

Vibrational Spectroscopy in the Study of Reaction Mechanism

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Abstract: The detailed investigation of reaction mechanisms is nowadays a requirement in nearly all fields of chemistry. Molecular spectroscopy can provide key pieces of information on the reaction scheme. Among all available techniques, vibrational spectroscopy ones (notably mid- IR absorption, Near-IR absorption, non-resonant Raman scattering, Resonance Raman scattering) represent possibly the most appropriate approaches to get valuable insight on reaction mechanism. In this account/perspective, following the research I have developed so far in different fields (ranging from photochemistry/photobiology to surface chemistry), I will describe the most appropriate vibrational spectroscopy techniques needed to get specific pieces of information. Emphasis will be put on the interplay between the different vibrational spectroscopies, and on the synergy with advanced data analysis techniques, quantum chemistry calculations, and other experimental approaches. After general conclusions, the perspectives in the field of vibrational spectroscopy applied to the study of reaction mechanisms—or, more broadly speaking, to the study of time-evolving systems—will be presented.

Keywords: Raman; time-resolved FTIR; photosynthetic reaction centers; flavonoids; electron transfer; proton transfer

1. Introduction

The study of reaction mechanisms has always been one of the key issues in physical chemistry. The use of spectroscopy in this field dates back to ~100 years ago (see for instance [1]). Vibrational spectroscopy is probably the best-suited among molecular spectroscopy techniques for investigating reaction mechanisms. Vibrational bands of a molecular species in fact reflect its structure, and in some cases their exact frequency depends on the conformation adopted and/or on their interactions with the surrounding environment (e.g., solvent). Therefore, molecular vibrations not only are intrinsic probes to investigate the appearance (products) or disappearance (reactants) of molecules, but, if bands due to intermediate states are detectable, they are already informative about their structure and/or conformation.

In addition, several vibrational spectroscopy techniques exist, which are complementary and can often be used in a synergistic way. In this perspective I will focus on mid/near infrared absorption, (non-resonant) Raman scattering and Resonance Raman scattering spectroscopies, which have been the main tools I have used in the last 25 years to investigate reaction mechanism in different research fields, ranging from photobiology to prebiotic chemistry, from organic photochemistry to surface chemistry, from hybrid materials to solution chemistry, from biomineralization processes to processes at the solid-liquid interface. This perspective article aims to show the enormous potential of vibrational spectroscopy in elucidating reaction mechanisms in basically all fields of chemistry, using the point of view of a physical chemist who had the opportunity to work in many different laboratories across Europe and many different research fields, who could get involved in setting up specific instrumentation and spectroscopic approaches, and who—during his career so far—could use many other



experimental approaches (mainly, but not exclusively, spectroscopic) in the quest for understanding reaction mechanism. This experience makes it possible to better understand the peculiarity of vibrational spectroscopies compared to other investigation techniques. Attention will be paid also to other aspects related to experimental techniques (e.g., Density Functional Theory—DFT—calculations or other computational tools for band assignment).

The order I will follow in the description of examples is (very) roughly chronological. However, at the beginning, a short presentation of the used experimental approaches and on techniques of data analysis is provided.

For detailed information on vibrational spectroscopy applied to specific areas of research, the interested reader is referred to specific reviews: some examples are [2–6].

2. How to Use Infrared, Non-Resonant Raman and Resonant Raman Spectroscopy in Studying Reaction Mechanisms

The most straightforward approach to use vibrational spectroscopy to study the mechanism of a chemical reaction is to record spectra at different times after the beginning of the reaction. This immediately brings us to the fundamental issue of reaction trigger. When the reaction is slow (minutes/hours/days) a simple manual mixing of reactants (when required; as we will see, in some cases the reaction can be studied just monitoring the evolution of the system under study) is sufficient. However, when the reaction is faster, the situation becomes more complicated. The situation is highly simplified when dealing with photo-induced reactions. In this case, light (for instance a laser flash) triggers the reaction and spectra are recorded at increasing times. Recording time-resolved vibrational spectra, notably at sub-second time-resolution, is not a trivial issue, especially in infrared spectroscopy. In fact, nowadays almost all infrared spectrometers are Fourier Transform (FT) Infrared instruments. In FTIR spectroscopy, a spectrum is not directly recorded, but it is the result of the FT of an interferogram, recorded through a Michelson interferometer. The key point is that a fixed amount of time (in the ms-second range) is needed to record this interferogram. When the conventional FTIR approach is used (i.e., the movable mirror is displaced continuously), therefore, the maximal time resolution depends on the maximal speed of the movable mirror in the interferometer.

