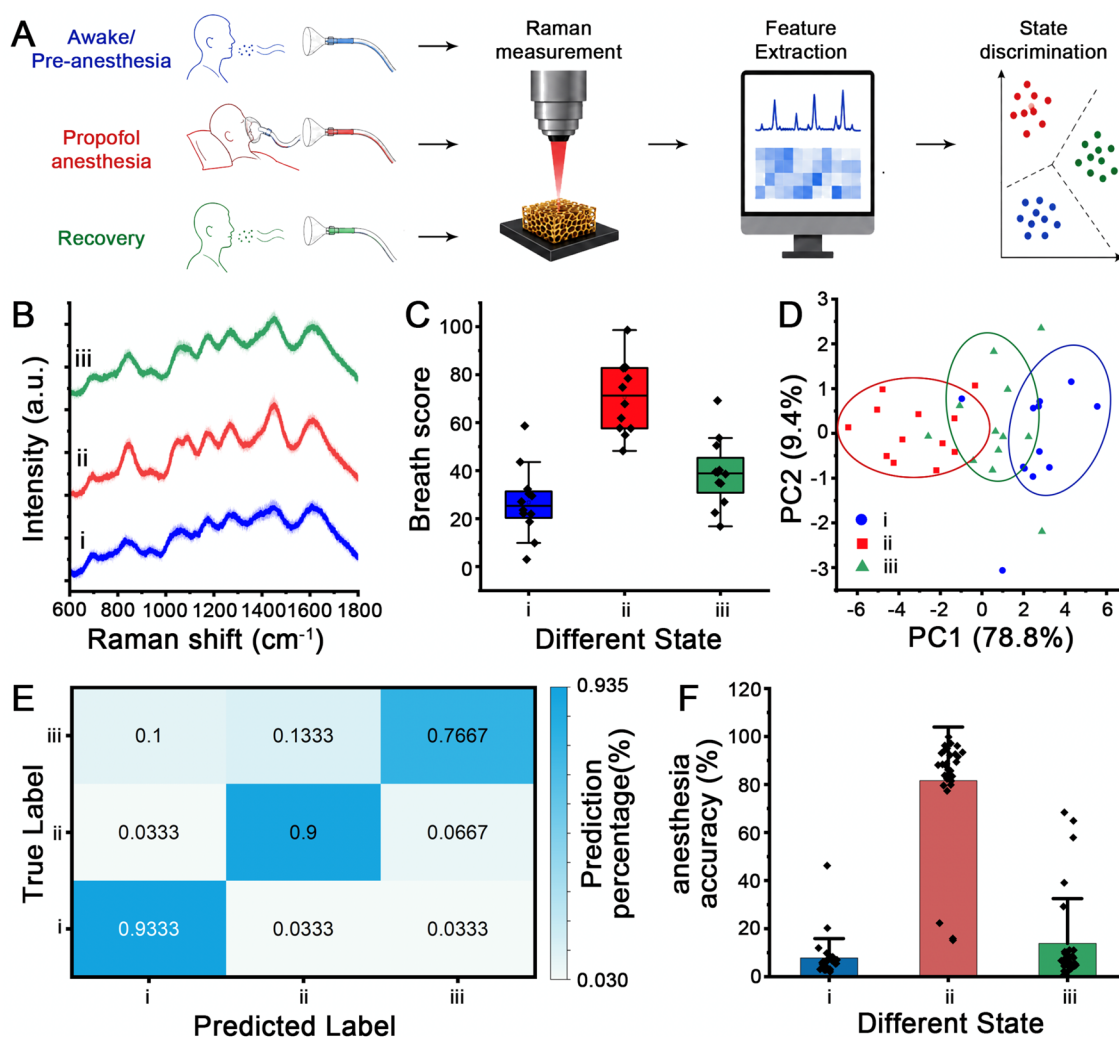


Figure 4. Breath SERS fingerprinting for discrimination of propofol anesthesia-related states. (A) Schematic workflow for breath-based anesthesia-state discrimination using the Au foam SERS platform. (B) Representative breath SERS spectra collected from the three states. (C) Distribution of breath SERS scores calculated from selected propofol-related spectral regions. (D) PCA score plot of breath SERS fingerprints. (E) Row-normalized confusion matrix for supervised classification of the three breath states using SERS-derived features ($n = 30$). (F) Predicted anesthesia-state probability extracted from the same classification. Unless otherwise indicated, labels i–iii correspond to awake/pre-anesthesia, propofol anesthesia, and recovery, respectively.

To quantitatively summarize the changes in these propofol-related spectral regions, a breath SERS score was calculated by integrating areas of the 842, 1048–1088, 1452, and 1600 cm^{-1} regions, which were first normalized and then averaged to generate a composite score. As shown in Figure 4C, the breath SERS score was significantly elevated in the propofol anesthesia group compared with the awake/pre-anesthesia group, while the recovery group showed a decreased score relative to the anesthesia group, which is consistent with the spectral changes observed before, indicating that the selected spectral regions collectively reflect anesthesia-associated breath molecular signatures. Subsequently, PCA analysis in an unsupervised manner was performed to evaluate the distinguish ability on different anesthesia-related states using the breath SERS fingerprints. As shown in Figure 4D, the awake/pre-anesthesia, propofol anesthesia, and recovery samples formed distinguishable clusters in the PCA score space. PC1 explained 78.8% of the total variance, whereas PC2 explained 9.4%, indicating that the major spectral variance was associated with state-dependent differences in the breath SERS fingerprints. Importantly, the anesthesia group was separated from the awake/pre-anesthesia group, while the recovery group was positioned between or partially overlapping with the other two states, consistent with a transitional molecular profile during recovery from anesthesia.

To further assess the state-discrimination capability of the SERS fingerprints, a supervised classification model was constructed using SERS-derived spectral features. The row-normalized confusion matrix in Figure 4E showed that the model achieved an overall classification accuracy of 86.7% using a balanced test set containing 30 spectra per state. The awake/pre-anesthesia and propofol anesthesia states were classified with high accuracies

of 93.3% and 90.0%, respectively. Notably, the recovery state showed a lower classification accuracy of 76.7%, with partial misclassification into awake/pre-anesthesia and anesthesia groups, which due to the recovery state was expected to represent an intermediate physiological and metabolic state rather than a completely independent molecular condition. Finally, the predicted probability assigned to the anesthesia class was extracted as a continuous anesthesia-state probability. As shown in Figure 4F, the propofol anesthesia group showed markedly higher predicted anesthesia probability over 80% than the awake/pre-anesthesia and recovery groups. In contrast, the awake/pre-anesthesia group showed low anesthesia probability, while the recovery group displayed reduced values compared with the anesthesia group, suggesting that breath SERS fingerprints can not only classify anesthesia-related states but also generate a continuous model output reflecting the likelihood of propofol anesthesia-associated breath signatures.

4. Conclusions

In this work, we developed a mesoporous Au foam-based SERS platform for noninvasive breath analysis of propofol metabolites signatures and anesthesia-state discrimination. Firstly, the Au foam chips were fabricated through selective dealloying of Au-Ag alloy films, generating a three-dimensional interconnected ligament-pore architecture with optimized pore size and ligament width of 55 ± 9 nm and 24 ± 4 nm, respectively, exhibiting strong SERS enhancement and high operational robustness with enhancement factor of 6.84×10^5 using 4-MBN as a Raman reporter. The dealloyed Au foam with homogeneous signal distribution (RSD < 9%) and batch-to-batch reproducibility (RSD < 2.5%) showed excellent signal stability output under dry N₂, high-humidity atmospheres, and artificial breath conditions within over 30 days of storage, providing a stable, reproducible, and breath-compatible SERS interface for gas-phase molecular sensing. Then, the molecular fingerprinting capability of the Au foam chip was further validated using propofol and representative propofol metabolites. Distinct SERS fingerprints were obtained for these structurally related molecules. Based on the specific Raman peak assignments, including 1452 cm⁻¹ for propofol, 842 cm⁻¹ for 4-hydroxypropofol, 1048 cm⁻¹ for propofol glucuronide, and 1088 cm⁻¹ for propofol sulfate, the PCA analysis of Au foam-derived SERS fingerprints showed clear separation of the four molecules, with PC1, PC2, and PC3 explaining 64.3%, 19.4% and 8.74% of the spectral variance, respectively, which finally achieved an overall accuracy above 85%, supporting reliable molecular recognition of structurally related propofol derivatives. Quantitatively, the SERS responses showed good linearity over 2–75 ppb for all four propofol-related molecules, with low LODs of 0.847 ppb for propofol, 0.663 ppb for 4-hydroxypropofol, 1.259 ppb for propofol glucuronide, and 1.035 ppb for propofol sulfate. Moreover, taking advantage of the high selectivity of the Raman feature-driven Au foam breath analysis platform, recovery rates above 90% was achieved in spike-recovery experiments at multiple concentration, further confirming the quantitative reliability of the platform in artificial breath matrix. Finally, in clinical application of the discrimination of propofol anesthesia-related states, it was found that the breath SERS score was significantly elevated in the anesthesia state and decreased during recovery, which achieved an overall accuracy of 86.7% for supervised classification, with classification accuracies of 93.3% for awake/pre-anesthesia, reaching an anesthesia-state probability over 80%. The proposed Au foam SERS platform provided a promising noninvasive analytical strategy for perioperative breath analysis, which offered complementary molecular information related to propofol exposure, metabolism, and recovery, supporting future development of compact optical sensing tools for anesthesia-state assessment.

Supplementary Materials: The following supporting information can be downloaded at: <https://media.scilit.com/articles/others/2606241446285739/MI-26050200-SI.pdf>.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, approved by the Ethics Committee of Shanghai General Hospital (approval number 2025-192, 22 September 2025).

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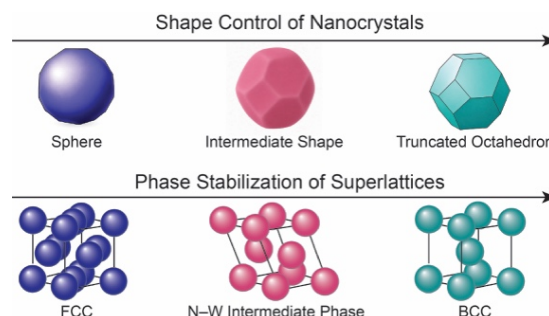


Capturing the Transient Phases of Nanocrystal Superlattice via Shape-Controlled Synthesis

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Abstract: Nanocrystal superlattices are ordered arrays of colloidal nanocrystals, often viewed as artificial solids in which nanocrystals replace atoms as the structural units. This article highlights a recent study in which the shape of silver nanocrystals was finely tuned to direct their assembly into superlattices from face-centered cubic to body-centered cubic structures, while accessing the transient intermediate structures along the Nishiyama-Wassermann martensitic pathway. By tuning nanocrystal sphericity and revealing the role of soft ligand-mediated interactions through computational simulation, this work shows how the shape of nanocrystal can be tailored to stabilize an intermediate superlattice phase and modulate its optical response.