In rapid-scan FTIR spectroscopy [7,8] the movable mirror of the interferometer moves at its maximum speed (~5 or 10 cm/s). For maximal time resolution, each interferogram is stored in a separate memory, and undergoes FT individually. Rapid-scan FTIR spectroscopy is limited in time resolution (milliseconds) due to mechanical limitations on mirror speed. Nowadays (the situation was different—and better—until ~20 years ago), an often-overlooked asset of several commercial instruments is the possibility to precisely synchronize external triggers—such as a laser flashing, fast mechanical shutter opening, or other electronic signal-controlled events—with data acquisition (the jitter being lower than 1 ms). This aspect can improve the time resolution in rapid-scan FTIR to 5–10 ms (depending on explored spectral range, and mirror velocity) [8].

Other parameters such as spectral resolution, interferogram recording mode (single sided, double sided...), ADC (Analog-to-Digital Converter) speed, also have an influence on the time resolution that can be attained and should be chosen with care [9].

For rapid-scan FTIR spectroscopy applied to photo-induced reactions, two main experimental approaches are used: spectra can be either recorded after one (or several) laser flashes, or they can be recorded under (and/or after) continuous illumination [5].

To achieve a higher time resolution while still using a FTIR spectrometer (and therefore exploiting the advantages of the FTIR approach) the time-resolved step-scan FTIR technique has been developed [10].

In step-scan FTIR spectroscopy, the movable mirror moves stepwise. At each position, a reaction (usually photoinduced) is triggered. Interferograms are built from time-resolved IR transients (one transient for each mirror stop position). This allows time resolutions down to 30–100 ns, depending on the IR detector used and on the speed of the ADC of the spectrometer [9]. When the internal ADC is used, a time resolution of ~10 μ s is usually obtained.

Step-scan FTIR spectroscopy is well-suited to investigate fully and rapidly reversible photo-induced processes. Irreversible or slow-relaxing systems can also be studied, but sample replacement is required.

Time-resolved IR measurements are possible with several other instrumental approaches (e.g., tunable IR lasers), and also at higher time resolution (ps-fs), but they do not rely on FTIR instruments. The interested reader is referred to [5].

In most cases, the spectral changes due to the reaction are so important that absolute IR spectra are already informative. However, in complex systems like enzymes, spectral changes are so small that it is necessary to use a difference approach. In other words, it is necessary to subtract the spectrum of the system before the reaction from each spectrum recorded after the beginning of the reaction. The term reaction-induced FTIR difference spectroscopy is often used [11]. It is interesting to note that also a “static” reaction-induced FTIR difference spectrum (obtained by subtracting the initial state from the final state) also provides a wealth of information on

the reaction mechanism in complex systems. In fact, in the obtained difference spectrum only bands of molecular species involved in the reaction appear.

Finally, it should be underlined that also in the framework of reaction-induced FTIR difference spectroscopy studies (both “static” or time-resolved), absolute IR spectra can also provide important pieces of information in product identification (including *in situ*, if the vibrational bands lie in a spectral region free from other spectral contributions) and in the assessment of starting conditions (or conditions after a given time). For instance, IR water bands are informative of the hydration state of the sample and on how water molecules interact with each other or with the solute.

A special case is the one of reactions at the solid-liquid interface. In this case, usually the time resolution is not a critical point. The biggest challenge is to find the appropriate conditions for this kind of measurements. The most widely used approach is time-resolved Attenuated Total Reflection (ATR) FTIR spectroscopy under continuous flow conditions of the liquid phase, the solid phase being deposited on the ATR crystal [6].

Near Infrared (NIR) spectroscopy is a technique which has defined peculiarities in mechanistic studies (for interesting examples see [12–14]). In the NIR region several interesting combination and overtone bands absorb, with the additional advantage that a thicker optical path can be used compared to mid-IR. Interestingly, NIR spectroscopy concerns mainly X-H bonds, and often bands which are overlapped in the mid-IR region are well-separated in the NIR region (for instance, the OH stretching band of alcohols superimposes to OH stretching band of water, but the combination band of OH of alcohols is well-separated from the combination band of water [14]). Actually, the combination band of water (at $\sim 5150\text{ cm}^{-1}$) is essentially free from other spectral interferences, and therefore it can be used as a reporter to quantify the amount of water molecules present in the sample. In addition, the shape of this band brings detailed information on the hydration state of a system (a protein, an inorganic surface, etc.), making it possible to distinguish strongly hydrogen-bonded water molecules from weakly hydrogen bonded ones [14].

Non-resonant Raman spectroscopy is also very useful when studying reaction mechanisms. A definite advantage of Raman spectroscopy is that solutes in (relatively diluted) aqueous solution can be studied, as the water bending band ($\sim 1640\text{ cm}^{-1}$) is extremely low. Another side advantage is that Raman bands usually have a narrow bandwidth, so that spectral overlap between bands is limited. Reaction monitoring by non-resonant Raman has been reported (see for instance [15]). Interestingly, Raman spectroscopy can be applied to obtain precise information on solute-solvent interactions (see for instance [16,17]) which are a key point in understanding the influence of the solvent on reaction mechanism. In non-resonant Raman, the use of FT-Raman (1064 nm laser) is recommended to avoid fluorescence interference either from the system under study or from fluorescent impurities.

Resonance Raman is the method of choice when a chromophore is present. Various approaches are possible for time-resolved experiments (including at high time resolution [18,19]). However, as for non-resonant Raman spectroscopy, often static measurements provide valuable information on how the molecule endowed with a chromophore (usually the reactant) interacts with its environment.

3. Data Analysis and Band Assignment

3.1. Data Analysis

Normally experimental data need some data correction. For instance, the most common issues in IR spectroscopy are baseline drifts and water vapor bands contamination, for which software corrections are available. It is however recommended, especially in IR difference spectroscopy, to perform these operations manually.

Another important issue in vibrational spectroscopy is the signal-to-noise ratio. Differently from UV-vis or fluorescence spectroscopy, this point is particularly relevant in vibrational spectra because identification of all bands (with the exact intensity and spectral position) is a key issue. A particularly nagging effect is observed in IR difference spectra, where if the noise level is too high, the “apparent bandwidth” of noise bumps is roughly the same of difference bands (especially if positive and negative bands are partially superimposed).

A different topic is how to analyze the obtained data, especially time-resolved vibrational spectra. If the main interest is in a kinetic study, single wavenumber kinetics analysis (especially for bands that are not subject to partial overlap from other bands) is a simple but direct way to follow kinetics of reactants, intermediates, and products. Such an approach is particularly useful if the reaction scheme is already known, or in case of a preliminary assessment (e.g., to evaluate the disappearance rate of reactants).

If the system under study has to be analyzed from zero, a first issue is to assess the number of species involved. Classical approaches are Singular Value Decomposition (SVD), Principal Component Analysis (PCA) or similar techniques [20].

A second issue is to evaluate the order of appearance (or disappearance) of given bands. To this aim, a particularly powerful technique is Two-dimensional Correlation spectroscopy (2D-COS) [21].

Is important to keep in mind that these approaches (SVD and assimilates, 2D-COS) are purely mathematical, and they do not suffer from any bias coming from the introduction of any hypothesis (kinetics, band spectral shape etc.).

Different is the case of other chemometric approaches, that can roughly be divided into soft-modelling strategies (like Multivariate Curve Resolution-Alternate Least Squares—MCR-ALS), where no hypothesis on the kinetic scheme is introduced, and hard modelling strategies, where the assumption of a kinetic scheme is the key point. Care is advised when such approaches are employed, especially when dealing with difference spectra (for more information see [20] and references therein).

3.2. Band Assignment

A completely different issue is how to assign vibrational bands in IR and Raman spectra. Strategies are different according to the peculiar system under study and the used technique (IR, non-resonant Raman, Resonance Raman).

A common strategy is to perform (when possible) replica experiments using isotopically-labelled molecules. Similarly, comparison with quantum chemistry calculations can be extremely useful. Also experiments performed in different solvents (or at different pH) often provide useful pieces of information. For time-resolved vibrational spectra, the effect of temperature can also be important (for instance in time-resolved studies the kinetics of appearance/disappearance of an intermediate can be slowed down, making its bands easier to identify; in some cases, intermediates can also be trapped).