Keywords: nanocrystal superlattice; Nishiyama-Wassermann pathway; phase-intermediate structure

Colloidal nanocrystals (NCs) can serve as “artificial atoms” to crystallize into periodic superlattices (SLs), in which the arrangement of NC centers defines lattice symmetry, unit cells, and diffraction features analogous to those of atomic crystals [1,2]. This analogy has made NC SLs a useful platform for translating crystallographic structures, such as face-centered cubic (FCC) and body-centered cubic (BCC) lattices, into mesoscale materials assembled from colloidal particles [3]. At the same time, NCs differ from atoms because they possess controllable size, shape, crystallographic facets, surface ligands, and degrees of rotational freedom, all of which provide additional handles for controlling SL structures [4–6].

The programmability makes NC SLs attractive for probing structural intermediates that are difficult to access in atomic crystals. During solid-state phase transitions, especially diffusionless martensitic transformations between high-symmetry lattices, intermediate configurations determine the transformation pathway and energy landscape [7]. Yet the intermediates are usually transient and unstable, making them difficult to isolate under ambient conditions. In NC assemblies, the larger size of the building blocks, together with tunable particle geometry and surface chemistry, make it possible to stabilize and structurally resolve such transition-related states. The technical challenge is to convert a normally transient crystallographic intermediate into a persistent and well-defined SL phase.

Writing in *Science*, Nagaoka and co-workers addressed this challenge using shape-controlled Ag-NCs as SL building blocks [8]. These SLs were obtained through slow evaporation of dispersions Ag-NCs in hexane over several weeks, a condition identified as critical for producing structurally rigid SLs with sharp and well-defined small-angle X-ray scattering (SAXS) peaks [9]. As shown in Figure 1A,B, spherical Ag-NCs crystallized into FCC SLs, whereas truncated octahedra (mecon)-shaped Ag-NCs assembled into BCC SLs. Most interestingly, when the shape of the NCs was tuned to an intermediate sphericity, synchrotron-based SAXS analysis identified a Nishiyama-Wassermann (N-W) phase-intermediate SL described by a base-centered monoclinic unit cell (Figure 1C). Taken together, by varying the shape of the Ag-NCs from sphere-like to mecon-like, the authors were able to trace a structural evolution from FCC to BCC and, most excitingly, stabilize an otherwise transient intermediate SL phase under ambient conditions.

As expected, the stabilization of the intermediate phase could not be explained by particle shape alone. In simulations using only the stiff interaction model, the SL showed an abrupt transition between FCC and BCC lattices as the sphericity parameter was changed (Figure 1D). In contrast, the total potential model, which combines



stiff and soft interactions, reproduced the experimentally observed continuous structural evolution. This result highlights the importance of soft ligand-mediated interactions in addition to NC geometry. Computational modeling further elucidated the evolution of orientational order and soft ligand-induced facet-to-facet interactions among neighboring Ag-NCs as the SL evolved from FCC to BCC through the intermediate phase (Figure 1E). These combined effects allow Ag-NCs with intermediate sphericity to access and stabilize the N-W intermediate phase.

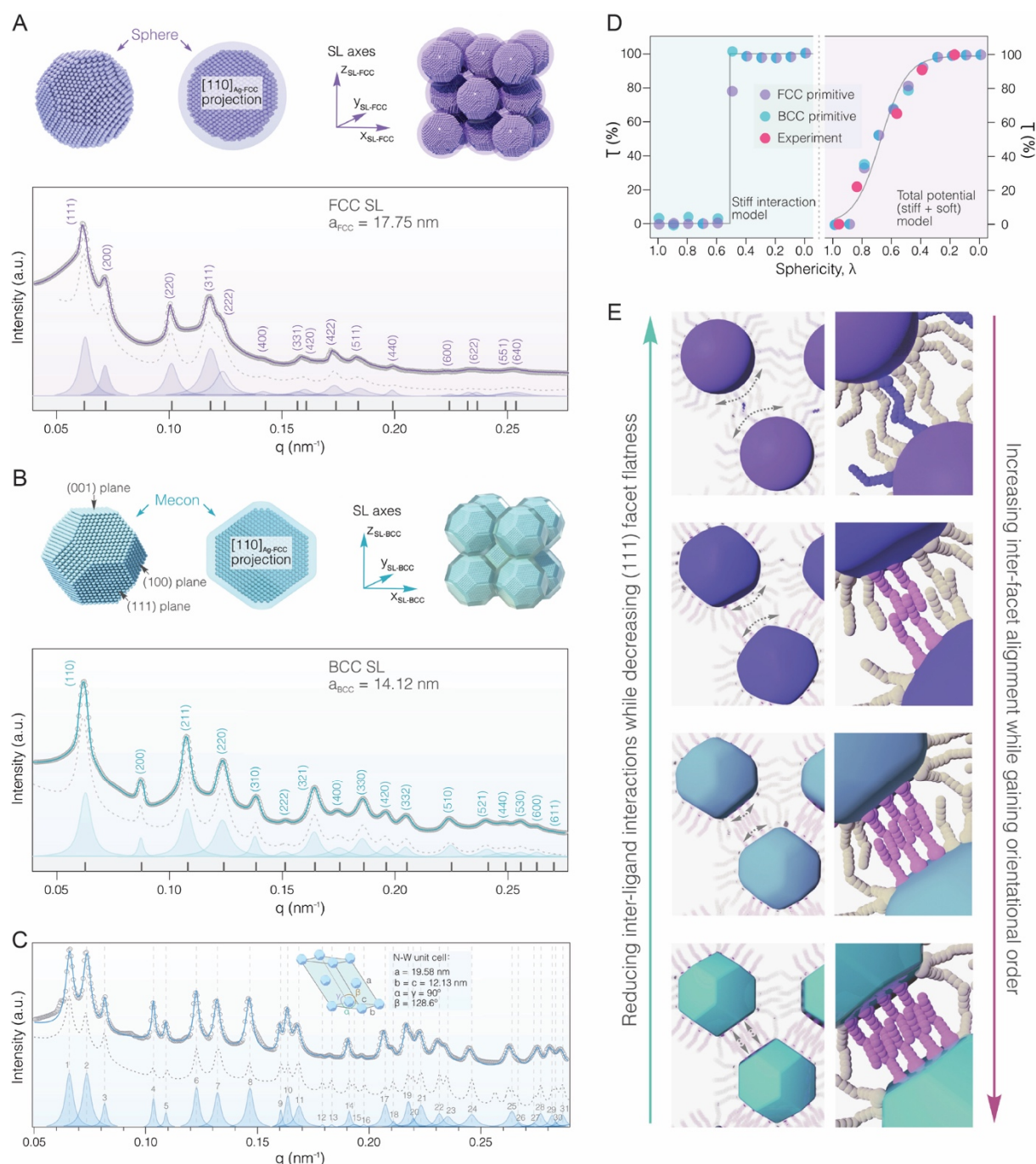


Figure 1. Stabilization of SLs comprised of shape-controlled Ag-NCs. **(A,B)** Spherical and mecon-shaped Ag-NCs crystallized into FCC and BCC SLs, respectively, as confirmed by SAXS analysis. **(C)** Ag-NCs with intermediate sphericity formed an N-W phase-intermediate SL described by a base-centered monoclinic unit cell. **(D)** Simulations showed that the stiff interaction model gave an abrupt FCC-to-BCC transition, whereas the total potential model, which combined stiff and soft interactions, reproduced the experimentally observed structural evolution. **(E)** Computational models of neighboring Ag-NCs in FCC, intermediate phase, and BCC SLs, highlighting the evolution of orientational order of the constituent Ag-NCs and soft ligand-induced facet-to-facet interactions. Modified from Ref. [7] with permission. Copyright 2026, The American Association for the Advancement of Science.

Beyond structural control, the ability to stabilize SLs in intermediate phases may create opportunities for tuning their collective properties [10]. In the reported Ag-NC SLs, FCC, N-W intermediate phase, and BCC structures all exhibited plasmon-photon hybridization and reached the deep-strong light-matter coupling regime [8,11]. The stable intermediate structure therefore provides an experimentally accessible bridge for examining how optical coupling evolves between the two endpoint lattices. To sum up, this work shows that shape control of NCs can stabilize otherwise transient intermediate structures while preserving tunable collective optical responses.

Looking forward, the broader impact of this work will depend on both conceptual generality and operational stability. It remains unclear whether shape control can stabilize intermediate phases beyond the Ag-NC FCC-to-BCC/N-W pathway, particularly for other NC compositions or transformation pathways. In addition, although the N-W phase-intermediate SLs remained stable for at least 6 months under ambient conditions, their use as optical metamaterials or in polaritonic devices will require stability under thermal cycling, mechanical vibration, light exposure, and large-area film-processing conditions. Addressing these issues would move transient SL phases from structural curiosities toward practical functional materials.

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Au-Fe Separates Enable CO-Assisted Methane Oxidation to Acetic Acid with O₂ via Tandem Catalysis

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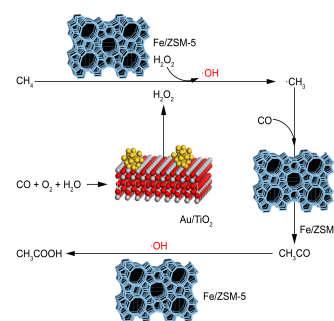
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Abstract: Direct conversion of methane to acetic acid with molecular O₂ at room temperature remains as a grand challenge. Herein, we achieved room-temperature oxidation of CH₄ into acetic acid with O₂ and CO over structurally optimized Au/TiO₂ and Fe/ZSM-5 through tandem catalysis. The process decouples H₂O₂ formation and migration, H₂O₂ activation to hydroxyl radicals for CH₄ oxidation, and carbon-carbon coupling to acetic acid. The physical mixture of Au/TiO₂ and Fe/ZSM-5 yielded 55.6 μmol g_{cat}⁻¹ with the acetic acid selectivity in liquid of 64.9% in H₂O under 61 bar (CH₄:CO:O₂ = 15:40:6) at 30 °C for 6 h without any external energy. *In situ* synchrotron-based vacuum ultraviolet photoionization mass spectrometry demonstrates a closer spatial interval is critical for *in situ* generated H₂O₂ migrates from Au/TiO₂ to Fe/ZSM-5, which can be readily used by Fe/ZSM-5 to produce hydroxyl radicals, enabling the maximum liquid-phase product accumulation.