In IR and non-resonant Raman spectroscopy a rough distinction (for reactions in condensed phase) can be made between simple systems (e.g., solutions, solid-liquid interfaces) where the number of involved molecular species is limited and spectra are in most cases absolute spectra, and complex systems (e.g., enzymes, DNA-protein complexes) where the number of involved species is high and difference vibrational spectra are involved.

In simple systems, often comparison with data from other techniques (e.g., Gas Chromatography coupled with Mass Spectrometry, GC-MS) can be extremely useful. The use of specific conditions can also reduce spectral interferences (e.g., if deuterated acetonitrile—ACN- d_6 —can be used as solvent, this provides a 1800–1200 cm^{-1} spectral interval free from bands coming from the solvent).

In complex systems, comparison with data from usual analytical chemistry techniques is often not possible and the solvent cannot be easily changed (e.g., reactions involving biomolecular systems). Conversely, other tools may become available: for instance, in the case of an enzymatic reaction, if site-directed mutagenesis techniques are available for the system under study, specific amino acids can be changed and spectra can be obtained on different mutants. This greatly helps the assignment of vibrational bands to specific amino acids (e.g., one specific Glutamic acid side chain among several Glu amino acid present in the protein). Another example comes from the use of D_2O : all the water-exposed moieties with exchangeable protons will undergo H/D exchange, deeply modifying the resulting vibrational spectra. Comparison of spectra recorded in H_2O and D_2O can provide important pieces of information. No standard strategy holds valid for any studied system; among the possible techniques, isotope labelling (when possible) and comparison with quantum chemistry-calculated spectra are the most appropriate.

4. Examples from Different Research Fields

4.1. Photosynthetic Reaction Centers

The photochemistry of photosynthesis takes place in special membrane proteins called reaction centers (RCs). In the so-called oxygenic photosynthesis (performed by e.g., plants and algae) two different RCs belonging to two different photosystems (called I and II) work in series. Conversely, the situation is simpler in anoxygenic photosynthesis (performed by bacteria), where only one RC is involved. The most studied RC is the one from the purple bacterium *Rhodobacter sphaeroides* (since 2020 renamed *Cereibacter-C. sphaeroides*; it is one of the first membrane proteins for which the structure was solved), whose schematic structure is shown in Figure 1. The initial light-induced event is the absorption of a photon by a dimer of Bacteriochlorophylls, named P, that in its excited state gives an electron to a primary acceptor A. The electron is then transferred to a ubiquinone molecule named Q_A within 200 ps, and then from Q_A to a second ubiquinone Q_B on a 100 μs timescale [22]. The formed P^+ is reduced to P by an external cytochrome. This makes it possible to repeat this series of electron transfer reactions when a second photon is absorbed by P. The consequence is the formation of doubly-reduced Q_B that takes two H^+ from the cytoplasm (through a proton transfer channel) forming the ubiquinol QH_2 that leaves the Q_B binding pocket to diffuse in the membrane. The Q_B site is refilled by another oxidized ubiquinone Q from the so-called membrane pool.

Site-directed mutagenesis is well established in *C. sphaeroides*, so that it is possible to change specific amino acids in the RC. Furthermore, it is possible to exchange naturally-occurring ubiquinones at the Q_A and Q_B site by isotope-labelled ubiquinones. This allowed in the past the identification of a large number of IR marker bands (for

ubiquinones and P in different redox states, specific amino acid side chain, localized conformational changes, internal water molecules).

On this ground, a series of FTIR difference spectroscopy studies have concerned the light-induced charge separation $PQ_A \rightarrow P^+Q_A^-$ and the following charge recombination $P^+Q_A^- \rightarrow PQ_A$ (see Figure 1a).