Keywords: methane oxidation; acetic acid; hydroxyl radicals; tandem catalysis

1. Introduction

Direct conversion of methane to methanol with O₂ (CH₄ + 1/2 O₂ → CH₃OH) at room temperature remains a grand challenge due to the chemical inertness of both CH₄ and O₂ [1–4]. In nature, methane monooxygenase (MMO), including soluble MMO (sMMO) and particulate MMO (pMMO), efficiently catalyzes this reaction at room temperature [5–7]. The enzymatic processes inspired the researchers to construct partially reduced metal sites [8,9]. Converting methane to acetic acid via a carbon-carbon coupling pathway with O₂ at room temperature is highly challenging, typically requiring elevated temperatures (≥80 °C) or strong oxidants such as H₂O₂ or K₂S₂O₈ [10–21].

Room-temperature conversion of methane into acetic acid necessitates external energy sources including light [22], plasma, and γ-ray radiation. Under UV irradiation, photoexcited electrons from TiO₂ moved to Pt/NPW clusters, while holes on TiO₂ reacted with H₂O to produce hydroxyl radical (·OH). These ·OH radicals activated methane and reacted with CO adsorbed on Pt sites, forming acetyl groups oxidized to acetic acid [23]. Using dielectric barrier discharge plasma over Cu/γ-Al₂O₃, the reaction of CO₂(v) + H → CO + OH could be one of the major routes for ·OH radicals formation, collectively forming acetic acid from methyl radicals, CO, and ·OH radicals [24]. Under γ-rays, H₂O radiolysis produced ·OH radicals and hydrated electrons, interacting with CH₄ and CO₂ to form methyl and ·CO₂⁻ radicals, coupling into acetic acid [25]. Based on these findings, successfully converting methane to acetic acid at room temperature depended critically on generating reactive hydroxyl radicals.



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Based on previous findings, our focus shifts to addressing the key challenge of efficiently generating and utilizing hydroxyl radicals at room temperature. To tackle this, we propose a tandem catalytic strategy that integrates two functions, constructing components capable of efficient H_2O_2 synthesis at room conditions and enabling the efficient utilization of *in situ* generated H_2O_2 to produce hydroxyl radicals for subsequent methane oxidation. By investigating the proximity and interaction between these components, we aim to maximize the overall efficiency of methane selective oxidation to acetic acid. We demonstrate that tandem catalysis enables efficient methane oxidation to acetic acid with O_2 at room temperature without external energy input. Using a physical mixture of Au/TiO_2 and $\text{Fe}/\text{ZSM-5}$ catalysts, we achieved $55.6 \mu\text{mol g}_{\text{cat}}^{-1}$ acetic acid yield with 64.9% selectivity in aqueous solution under mild conditions (61 bar, 30 °C, 6 h, $\text{CH}_4:\text{CO}:\text{O}_2 = 15:40:6$). *In situ* synchrotron-based vacuum ultraviolet photoionization mass spectrometry reveals that close spatial proximity between catalysts is essential for efficient migration of *in situ* generated H_2O_2 from Au/TiO_2 to $\text{Fe}/\text{ZSM-5}$, where it decomposes into hydroxyl radicals for subsequent methane oxidation, thereby maximizing liquid-phase product formation.

2. Experimental Section

2.1. Catalyst Preparation

TiO_2 was synthesized using a hydrothermal method. In a typical procedure, 1.8 g of $\text{CO}(\text{NH}_2)_2$ and 30 mL of Titanium(IV) bis(ammonium lactato) dihydroxide solution (TALH) were dissolved in 270 mL of deionized water. After stirring at room temperature for 30 min, the solution was transferred to a 200-mL Teflon-lined autoclave. The autoclave was then heated to 160 °C and maintained at this temperature for 24 h. The resulting precipitate was collected by centrifugation, washed thoroughly with deionized water, and subsequently dried in an oven at 80 °C for 12 h. The dried powders were ground and calcined in a muffle furnace, heating from room temperature to 300 °C at a ramp rate of 10 °C min^{-1} , and held at 300 °C for 3 h.

Au/TiO_2 was prepared using the deposition-precipitation method, employing NaOH aqueous solution to control the pH. In a typical synthesis, 2.5 g of TiO_2 dispersed in 200 mL of deionized water was mixed with 2.1 mL of HAuCl_4 aqueous solution ($23.5 \text{ g}_{\text{Au}} \text{ L}^{-1}$) in a beaker, stirred at 750 rpm for 30 min. Subsequently, 17.0 mL of NaOH aqueous solution (0.2 mol L^{-1}) was slowly added to adjust the pH to approximately 9. The resulting mixture was aged in a water bath at 80 °C for 2 h. The precipitate was collected by centrifugation, washed thoroughly with deionized water, and dried in an oven at 80 °C for 12 h. The dried powders were then ground and calcined in a muffle furnace, heating from room temperature to 300 °C at a ramp rate of 2 °C min^{-1} , and maintained at 300 °C for 2 h. For references, $\text{Au}/\text{TiO}_2\text{-A}$, $\text{Au}/\text{TiO}_2\text{-P25}$, and $\text{Au}/\text{TiO}_2\text{-R}$ were synthesized using the identical preparation method as Au/TiO_2 . TiO_2 -supported Pd, Pt, and Cu catalysts were prepared using the same synthesis method as Au/TiO_2 for comparison.

$\text{Fe}/\text{ZSM-5}$ was prepared using the ball milling method. In a typical procedure, 10.0 g of $\text{NH}_4\text{-ZSM-5}$ and 0.48 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were placed in hardened steel jars, and ball milling was conducted at 800 rpm at room temperature for 30 min using a planetary ball mill (Retsch GmbH, Emax). The resulting powders were calcined in a muffle furnace, with the temperature increased from room temperature to 400 °C at a rate of 2 °C min^{-1} , and held at 400 °C for 4 h.

2.2. Catalyst Characterization

The inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (710-ES, Varian, Palo Alto, CA, USA) determined the actual metal loadings of Au and Fe. X-ray diffraction (XRD) patterns were recorded by using a Philips X'Pert Pro Super diffractometer (Malvern Panalytical, Almelo, The Netherlands) with $\text{Cu-K}\alpha$ radiation ($\lambda = 1.54178 \text{ \AA}$). Aberration-corrected high-angle annular dark field scanning transmission electron microscopy (HAADF-STEM) images were carried out on a JEOL ARM-200F (JEOL Ltd., Tokyo, Japan) operating at an accelerating voltage of 200 kV. XPS measurements were conducted on an X-ray photoelectron spectrometer (VG ESCALAB MK II, Thermo Fisher Scientific, Waltham, MA, USA) with $\text{Mg K}\alpha = 1253.6 \text{ eV}$ as the exciting source. Ultraviolet-visible (UV-Vis) spectroscopy spectra were recorded on a Shimadzu Solid 3700 DUV (Shimadzu, Kyoto, Japan). The X-ray absorption spectroscopy (XAS) measurements were carried out at the BL11B beamline of the Shanghai Synchrotron Radiation Facility (SSRF, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201204, China) in the fluorescence mode (<https://cstr.cn/31124.02.SSRF.BL11B> (accessed on 6 October 2023)).

Fluorescence emission spectroscopy was used to detect hydroxyl radical ($\cdot\text{OH}$), employing terephthalic acid as a probe molecule. Terephthalic acid readily reacts with $\cdot\text{OH}$ radicals to form the highly fluorescent 2-hydroxyterephthalic acid. For this analysis, a fixed amount of terephthalic acid was dissolved in $2 \times 10^{-3} \text{ mol L}^{-1}$ NaOH to prepare a $5 \times 10^{-4} \text{ mol L}^{-1}$ terephthalic acid solution. The terephthalic acid solution was used instead of

H₂O to disperse the catalyst. After the reaction at 30 °C for 2 h and the removal of solid catalyst, the liquid solution was subjected to fluorescence spectroscopy measurements. The spectrum was recorded on a Horiba Fluorolog-3-Tau and deltaflex fluorescence spectrophotometer with an excitation wavelength of 315 nm.

High resolution mass spectrometry (HRMS) measurement was conducted on a LTQ-Orbitrap XL using the ESI technique. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 2 h. During the reaction, 100 mg of TEMPO was added into the reaction solution. Following the completion of the reaction, the mixture was promptly centrifuged and used for further HRMS measurement.

In situ diffuse reflectance Fourier transform spectroscopy (DRIFTS) measurement was conducted in a cell with a Fourier transform infrared spectrometer (TENSOR II Sample Compartment) and a liquid nitrogen-cooled detector. The catalyst was pretreated by 1 bar of argon (30 mL min⁻¹) at 150 °C for 30 min, followed by cooling to 30 °C, the background spectra of the sample were acquired. For all DRIFTS measurements, the test temperature was maintained at 30 °C. This temperature has been explicitly noted for each individual adsorption measurement. For CH₄, CO, O₂, and H₂O co-adsorption, 10 mg of Au/TiO₂ and 20 mg of Fe/ZSM-5 were used for measurement. 80% CH₄ (CO as a balanced gas, 15 mL min⁻¹) and 20% O₂ (argon as a balanced gas, 15 mL min⁻¹) were used as the co-fed gases. For CH₄, CO, H₂O₂, and H₂O co-adsorption, 30 mg of Fe/ZSM-5 was used for measurement. 80% CH₄ (CO as a balanced gas, 15 mL min⁻¹) and argon (15 mL min⁻¹) were used as the reactant gaseous. H₂O₂ solution was brought into the chamber by co-fed gases.

Synchrotron vacuum ultraviolet photoionization mass spectrometry (SVUV-PIMS) measurements were conducted at the combustion beamline (BL03U) of the National Synchrotron Radiation Laboratory (Hefei, China). In each experiment, 100 mg of 20–40 mesh catalyst (or quartz for blank tests) was loaded into a quartz reactor fitted with a ~0.1 mm nozzle. The Au/TiO₂ and Fe/ZSM-5 mass ratio was fixed at 1:2. The reactor was coupled to an online SVUV-PIMS spectrometer, and the gas flow was switched to the reaction mixture (15 mL min⁻¹ of CH₄, 40 mL min⁻¹ of CO, and 6 mL min⁻¹ of O₂), water was introduced by the feed gas. Data were acquired at 30 °C in 600 s intervals (200 s sampling time) under constant 11.5 eV photon energy and 2 Torr pressure.

2.3. Catalyst Performance Test

Methane oxidation reactions with O₂ as an oxidant in the presence of CO were carried out in a 50-mL Hastelloy slurry reactor (Shanghai Yanzheng Experiment Instrument Co., LTD, Shanghai, China). In a typical process, the reaction was conducted over 50 mg of Au/TiO₂ and 100 mg of Fe/ZSM-5 (denoted as Au/TiO₂ + Fe/ZSM-5) with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 6 h. After the reaction, the autoclave was cooled down to below 10 °C and filtered to remove the catalyst before analyses. Unless otherwise specified, 150 mg of Au/TiO₂ + Fe/ZSM-5 refers to a combination of 50 mg of Au/TiO₂ and 100 mg of Fe/ZSM-5.