Malferrari et al. [23] used static “light-minus-dark” FTIR difference spectroscopy (spectrum recorded under continuous illumination minus spectrum recorded in the dark) and time-resolved Vis spectroscopy to investigate these reactions at different hydration levels; for time-resolved Vis experiments, two different illumination protocols were used (1 laser flash vs. 20 s continuous illumination). The authors found that both hydration and illumination period influence the kinetics of charge recombination deeply. Static “light-minus-dark” FTIR difference spectroscopy showed that the $P^+Q_A^-$ minus PQ_A spectrum (hereafter, following common practice, indicated as $P^+Q_A^-/PQ_A$) is strongly dependent on the hydration level. In particular, strong differences were observed at frequencies $>3100\text{ cm}^{-1}$, with spectra showing different behaviour of IR bands of specific weakly hydrogen-bonded internal water molecules, which could be identified through $H_2^{16}O/H_2^{18}O$ exchange. A subsequent Vis-NIR spectroscopy/FTIR difference spectroscopy paper showed that hydration also strongly influences the structure of P, especially in the $P^+Q_A^-$ charge-separated state [24].

The issue of the influence of hydration and illumination protocol on the $P^+Q_A^- \rightarrow PQ_A$ recombination reaction was recently investigated by time-resolved FTIR difference spectroscopy [25,26]. First, time-resolved FTIR difference spectra confirmed the results obtained from time-resolved Vis spectroscopy, i.e., dehydration induces a faster charge recombination reaction, whereas continuous illumination induced a slower charge recombination.

A detailed comparison was carried out between $P^+Q_A^-/PQ_A$ FTIR difference spectra recorded under (or 650 ms after) 20.5 s of continuous illumination, and spectra recorded after one 532 nm laser flash, at RH = 76% and T = 281 K [26]. The double difference spectrum (continuous illumination spectrum minus flash-induced spectrum) pinpointed meaningful (albeit small) different spectral features in the two situations. These features reflect variation in internal conformation in the so-called “light-adapted state” which is obtained after 20 s of continuous illumination. Very interestingly, these differences are very similar to those appearing in the double difference spectrum obtained by comparing FTIR difference spectra reflecting photo-induced charge separation in photosystem II in “light-adapted states” (recorded after 20 saturating flashes) and non-light-adapted states (recorded after a single saturating flash) [27]. It is also interesting to notice that also in photosystem II the light-adapted charge-separated state (produced after 20 saturating flashes at 5 Hz) recombines to the neutral resting state with a 3-time slower kinetics compared to “normal” charge-separated state (produced after one saturating flash) [27].

2D-COS analysis of time-resolved FTIR difference spectra obtained in *C. sphaeroides* RCs showed that in all the observed series of time-resolved spectra (under illumination, after illumination, after one laser flash), literature marker bands for P^+ and for protein response all evolve in synchronicity [26]. This shows that electron transfer charge recombination reactions and protein backbone response (at least the response indicated by a negative band at 1668 cm^{-1}) are deeply correlated. However, in time-resolved spectra under/after continuous illumination, several asynchronicities appear in the 2D-COS analysis, notably for several bands for which attribution is still lacking in the whole $1740\text{--}1200\text{ cm}^{-1}$ spectral region [26]. This indicates a complicated molecular scenario inside the RC during the continuous illumination-driven construction of the light-adapted state, as well as during its relaxation (related to the charge recombination) to the neutral PQ_A state.

Time-resolved rapid-scan FTIR difference spectroscopy was also used to investigate the protein response under strongly dehydrated conditions (RH = 11% at 281 K) [25]. As in the previous case, two illumination schemes were employed, i.e., (1) after a saturating flash, and (2) under/after 20 s continuous illumination. Spectra recorded after the laser flash showed that the protein response to charge separation recovers nearly synchronously (within the limited time resolution of the rapid-scan FTIR technique) to the $P^+Q_A^- \rightarrow PQ_A$ charge recombination. Unfortunately, in this case a detailed 2D-COS analysis was hampered by baseline drift issues. Time-resolved FTIR difference spectra recorded after continuous illumination (20 s) showed roughly the same kinetics of electron transfer $P^+Q_A^- \rightarrow PQ_A$ compared to time-resolved spectra obtained after one flash. This fact suggests that mechanism of formation of the light-adapted state deeply depends on the hydration level of the RC, the stabilization being less efficient under strongly dehydrated condition. Time-resolved FTIR difference spectra recorded after continuous illumination shows some indications that the 1666 cm^{-1} protein backbone band (corresponding to the 1668 cm^{-1} band at higher hydration) could decay faster compared to marker bands for the charge recombination reaction $P^+Q_A^- \rightarrow PQ_A$.