Liquid products were analyzed by ¹H nuclear magnetic resonance (¹H-NMR). 66.7 μL of DMF was introduced into 100 mL of D₂O as an internal standard solution. 100 μL of the internal standard solution and 600 μL of the reaction sample were added into NMR tubes for measurement. The signal of protons from the solvent H₂O is much stronger than that from the products. Therefore, all ¹H NMR spectra were recorded using a pre-saturation solvent suppression technique to suppress the dominant H₂O signal. The amount of HOCH₂OH was quantified via the acetylacetone color-development method (UV-Vis spectrophotometer, Cary 60 UV-Vis, Agilent, Santa Clara, CA, USA). In this work, CH₃OH, HOCH₂OH, and HCOOH originated from CH₄, while CH₃COOH was sourced from both CH₄ and CO. Therefore, we calculated the product yield (micromoles of acetic acid per gram of the catalyst, μmol g_{cat}⁻¹) and CH₃COOH selectivity (% in liquid) on a molecule basis using Equations (1) and (2).

$$\text{Products yield } (\mu\text{mol g}_{\text{cat}}^{-1}) = \frac{n_{\text{product}}}{g_{\text{cat}}} \quad (1)$$

$$\text{CH}_3\text{COOH selectivity } (\%) = \frac{n_{\text{CH}_3\text{COOH}}}{n_{\text{product}}} \times 100\% \quad (2)$$

In Equation (1), n_{product} represented the total moles of liquid products, g_{cat} was the mass of the catalysts. In Equation (2), $n_{\text{CH}_3\text{COOH}}$ was the mole of produced CH₃COOH.

Elevated-pressure operation requires specialized equipment and strict CO safety protocols. Although high pressure increases cost, it is common in industry. Ongoing catalyst optimization aims to improve CO utilization and reduce required pressure while maintaining performance for practical application.

An H₂O₂ synthesis experiment was carried out in a 50-mL Hastelloy slurry reactor. 15 mg of catalyst was added into the reaction reactor with 15 mL of H₂O. The reaction was conducted with mechanical stirring (1000 rpm) under

40 bar of CO, 6 bar of O₂, and 15 bar of N₂ at 30 °C for 1 h. After completion of the reaction, 1 mL of the sample was combined with 20 mL of H₂O and 10 mL of 3 mol L^{−1} sulfuric acid in a titration vessel. Subsequently, 20 µL of Ferroin solution was added to the mixture under continuous stirring. The resulting solution was then gradually titrated with 0.002 mol L^{−1} Ce(SO₄)₂ solution until a persistent pale blue endpoint was observed.

For methane oxidation with H₂O₂ in the presence of CO, the experiments were conducted in a 50-mL Hastelloy slurry reactor. The reactor contained 15 mg of catalyst, 15 mL of H₂O containing 1500 µmol of H₂O₂. The reaction was conducted with mechanical stirring (1000 rpm) under 15 bar of CH₄, 40 bar of CO, and 6 bar of N₂ at 30 °C for 1 h.

H₂O₂ decomposition experiment was carried out in a 50-mL Hastelloy slurry reactor. 15 mg of catalyst was added into the reaction reactor with 15 mL of H₂O containing 1500 µmol of H₂O₂. The reaction was conducted with mechanical stirring (1000 rpm) under 40 bar of CO and 21 bar of N₂ at 30 °C for various time. After completion of the reaction, 1 mL of the sample was combined with 20 mL of H₂O and 10 mL of 3 mol L^{−1} sulfuric acid in a titration vessel. Subsequently, 20 µL of Ferroin solution was added to the mixture under continuous stirring. The resulting solution was then gradually titrated with 0.002 mol L^{−1} Ce(SO₄)₂ solution until a persistent pale blue endpoint was observed.

To trace the fate of carbon atoms from CH₄ and CO, ¹³C labeled CH₄ (¹³CH₄) with 99% natural abundance was used in the coreaction with CO and O₂. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 8 bar of ¹³CH₄, 8 bar of CO, and 4 bar of O₂ at 30 °C for 6 h. ¹³C labeled CO (¹³CO) with 99% natural abundance was used in the coreaction with CH₄ and O₂. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 8 bar of CH₄, 8 bar of ¹³CO, and 4 bar of O₂ at 30 °C for 6 h.

3. Results and Discussion

3.1. Design, Synthesis, and Characterization of the Catalysts

Au/TiO₂ was synthesized via hydrothermal treatment followed by deposition-precipitation processing. The content of Au in Au/TiO₂ was 1.7 wt%, as determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Table S1). X-ray diffraction (XRD) analysis showed that no detectable metallic Au or Au oxide in Au/TiO₂ (Figure S1). As references, Au/TiO₂-A, Au/TiO₂-P25, Au/TiO₂-R (1.6–1.7 wt% Au) prepared using the identical preparation method as Au/TiO₂ (Table S1). Aberration-corrected high-angle annular dark field scanning transmission electron microscopy (HAADF-STEM) confirmed the presence of Au nanoparticles in Au/TiO₂ with an average size of 5.7 nm (Figure 1a). The homogeneous distribution of Au species was further corroborated by energy-dispersive X-ray spectroscopy (EDS) mapping results (Figure 1b). The lattice spacing of the Au nanoparticles was measured at 0.24 nm, corresponding to (111) face-centered cubic (fcc) Au (Figure 1c) [26,27]. In addition, TEM analyses revealed that Au formed relatively large nanoparticles on Au/TiO₂-A, Au/TiO₂-P25, and Au/TiO₂-R (Figure S2). For all samples, the peaks observed at approximately 83.1 eV and 86.9 eV correspond to the Au 4f_{7/2} and Au 4f_{5/2} binding energies of Au⁰ (Figure S3) [28–30], respectively.

To synthesize Fe/ZSM-5 catalyst, we adopted a ball-milling approach, while preparing Fe/ZSM-5-imp via conventional incipient wetness impregnation for comparative studies. XRD analysis revealed no detectable peaks attributable to crystalline Fe or Fe oxide phases in Fe/ZSM-5 (Figure S4). HAADF-STEM images of Fe/ZSM-5 showed the presence of atomically dispersed Fe species, as highlighted by yellow cycles (Figure 1d). The EDS mapping results further confirmed the homogeneous distribution of Fe species (Figure 1e). In contrast, Fe/ZSM-5-imp exhibited coexistence of atomically dispersed Fe species and FeO_x nanoparticles (Figure S5). XPS analysis reveals binding energies at 711.0 eV and 724.6 eV for Fe/ZSM-5, which are characteristic of Fe³⁺ species [31–33]. The corresponding peaks for Fe/ZSM-5-imp appeared at lower binding energies of 710.8 eV and 724.4 eV, respectively (Figure S6). This shift to lower binding energies indicates that the Fe species in Fe/ZSM-5-imp possessed a lower average oxidation state compared to those in Fe/ZSM-5. Ultraviolet-visible spectroscopy (UV-Vis) analysis revealed trace Fe impurities (<0.03 wt%) in ZSM-5 as monomeric tetrahedral (MT) and octahedral (MO) Fe³⁺ (Figure 1f) [34–36]. Fe incorporation (1 wt%) intensified these signals and introduced polynuclear clusters (360 nm) and Fe₂O₃ nanoparticles (>450 nm). Deconvolution showed ball-milled Fe/ZSM-5 retained 62.7% monomeric Fe versus 41.3% in impregnated Fe/ZSM-5-imp (41.2% Fe₂O₃) (Table S2), confirming superior Fe dispersion in ball-milled Fe/ZSM-5, consistent with HAADF-STEM results.

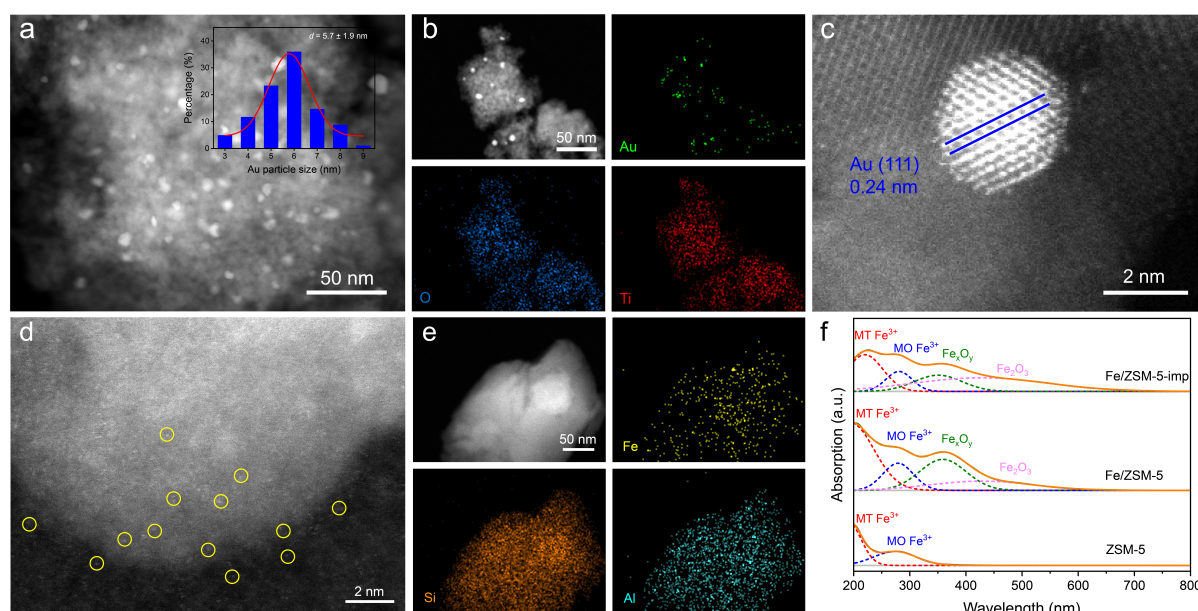


Figure 1. Identification of the active sites. (a) Aberration corrected HAADF-STEM image and particle size distribution of Au nanoparticles. (b) and (c) EDS mapping images and HAADF-STEM image of Au/TiO₂. (d) and (e) HAADF-STEM image and corresponding EDS mapping images. (f) UV-Vis spectral profiles of ZSM-5, Fe/ZSM-5, and Fe/ZSM-5-imp samples.