An interesting issue concerns the FTIR double-difference spectrum [25] obtained as in the previous work [26] (meaning FTIR difference spectrum recorded at the end of the continuous illumination period minus the flash-induced FTIR difference spectrum). Comparison with the same spectrum obtained at RH = 76% suggest that at RH = 11% a different light-adapted state is formed, compared to the light-adapted state at RH = 76%. Possibly, at

RH = 11% the response of the RC is restricted by limited movements of internal water molecules. This is also suggested by static “light-minus-dark” FTIR difference spectra ($3800\text{--}3000\text{ cm}^{-1}$) obtained with 20 s continuous illumination period at the two hydration levels [23]. Under strongly dehydrated conditions, only H^+ can move easily, whereas H_2O molecules cannot displace (and possibly cannot even rotate). This is likely to be the physical reason that makes an effective and long-lived light-adapted $\text{P}^+\text{Q}_\text{A}^-$ stabilized state unfeasible at RH = 11%.

As a final remark, it is noteworthy that these series of studies prompted also new approaches for H/D and $\text{H}_2^{16}\text{O}/\text{H}_2^{18}\text{O}$ exchange in bacterial RCs, based on the so-called isopiestic approach (i.e., dehydration/rehydration procedure carried out by changing the relative humidity thanks to saturated salt solutions). These approaches were described in two papers where both the efficiency and the kinetics of isotopic exchange were investigated by static and time-resolved FTIR and NIR spectroscopies [28,29]. The combination band of water centered at $\sim 5150\text{ cm}^{-1}$ turned out to be an excellent NIR marker band to follow hydration/dehydration and isotopic exchange processes [29].

Time-resolved FTIR spectroscopy has also been applied to the electron transfer reaction between the two ubiquinones ($\text{Q}_\text{A}^-\text{Q}_\text{B} \rightarrow \text{Q}_\text{A}\text{Q}_\text{B}^-$). A difficulty in this study is that normally, in isolated RCs no Cytochrome is present to reduce the oxidized primary donor P^+ . This fact is not only different compared to what happens under physiological conditions, but also has a practical problem: if P^+ is present, the observed reaction is actually $\text{P}^+\text{Q}_\text{A}^-\text{Q}_\text{B} \rightarrow \text{P}^+\text{Q}_\text{A}\text{Q}_\text{B}^-$ (see for instance [30]). IR bands arising from P^+ are present in the whole $1750\text{--}1200\text{ cm}^{-1}$ region and are roughly ~ 5 times more intense than those arising from Q_A^- and Q_B^- . In addition, spectra will also contain spectral contributions arising from the protein response to P^+ formation.

That's why Mezzetti et al. [7] proposed to use Diaminodurene (DAD) as an external donor to rapidly reduce P^+ (see Figure 1b; notice that $\text{DAD}^{\text{ox}}/\text{DAD}$ spectral contributions to time-resolved FTIR difference spectra are negligible). The $\text{Q}_\text{A}^-\text{Q}_\text{B} \rightarrow \text{Q}_\text{A}\text{Q}_\text{B}^-$ reaction could be followed in wild-type RCs and specific mutant RCs where the electron transfer reaction was slowed down. In fact, the electron transfer between quinones is rate-limited by a proton uptake from the cytoplasm towards the Q_B site, so that any mutation on the proton transfer channel slows down the electron transfer reaction. In the DN(L210)/DN(M17) double mutant, it was possible to record spectra corresponding to a pure $\text{Q}_\text{A}^-\text{Q}_\text{B}$ state (It should be noticed that the corresponding $\text{Q}_\text{A}^-/\text{Q}_\text{A}$ difference spectrum is recorded in the presence of a functional Q_B , and not in the presence of an inhibitor occupying the Q_B site) and to its evolution towards the $\text{Q}_\text{A}\text{Q}_\text{B}^-$ state. More importantly, it was possible to conclude that protonation of the side chain of a specific Glu residue called Glu-L212 (close to the Q_B pocket) is concomitant with Q_B reduction. A similar effect was also observed in other mutants, and upon heavy metal binding to the proton channel entry point [7,31].