To further study the chemical state and coordinated environment of Fe in the Fe-containing samples, X-ray absorption near edge spectroscopy (XANES) showed that the absorption edge of Fe/ZSM-5 was shifted to higher energy relative to Fe foil and closely resembled that of Fe₂O₃ (Figure S7a). A pre-edge feature at 7114 eV, characteristic of the $1s \rightarrow 3d$ transition of Fe³⁺ in tetrahedral or distorted octahedral coordination, further indicated that Fe predominantly existed as Fe³⁺ species [37]. The Fourier-transformed k^3 -weighted extended X-ray absorption fine structure (EXAFS) spectrum of Fe/ZSM-5 exhibited a dominant peak at ~ 1.6 Å, corresponding to the Fe-O first shell, and a weak peak at ~ 2.6 Å assigned to the Fe-Fe second shell (Figure S7b) [38]. Notably, no distinct Fe-Fe first-shell scattering was observed, in contrast to Fe foil. EXAFS fitting yielded an Fe-O coordination number (CN) of 5.3 ± 0.3 with a bond length of 2.04 ± 0.01 Å and an Fe-Fe CN of only 0.4 ± 0.7 at 3.06 ± 0.13 Å (Table S3), confirming the atomic dispersion of Fe species in Fe/ZSM-5. By comparison, Fe/ZSM-5-imp exhibited a substantially higher Fe-Fe CN of 1.7 ± 0.6 with a bond length of 3.03 ± 0.06 Å, indicative of the formation of large FeO_x particles.

3.2. Optimization of Tandem Catalysis Enables Methane Oxidation to Acetic Acid

Building on insights into H₂O₂ synthesis and acetic acid formation, we developed a tandem route for methane conversion by integrating Au/TiO₂ and Fe/ZSM-5 (Figure 2a). H₂O₂ forms on Au/TiO₂ and subsequently migrates to Fe/ZSM-5 for methane oxidation to acetic acid [15,23]. To validate the effectiveness of tandem catalytic mechanism, we first examined H₂O₂ synthesis, confirming CO is essential, as O₂ and H₂O alone produced no H₂O₂ over Au/TiO₂ or Fe/ZSM-5. Au/TiO₂ catalysts screened for H₂O₂ synthesis from CO/O₂/H₂O via colorimetry. Among these catalysts, Au/TiO₂ exhibited the highest H₂O₂ production ($2600 \mu\text{mol g}_{\text{cat}}^{-1}$), outperforming Au/TiO₂-A ($2200 \mu\text{mol g}_{\text{cat}}^{-1}$), Au/TiO₂-P25 ($1600 \mu\text{mol g}_{\text{cat}}^{-1}$), and Au/TiO₂-R ($100 \mu\text{mol g}_{\text{cat}}^{-1}$) (Figure 2b), highlighting that optimal Au dispersion and electronic structure enhance catalytic activity. In contrast, Fe/ZSM-5 produced a low H₂O₂ yield of $30 \mu\text{mol g}_{\text{cat}}^{-1}$ (Figure S8).

We then screened catalysts for methane oxidation with H₂O₂ in the presence of CO over Fe species on ZSM-5 supports. ¹H nuclear magnetic resonance (NMR) and UV-Vis were employed to quantify the liquid products (Figures S9 and S10). We found that ball-milled Fe/ZSM-5 (predominantly featuring isolated Fe sites) significantly enhances the yield of acetic acid, reaching $817.6 \mu\text{mol g}_{\text{cat}}^{-1}$ (Figure 2c). In contrast, although Fe/ZSM-5-imp (containing more FeO_x particles) exhibited a slightly higher selectivity towards acetic acid (93.6% vs. 86.2%), its total oxygenate yield was markedly lower ($610.2 \mu\text{mol g}_{\text{cat}}^{-1}$ vs. $948.5 \mu\text{mol g}_{\text{cat}}^{-1}$). Consequently, its acetic acid yield ($571.1 \mu\text{mol g}_{\text{cat}}^{-1}$) was actually lower than that achieved over the Fe/ZSM-5. These results demonstrated that isolated Fe sites are more effective than FeO_x particles in promoting the conversion of CH₄ to oxygenates, particularly in enhancing acetic acid yield. The presence of FeO_x particles may be associated with

lower overall catalytic activity, thereby limiting the actual production of acetic acid. Conversely, no liquid products were detected for Au/TiO₂ (Figure S11). Collectively, the structurally optimized Au/TiO₂ and Fe/ZSM-5 were selected for spatial coupling due to peak catalytic performance. Based on above-mentioned findings, we performed methane oxidation with O₂ in the presence of CO in H₂O solvent. The standard reaction was conducted over 150 mg of catalyst with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 6 h. Neither Au/TiO₂ nor Fe/ZSM-5 alone produced detectable oxygenates. To clarify this, CH₄ oxidation was examined over Au/TiO₂ with varying H₂O₂ dosages. Despite minimal residual H₂O₂ after reaction, no liquid oxygenates were detected (Figure S12), suggesting rapid, nonproductive H₂O₂ decomposition to H₂O and O₂, which prevents oxygenate accumulation.

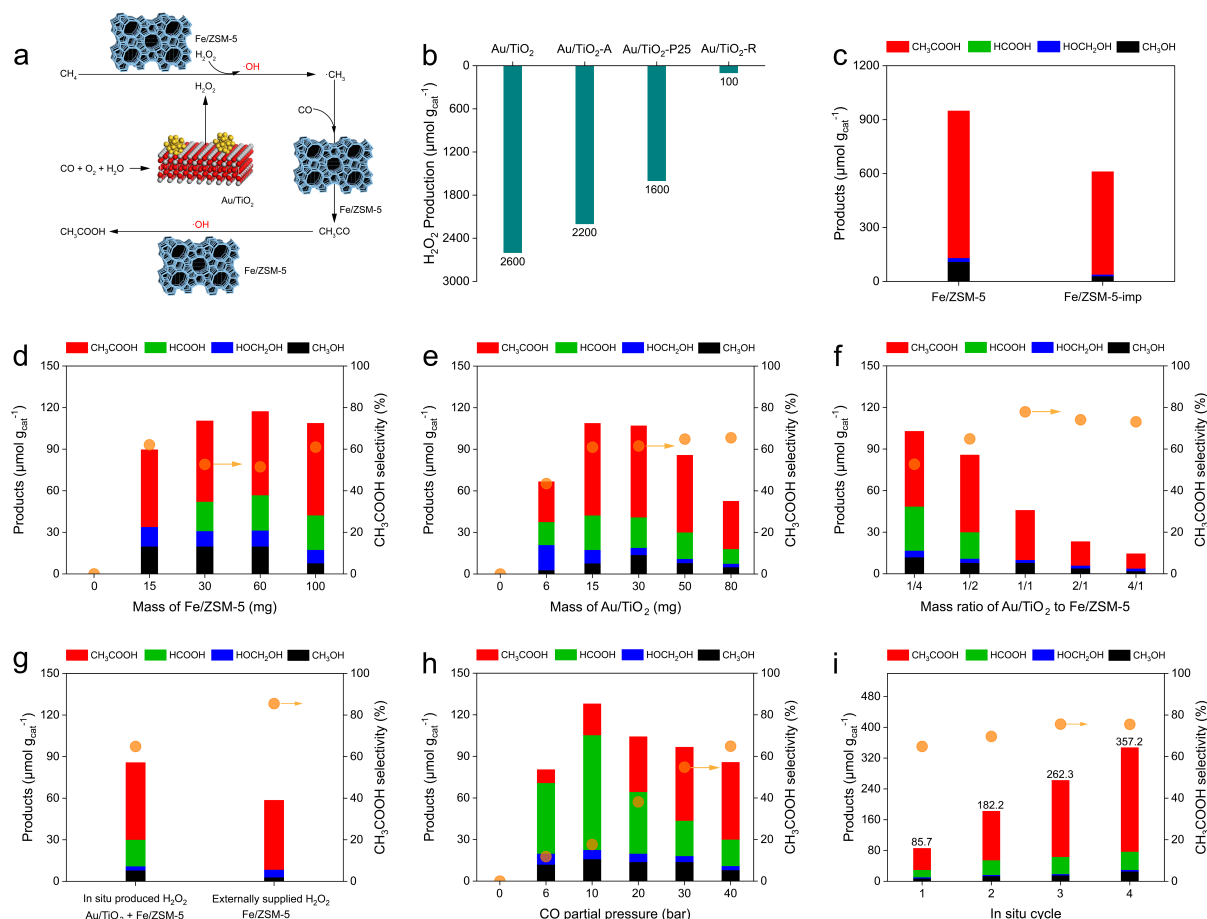


Figure 2. Tandem catalysis enables methane oxidation to acetic acid. (a) Concept for acetic acid synthesis from CH₄ + CO + O₂ + H₂O system. (b) H₂O₂ synthesis from the reaction of CO + O₂ + H₂O. The reaction was conducted over 15 mg of catalyst with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of N₂, 40 bar of CO, and 6 bar of O₂ at 30 °C for 1 h. (c) Acetic acid synthesis from the reaction of CH₄ + CO + H₂O₂. The reaction was conducted over 15 mg of catalyst with mechanical stirring (1000 rpm) in 15 mL of H₂O containing 1500 μmol of H₂O₂ under 15 bar of CH₄, 40 bar of CO, and 6 bar of N₂ at 30 °C for 1 h. (d–i) Acetic acid synthesis from CH₄ + CO + O₂ + H₂O system by verifying (d) Fe/ZSM-5 loading, (e) Au/TiO₂ loading, (f) Mass ratio of the Au/TiO₂ to Fe/ZSM-5. The standard reaction was conducted over 150 mg of catalyst with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 6 h. (g) Methane oxidation with *in situ* produced H₂O₂/externally supplied H₂O₂ in the presence of CO. For externally supplied H₂O₂, the reaction was conducted over 100 mg of the Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O containing 234 μmol of H₂O₂ under 15 bar of CH₄, 40 bar of CO, and 6 bar of N₂ at 30 °C for 6 h. (h) CO partial pressure-dependent product yields over Au/TiO₂ + Fe/ZSM-5 under standard conditions. CO dependent catalytic performance under standard reaction conditions. (i) *In situ* cycle test under standard reaction conditions.