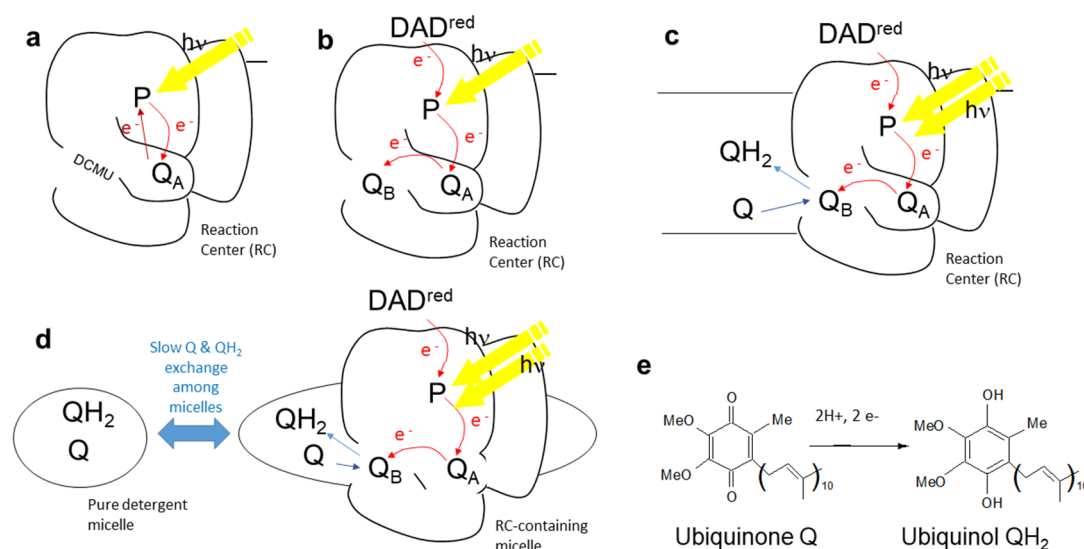


Figure 1. Reactions studied in Reaction Centers (RCs) from *C. sphaeroides* in different works. **(a)** Photo-induced reaction $\text{PQ}_\text{A} \rightarrow \text{P}^+\text{Q}_\text{A}^-$ and the following charge recombination $\text{P}^+\text{Q}_\text{A}^- \rightarrow \text{PQ}_\text{A}$. This is the situation studied in [23–26]. **(b)** Approach followed to investigate the reaction taking place between the two ubiquinones $\text{Q}_\text{A}^-\text{Q}_\text{B} \rightarrow \text{Q}_\text{A}\text{Q}_\text{B}^-$ used in [7,31]. **(c)** Approach followed (mainly under continuous illumination) to investigate the $\text{Q} \rightarrow \text{QH}_2$ reaction in chromatophores from purple bacteria [32–36]. **(d)** Approach used to investigate the $\text{Q} \rightarrow \text{QH}_2$ reaction (under continuous illumination and in presence of an excess of exogenous ubiquinone [37,38]) and the $\text{Q}_\text{A}^-\text{Q}_\text{B} \rightarrow \text{Q}_\text{A}\text{Q}_\text{B}^-$ reaction (using two consecutive flashes [31,37]) in isolated RCs. **(e)** Structural formulas of ubiquinone and ubiquinol and the reaction linking them.

The same approach was also used to investigate the $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ reaction and ubiquinol Q_BH_2 release. Rapid-scan FTIR difference spectra recorded between and after two consecutive flashes showed the formation and the displacement of Q_BH_2 from the Q_B site towards the outside [37].

The detailed investigation of the H^+ transfer processes related to the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ and $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-} \rightarrow Q_AQ_BH_2$ reaction required time-resolved FTIR difference spectra to be recorded at lower temperature (268 K). Under these conditions, reduction of P^+ by DAD is severely slowed down, and is therefore necessary to use a different external electron donor, tetramethyl-*p*-phenylenediamine (TMPD) which however gives intense spectral contributions in the 1630–1380 cm^{-1} region [31,37]. The influence of external redox mediators on P^+ reduction and quinol reoxidation has been studied in detail by time-resolved FTIR difference spectroscopy in [39].