To further explore how the mass ratio of Au/TiO₂ to Fe/ZSM-5 influenced the catalytic performance. We firstly optimized the Fe/ZSM-5 content while maintaining a fixed Au/TiO₂ loading of 15 mg. As shown in Figure 2d, no oxygenated products were detected in the absence of Fe/ZSM-5. Increasing the Fe/ZSM-5 mass from 15 to 100 mg resulted in oxygenate yields ranging from 89.6 to 117.2 μmol g_{cat}⁻¹, with acetic acid selectivity between 62.1%

and 51.5% (Table S4). Based on these results, 100 mg of Fe/ZSM-5 was selected for further optimization. Subsequent tests with fixed Fe/ZSM-5 (100 mg) and varying Au/TiO₂ loadings revealed that oxygenate formation required Au/TiO₂, with the highest yield (108.7 $\mu\text{mol g}_{\text{cat}}^{-1}$) and acetic acid selectivity (61.0%) achieved at 15 mg of Au/TiO₂ (Figure 2e and Table S5). While higher Au/TiO₂ mass loadings (50–80 mg) improved acetic acid selectivity (up to 65.5%), the oxygenate yield dropped significantly (52.5 $\mu\text{mol g}_{\text{cat}}^{-1}$ at 80 mg), prompting the selection of 50 mg of Au/TiO₂ for balance. Further investigations at a total catalyst mass of 150 mg demonstrated that increasing the mass ratio of Au/TiO₂ to Fe/ZSM-5 ranging from 1/4 to 4/1 sharply reduced oxygenate yields from 102.8 to 14.5 $\mu\text{mol g}_{\text{cat}}^{-1}$ but enhanced acetic acid selectivity from 52.7% to 77.9%, which plateaued at higher ratios (Figure 2f and Table S6). Physically mixing 50 mg of Au/TiO₂ and 100 mg of Fe/ZSM-5 (denoted as Au/TiO₂ + Fe/ZSM-5) yielded 85.7 $\mu\text{mol g}_{\text{cat}}^{-1}$ of liquid products, with the CH₃COOH selectivity up to 64.9%, corresponding to the CH₃COOH yield of 55.6 $\mu\text{mol g}_{\text{cat}}^{-1}$.

To provide a more quantitative comparison between methane oxidation using externally supplied H₂O₂ and *in situ* generated H₂O₂, we evaluated the performance of externally supplied H₂O₂ versus *in situ* generated H₂O₂ in methane oxidative coupling to acetic acid. The results revealed that the *in situ* H₂O₂ pathway achieved a higher oxygenate yield (85.7 $\mu\text{mol g}_{\text{cat}}^{-1}$) compared to the external H₂O₂ route (58.5 $\mu\text{mol g}_{\text{cat}}^{-1}$), despite the higher acetic acid (85.5%) for externally supplied H₂O₂ system (Figure 2g). These results supporting the intrinsic advantage of tandem catalysis in enhancing H₂O₂ utilization efficiency due to its proximity-driven delivery between Au/TiO₂ and Fe/ZSM-5, which aligns with our conclusion that spatially confined *in situ* H₂O₂ generation favors overall oxygenate production despite the selectivity trade-offs. Importantly, our current work represents a significant advance by enabling methane-to-acetic acid conversion at 30 °C using O₂ as the terminal oxidant in the presence of CO, whereas previous systems required both externally supplied H₂O₂ or elevated temperatures (Table S7). We then calculated the Fe-based turnover frequency (TOF) values, it was found that our tandem catalytic system exhibits TOF values ranging from 0.08 to 0.58 h^{−1} at low temperatures (30 to 60 °C). These values outperform those reported for Rh-Fe/MoS₂ (0.05 h^{−1} at 25 °C and 0.18 h^{−1} at 80 °C) and Au-Fe/ZSM-5 (0.41 h^{−1} at 60 °C), indicating enhanced low-temperature performance of tandem catalyst when using O₂ in the presence of CO, although its performance remains inferior to systems utilizing externally added H₂O₂. We prepared Pd/TiO₂, Pt/TiO₂, and Cu/TiO₂ using the same method as Au/TiO₂ for comparison. Under identical conditions, the physical mixture of Au/TiO₂ and Fe/ZSM-5 showed the best performance (85.7 $\mu\text{mol g}_{\text{cat}}^{-1}$), far exceeding Pd/TiO₂ (10.0 $\mu\text{mol g}_{\text{cat}}^{-1}$), while Pt/TiO₂ and Cu/TiO₂ exhibited no activity in this tandem system (Figure S13a). H₂O₂ measurements after reaction further show that Au is the most effective catalyst for H₂O₂ generation (Figure S13b), leading to higher local H₂O₂ concentration and thus improved methane oxidation to oxygenates.

Considering both yield and selectivity, a 1:2 mass ratio of Au/TiO₂ to Fe/ZSM-5 was used. No liquid products formed without CO. Increasing CO to 6 bar produced 80.4 $\mu\text{mol g}_{\text{cat}}^{-1}$, mainly HCOOH (63.4% selectivity). The maximum yield of 127.9 $\mu\text{mol g}_{\text{cat}}^{-1}$ with 17.6% CH₃COOH selectivity occurred at 10 bar, while further increasing CO to 40 bar reduced the yield to 85.7 $\mu\text{mol g}_{\text{cat}}^{-1}$ (Figure 2h and Table S8), likely due to active-site blockage [13,39]. However, the selectivity towards CH₃COOH increased significantly from 17.6% to 64.9%, indicating that higher CO partial pressures promoted the formation of CH₃COOH.

We further examined the effect of methane partial pressure on the performance of Au/TiO₂ + Fe/ZSM-5. Increasing the CH₄ pressure from 3 to 15 bar raised the product yield from 13.3 to 85.7 $\mu\text{mol g}_{\text{cat}}^{-1}$ (Figure S14 and Table S9), while CH₃COOH selectivity decreased from 83.5% to 64.9%. At low CH₄ pressure, abundant CO mainly promotes O₂ activation and favors CH₃COOH formation via methyl-CO coupling. As CH₄ pressure increases, more CO is consumed to generate reactive oxygen species for oxidizing additional methyl intermediates. This promotes competing pathways, including direct oxidation to C₁ products and methyl-CO coupling. Consequently, although CH₃COOH formation continues, the increased production of C₁ products lowers its selectivity, reflecting the balance between coupling and oxidation pathways. The time-dependent catalytic activity of Au/TiO₂ + Fe/ZSM-5 demonstrated a continuous increase in liquid products yield with reaction time, while the total oxygenates yield increased continuously with reaction time, the selectivity for CH₃COOH declined from 82.6% to 64.9% (Figure S15 and Table S10). We attribute this primarily to accumulation of *in-situ* generated H₂O₂ during extended reactions. Elevated H₂O₂ concentrations promote key side pathways, notably enhanced formation of C₁ oxygenates including CH₃OH, HOCH₂OH, and HCOOH.

We evaluated catalyst stability using an *in situ* recycling approach. Oxygenate yields increased over four cycles to 85.7, 182.8, 262.3, and 357.2 $\mu\text{mol g}_{\text{cat}}^{-1}$ (Figure 2i), corresponding to cumulative yields 2.1, 3.1, and 4.2 times higher than the first cycle. Meanwhile, acetic acid selectivity among oxygenates increased from 64.9% to 75.5%. TEM images of the physically mixed catalyst after four cycles showed an average Au particle size of 5.7 ± 1.2 nm, nearly identical to the fresh catalyst (5.7 ± 1.9 nm) (Figure S16a,b). XPS analysis further confirmed that the valence states of Au and Fe remained essentially unchanged (Figure S16c,d), demonstrating excellent

catalyst stability. The extremely low leaching (<0.01% for both metals) under mild conditions (30 °C) rules out significant cross-contamination between catalyst components (Table S11).

3.3. Mechanism Studies Towards Methane Oxidation by Tandem Catalysis

Isotopic labeling experiments were conducted to identify the carbon source of the oxygenates using $^{13}\text{CH}_4 + \text{CO} + \text{O}_2$ and $\text{CH}_4 + ^{13}\text{CO} + \text{O}_2$ in H_2O . With $^{13}\text{CH}_4$, ^{13}C NMR signals at -4.91 , 20.47 , 48.92 , 81.85 , and 166.98 ppm corresponded to $^{13}\text{CH}_4$, $^{13}\text{CH}_3\text{COOH}$, $^{13}\text{CH}_3\text{OH}$, $\text{HO}^{13}\text{CH}_2\text{OH}$, and H^{13}COOH (Figure 3a) [40,41], while ^1H NMR showed their characteristic satellite peaks (Figure S17) [42]. Using ^{13}CO , only $^{13}\text{CO}_2$ and $\text{CH}_3^{13}\text{COOH}$ signals appeared [43], with no $^{13}\text{CH}_3\text{OH}$ or H^{13}COOH detected [44]. These results confirm that CH_4 provides the carbon for oxygenates, with CH_4 and CO supplying the methyl and carbonyl groups, respectively, for CH_3COOH formation.

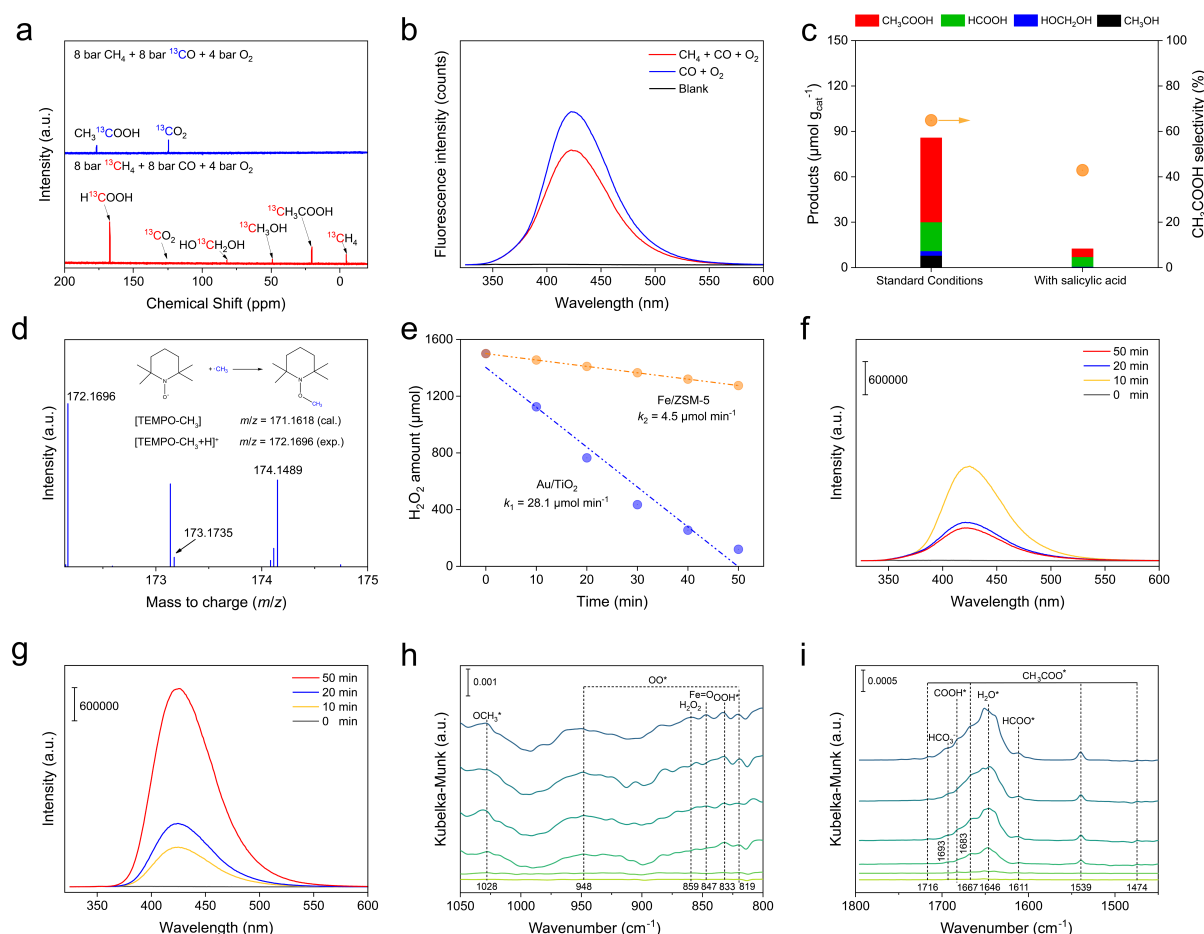


Figure 3. Reaction mechanism studies. **(a)** ^{13}C NMR spectrum. The reaction was conducted over 150 mg of $\text{Au/TiO}_2 + \text{Fe/ZSM-5}$ with mechanical stirring (1000 rpm) in 15 mL of H_2O under 8 bar of CH_4 (or 8 bar of $^{13}\text{CH}_4$), 8 bar of CO (or 8 bar of ^{13}CO), and 4 bar of O_2 at 30 °C for 6 h. **(b)** Fluorescence emission spectra. The reaction was conducted under standard reaction conditions except for 15 mL of NaOH -terephthalic acid solution added. **(c)** Quenching experiment. The reaction was conducted over 150 mg of $\text{Au/TiO}_2 + \text{Fe/ZSM-5}$ with mechanical stirring (1000 rpm) in 15 mL of H_2O under 15 bar of CH_4 , 40 bar of CO , and 6 bar of O_2 at 30 °C for 6 h. 1 mmol of salicylic acid as the scavenger. **(d)** HRMS spectra for TEMPO-trapped methyl radicals and hydroxyl radicals. The reaction was conducted under standard reaction conditions except for 100 mg of TEMPO added. **(e)** H_2O_2 decomposition experiments. **(f and g)** Fluorescence emission spectra over Au/TiO_2 and Fe/ZSM-5 . The reaction was conducted over 15 mg of catalyst with mechanical stirring (1000 rpm) in 15 mL of H_2O containing 1500 μmol H_2O_2 under 21 bar of N_2 and 40 bar of CO at 30 °C for different reaction time. **(h and i)** *In-situ* DRIFTS spectra of $\text{Au/TiO}_2 + \text{Fe/ZSM-5}$ at different wavenumber intervals after the exposure to CH_4 , CO , O_2 , and H_2O at 30 °C for 15 min (0 to 15 min, from bottom to top).

To probe the role of hydroxyl radicals, fluorescence spectroscopy was performed [45,46]. The $\cdot\text{OH}$ signal in the $\text{CO}/\text{O}_2/\text{H}_2\text{O}$ system was stronger than in the $\text{CH}_4/\text{CO}/\text{O}_2/\text{H}_2\text{O}$ mixture (Figure 3b), indicating $\cdot\text{OH}$ participation

in methane oxidation. Quenching experiments using salicylic acid further confirmed this role: the total oxygenate yield decreased from 85.7 to 12.3 $\mu\text{mol g}_{\text{cat}}^{-1}$, with acetic acid selectivity dropping from 64.9% to 42.9% (Figure 3c). High-resolution mass spectrometry with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) as a trapping agent detected TEMPO-CH₃ ([TEMPO-CH₃+H]⁺, m/z 172.1696) and TEMPO-OH ([TEMPO-OH+H]⁺, m/z 174.1489) (Figure 3d) [15,36]. These results confirm a free-radical pathway, where $\cdot\text{OH}$ radicals play a key role in methane activation and oxygenate formation. To investigate $\cdot\text{OH}$ formation over Au/TiO₂ and Fe/ZSM-5, H₂O₂ decomposition experiments were conducted to clarify their roles. Au/TiO₂ showed high H₂O₂ decomposition activity (28.1 $\mu\text{mol min}^{-1}$, Figure 3e), but fluorescence measurements revealed non-linear $\cdot\text{OH}$ generation (Figure 3f), indicating mainly non-productive decomposition to H₂O and O₂. In contrast, Fe/ZSM-5 displayed lower H₂O₂ decomposition activity (4.5 $\mu\text{mol min}^{-1}$) yet enabled sustained $\cdot\text{OH}$ accumulation (Figure 3g), suggesting efficient H₂O₂ activation. These results highlight the complementary roles of the two components. Au/TiO₂ efficiently produces H₂O₂, whereas Fe/ZSM-5 activates it to generate reactive $\cdot\text{OH}$ radicals, thereby explaining their synergistic cooperation.

To evaluate the role of water, we examined pH effects on catalytic performance. Under acidic conditions (HNO₃), the oxygenate yield increased from 85.7 to 105.1 $\mu\text{mol g}_{\text{cat}}^{-1}$ at pH = 3, while acetic acid selectivity remained at 64.9%, likely due to enhanced O₂ protonation and H₂O₂ formation (Figure S18). Under alkaline conditions (NaOH, pH~11), the yield further increased to 226.2 $\mu\text{mol g}_{\text{cat}}^{-1}$, but acetic acid selectivity decreased to 47.7%, indicating a shift toward C1 products favored by base-mediated pathways. Control experiments replacing H₂O with cyclohexane or acetonitrile gave no oxygenates (Figure S19), confirming that water is essential as both solvent and reactant. Mechanistically, H₂O participates in a water-gas shift reaction with CO to form COOH* and H*, where H* promotes O₂ protonation to generate H₂O₂ as the key oxidant. Thus, water plays a dual role as reaction medium and stabilizer.

To identify key intermediates, *in situ* diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) measurement was performed on a physical mixture of Au/TiO₂ and Fe/ZSM-5 exposed to CH₄, CO, O₂, and H₂O (1 bar, 30 °C, 15 min). Peaks at 948 and 819 cm⁻¹ correspond to M-OO* and M-OO-M* species (Figure 3h), indicating O₂ activation [47]. Signals at 859 cm⁻¹ (free H₂O₂) and 833 cm⁻¹ (M-OOH*) confirm H₂O₂ formation [45], while a band at 847 cm⁻¹ is assigned to Fe=O (Fe(IV)), a key oxidizing intermediate [4]. Peaks at 1693 and 1683 cm⁻¹ correspond to HCO₃⁻ and COOH* (Figure 3i) [48,49], typical intermediates in H₂O₂ formation from CO, O₂, and H₂O. For methane oxidation, bands at 1716, 1676, 1539, and 1474 cm⁻¹ indicate CH₃COO* [46,50], and a band at 1028 cm⁻¹ corresponds to OCH₃* (Figure 3h) [51]. These results show that Au/TiO₂ promotes O₂ activation and H₂O₂ formation, while Fe/ZSM-5 catalyzes methane oxidation to oxygenates in the presence of CO.

3.4. Effect of Integrated Manners on Methane Oxidation

The integrated manners of active sites in this tandem catalytic system plays a crucial role in determining the overall reaction performance. To systematically investigate the distance effect between Au/TiO₂ and Fe/ZSM-5 catalysts, we conducted *in situ* synchrotron vacuum ultraviolet photoionization mass spectrometry (SVUV-PIMS) experiments. As illustrated in Figure 4a, three distinct catalyst configurations were prepared by loading 100 mg of 20–40 mesh catalyst into a quartz reactor: (I) dual-bed layered packing configuration (Au/TiO₂ up and Fe/ZSM-5 bottom); (II) granule stacking after pelletization; and (III) mortar mixing before pelletization. Analysis focused on methane oxidation products CH₃OH (m/z = 32), HCOOH (m/z = 46), and CH₃COOH (m/z = 60), correlating with catalytic activity (Figure 4b). Under identical conditions, the mortar-mixed configuration (III) showed significantly stronger signal intensities than the other two. Time-dependent, background-subtracted data confirmed greater product accumulation in all cases, with configuration (III) exhibiting the highest activity (Figure 4c). The superior performance of the mortar-mixed catalyst stems from the minimal distance between active sites, which shortens H₂O₂ diffusion, maximizes its local concentration at Fe sites, and enables immediate activation for methane oxidation before decomposition. The superior performance of the mortar-mixed catalyst stems from the minimal distance between active sites, which shortens H₂O₂ diffusion, maximizes its local concentration at Fe sites, and enables immediate activation for methane oxidation before decomposition, as supported by experiments showing a critical H₂O₂ threshold for oxygenate formation (Figure S20).

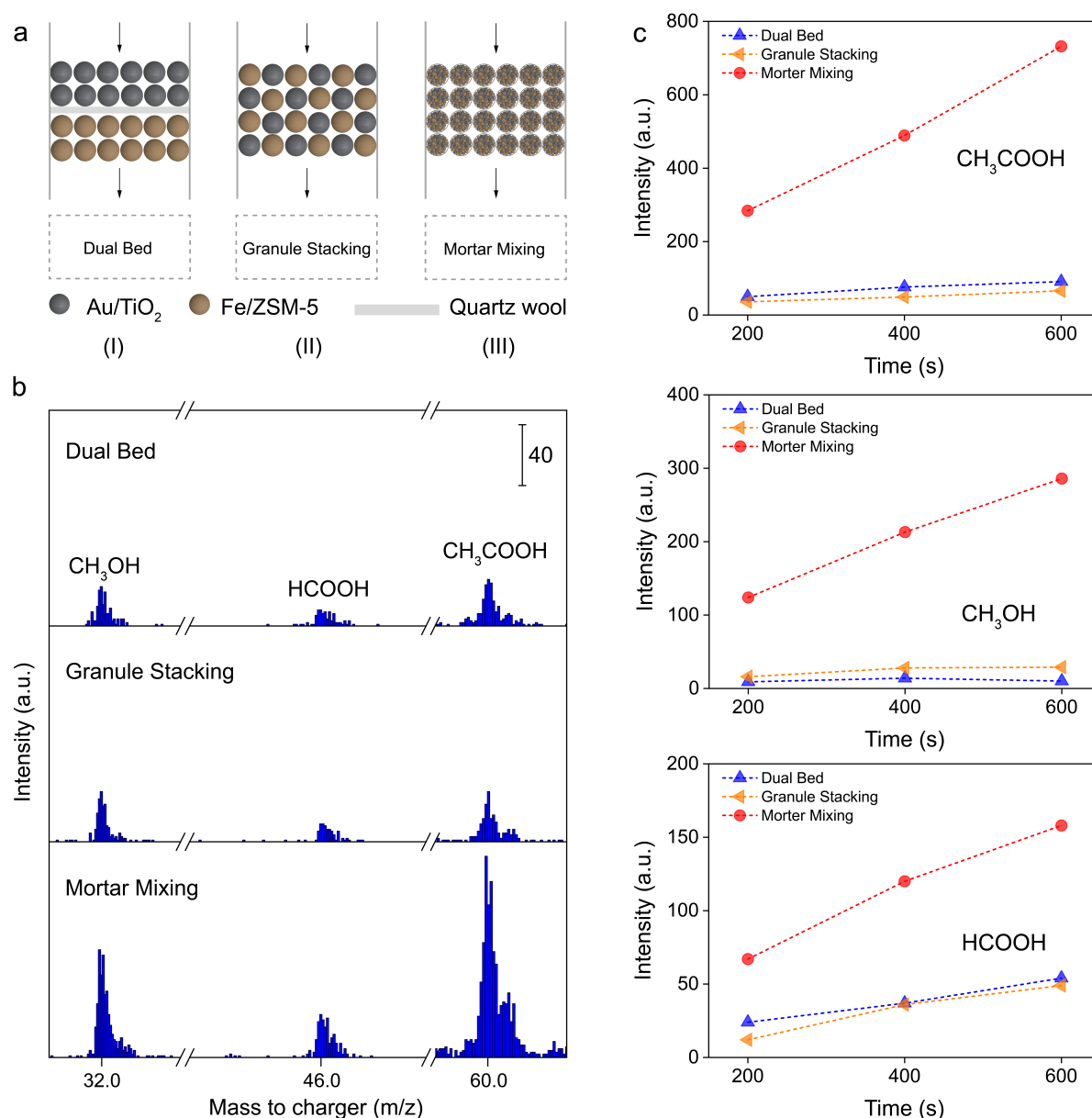


Figure 4. Effect of integrated manners on methane oxidation. (a) Detailed integration patterns. (b) SVUV-PIMS spectra of reaction intermediates during methane oxidation under the following reaction conditions: total pressure of 2 Torr at 30 °C with 11.5 eV photon energy, using gas flow rates of 15 mL min^{−1} of CH₄, 40 mL min^{−1} of CO, and 6 mL min^{−1} of O₂, where water was introduced by the feed gas. (c) Time-accumulated product distribution analysis.

4. Conclusions

In summary, we demonstrated a tandem catalysis system for methane conversion into acetic acid with O₂ in the presence of CO in H₂O solvent at room temperature, without the usage of an external energy source such as light, electricity, or γ -radiation. The process achieved an acetic acid yield of 55.6 $\mu\text{mol g}_{\text{cat}}^{-1}$ with a selectivity of 64.9% using the physical mixture of Au/TiO₂ and Fe/ZSM-5 at room temperature. The catalytic efficiency of this tandem system critically depends on the molecular-scale migration of *in situ*-generated H₂O₂ from Au/TiO₂ to Fe/ZSM-5. This close spatial proximity enables immediate activation of H₂O₂ at Fe sites to produce hydroxyl radicals, which drives the reaction toward optimal liquid-phase product formation. Our findings not only add a new dimension to research on the room-temperature conversion of methane, but also deepen our understanding of the proximity effect of multifunctional active sites in heterogeneous catalysis.

Supplementary Materials: The following supporting information can be downloaded at: <https://media.sciltp.com/articles/others/2607011053317055/MI-26060083-SM.pdf>. Figure S1. XRD patterns of TiO₂ and Au/TiO₂. Figure S2. TEM images for TiO₂ supported Au-based catalysts. (a) Au/TiO₂-A. (b) Au/TiO₂-P25. (c) Au/TiO₂-R. Figure S3. Au 4f XPS spectra of Au/TiO₂, Au/TiO₂-A, Au/TiO₂-P25, and Au/TiO₂-R. Figure S4. XRD patterns of ZSM-5 and Fe/ZSM-5. Figure S5. HAADF-STEM characterizations of Fe/ZSM-5-imp. (a) and (b) HAADF-STEM images of Fe/ZSM-5-imp. Figure S6. Fe 2p XPS spectra of Fe/ZSM-

5 and Fe/ZSM-5-imp. Figure S7. XAS measurements of Fe foil, Fe₂O₃, Fe/ZSM-5, and Fe/ZSM-5-imp. (a) Normalized Fe *K*-edge XANES spectra. (b) *k*³-weighted Fe *K*-edge EXAFS spectra. Figure S8. H₂O₂ synthesis over Au/TiO₂ and Fe/ZSM-5. The reaction was conducted over 15 mg of the catalyst with mechanical stirring (1000 rpm) in 15 mL of H₂O under 40 bar of CO, 6 bar of O₂, and 15 bar of N₂ at 30 °C for 1 h. Figure S9. Standard calibration curves for oxygenates quantification. (a) CH₃OH. (b) HCOOH. (c) CH₃COOH. (d) HOCH₂OH. Figure S10. (a) and (b) Typical ¹H NMR and UV-Vis spectra. Figure S11. Methane oxidation with H₂O₂ in the presence of CO over Au/TiO₂ and Fe/ZSM-5. The reaction was conducted over 50 mg of the catalyst with mechanical stirring (1000 rpm) in 15 mL of H₂O containing 1500 μmol of H₂O₂ under 15 bar of CH₄, 40 bar of CO, and 6 bar of N₂ at 30 °C for 1 h. Figure S12. Methane oxidation with H₂O₂ as the oxidant over Au/TiO₂. (a) Remaining H₂O₂ after reaction. (b) Amount of produced oxygenates. The reaction was conducted over 50 mg of Au/TiO₂ with mechanical stirring (1000 rpm) in 15 mL of H₂O containing 50–500 μmol of H₂O₂ under 15 bar of CH₄ and 46 bar of N₂ at 30 °C for 1 h. Figure S13. (a) Catalytic performance and (b) remaining H₂O₂ amount over M/TiO₂ (M= Au, Pd, Pt, Cu) and Fe/ZSM-5. The reaction was conducted over a total catalyst mass of 150 mg with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 6 h. Figure S14. CH₄ partial pressure-dependent product yields over Au/TiO₂ + Fe/ZSM-5 under a standard condition except for CH₄ partial pressure. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 61 bar containing 3–15 bar of CH₄, 40 bar of CO, 6 bar of O₂, and N₂ as a balanced gas at 30 °C for 6 h. Figure S15. Time-dependent product yields over Au/TiO₂ + Fe/ZSM-5 under a standard condition except for the reaction time. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 1–6 h. Figure S16. (a) TEM image and (b) Particle size distribution of Au nanoparticles for physically mixed Au/TiO₂ with Fe/ZSM-5 after fourth *in situ* cycles. (c) Au 4f XPS spectra for fresh and spent Au/TiO₂. (d) Fe 3d XPS spectra for fresh and spent Fe/ZSM-5. Figure S17. ¹H NMR spectrum for the isotopic labeling experiments. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 8 bar of CH₄ (or 8 bar of ¹³CH₄), 8 bar of CO (or 8 bar of ¹³CO), and 4 bar of O₂ at 30 °C for 6 h. Figure S18. Effect of pH on catalytic performance under standard reaction conditions. HNO₃ and NaOH were used to adjust the pH value of the system. Figure S19. Effect of solvent on catalytic performance over 50 mg of Au/TiO₂ and 100 mg of Fe/ZSM-5 under standard reaction conditions. Figure S20. Methane oxidation with H₂O₂ in the presence of CO over Fe/ZSM-5. The reaction was conducted over 100 mg of Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O containing 1.5–50 μmol of H₂O₂ under 15 bar of CH₄, 40 bar of CO, and 6 bar of N₂ at 30 °C for 1 h. Table S1. ICP-AES results of all the samples. Table S2. The deconvolution result of UV-Vis spectra of ZSM-5, Fe/ZSM-5, and Fe/ZSM-5-imp. Table S3. EXAFS fitting results of Fe foil and Fe-containing samples. *CN* refers to the coordination number. *R* refers to the bond length. *σ*² refers to the Debye-Waller factor. *ΔE*₀ refers to the inner potential correction to account for the difference in the inner potential between the sample and the reference compound. *R*-factor refers to the goodness of fit. Table S4. Adjusting the mass loading of Fe/ZSM-5 (0–100 mg) while maintaining a fixed Au/TiO₂ (15 mg) on methane oxidation. The reaction was conducted over Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 6 h. Table S5. Adjusting the mass loading of Au/TiO₂ (0–80 mg) while maintaining a fixed Fe/ZSM-5 (100 mg) on methane oxidation. The reaction was conducted over Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 6 h. Table S6. Mass ratio effect of the Au/TiO₂ to Fe/ZSM-5 on methane oxidation. The reaction was conducted over a total catalyst mass of 150 mg with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 6 h. Table S7. Comparison of methane oxidation with O₂/H₂O₂ as the oxidants in the presence of CO at low temperatures. Table S8. CO partial pressure-dependent product yields over Au/TiO₂ + Fe/ZSM-5 under a standard condition except for CO partial pressure. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 61 bar containing 15 bar of CH₄, 0–40 bar of CO, 6 bar of O₂, and N₂ as a balanced gas at 30 °C for 6 h. Table S9. CH₄ partial pressure-dependent product yields over Au/TiO₂ + Fe/ZSM-5 under a standard condition except for CH₄ partial pressure. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 61 bar containing 3–15 bar of CH₄, 40 bar of CO, 6 bar of O₂, and N₂ as a balanced gas at 30 °C for 6 h. Table S10. Time-dependent product yields over Au/TiO₂ + Fe/ZSM-5 under a standard condition except for the reaction time. The reaction was conducted over 150 mg of Au/TiO₂ + Fe/ZSM-5 with mechanical stirring (1000 rpm) in 15 mL of H₂O under 15 bar of CH₄, 40 bar of CO, and 6 bar of O₂ at 30 °C for 1–6 h. Table S11. ICP-AES analysis after methane oxidation under standard reaction conditions. References [52–55] are cited in supplementary materials.

Author Contributions: H.L. (Hongfei Lin) and J.Z.: conceived and designed the experiments. H.L. (Hongfei Lin), H.Y., C.M., C.L., L.Z. and H.L. (Hongliang Li): performed the catalyst preparation, characterization, and wrote the manuscript. H.L. (Hongfei Lin), H.Y., and H.L. (Hongliang Li): performed the catalytic tests. H.L. (Hongfei Lin), H.Y., and B.W.: prepared the manuscript and J.Z. and B.W.: revised the writing. The manuscript was written through the contributions of all authors. All authors have read and agreed to the published version of the manuscript.

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