The ubiquinol formation reaction was also followed by time-resolved FTIR difference spectroscopy during/after continuous illumination in the presence of exogenous quinones [37]. In a following paper the obtained spectra were also analysed by MCR-ALS coupled to 2D-COS analysis to better understand the overall reaction mechanism [38]. This work highlighted for the first time the possible formation of a new ubiquinol species (characterized by a strong positive band at 1565 cm^{-1} ; see below for further details) under intense illumination conditions (see Figure 2).

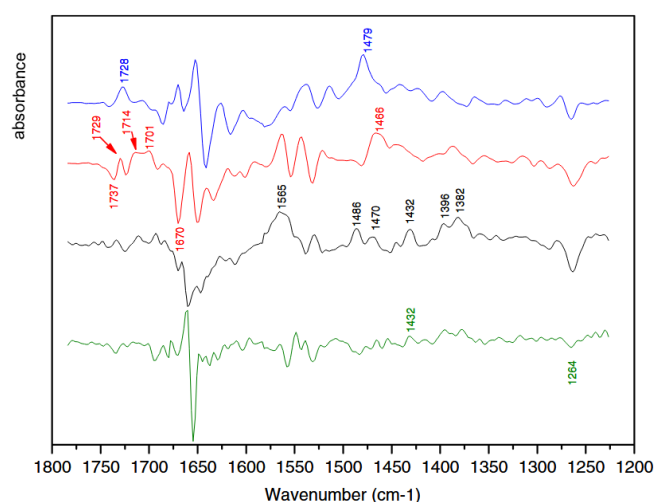


Figure 2. IR difference spectra extracted from the MCR-ALS procedure [38] applied to time-resolved FTIR difference spectra recorded during and after continuous illumination in isolated RCs from *C. sphaeroides* in presence of an excess of exogeneous ubiquinone. From top to bottom, the first spectrum represents Q_B^- formation (see characteristic positive band at 1479 cm^{-1}); the second reflects mainly Q_A^- formation (see characteristic positive band at 1467 cm^{-1}); the third reflects mainly $Q---QH_2$ (quinhydrone charge transfer) complex (see characteristic positive band at 1565 cm^{-1}); the fourth probably represents QH_2 formation (as suggested by the presence of the positive band at 1432 cm^{-1} and of the negative band at 1264 cm^{-1}). Figure adapted from Mezzetti et al. *Anal. Bioanal. Chem.* **2011**, 399, 1999–2014. <https://link.springer.com/article/10.1007/s00216-010-4325-0> (ref. [38]) and reproduced with permission from Springer Nature.

A following paper [32] showed that this peculiar ubiquinol species (characterized by the 1565 cm^{-1} positive band) is also formed after two flashes; quantum chemistry calculations associated to H/D exchange experiments demonstrated that it corresponds to a quinhydrone charge-transfer ubiquinone-ubiquinol complex $Q---QH_2$, as also observed in solution and in photosynthetic membranes (see later).

The investigation of the process of ubiquinol release in isolated RCs, especially in experiments under and after continuous illumination in presence of exogenous ubiquinones, is complicated by slow kinetics of ubiquinone and ubiquinol exchange between pure detergent micelles and RC-containing micelles. It is much more appropriate to investigate it in photosynthetic membranes (chromatophores), despite that fact that under these conditions is no longer possible—due to sensitivity issues—to investigate electron transfer, proton transfer and related events inside the RCs. Experiments on chromatophores also require the use of specific inhibitors for the two quinone binding sites of the so-called cytochrome bc_1 complex, to avoid further biochemical reactions involving quinones.

In [33] multiple quinone reduction in chromatophores was monitored by time-resolved FTIR difference spectroscopy, without any spectral interference coming from other biochemical reactions. In the spectra, a strong photoinduced signal indicative of protein conformational change (most likely due to a heating effect), was observed. This signal had different kinetics compared to ubiquinone reduction (and following ubiquinol

reoxidation by external redox mediator). To better understand the origin of this signal, spectra were analyzed by MCR-ALS using different strategies, like matrix augmentation [34] and so-called hard-soft modelling approaches (where a kinetic model can be applied selectively to one or more evolving species) [35]. Whereas this signal appeared disconnected with respect to ubiquinol formation and release, under intense light illumination bands due to a new ubiquinol species was identified (see Figure 3). In a following paper [32] this species was demonstrated to be the quinhydrone charge-transfer $Q\cdots QH_2$ complex, as observed also in isolated RCs.

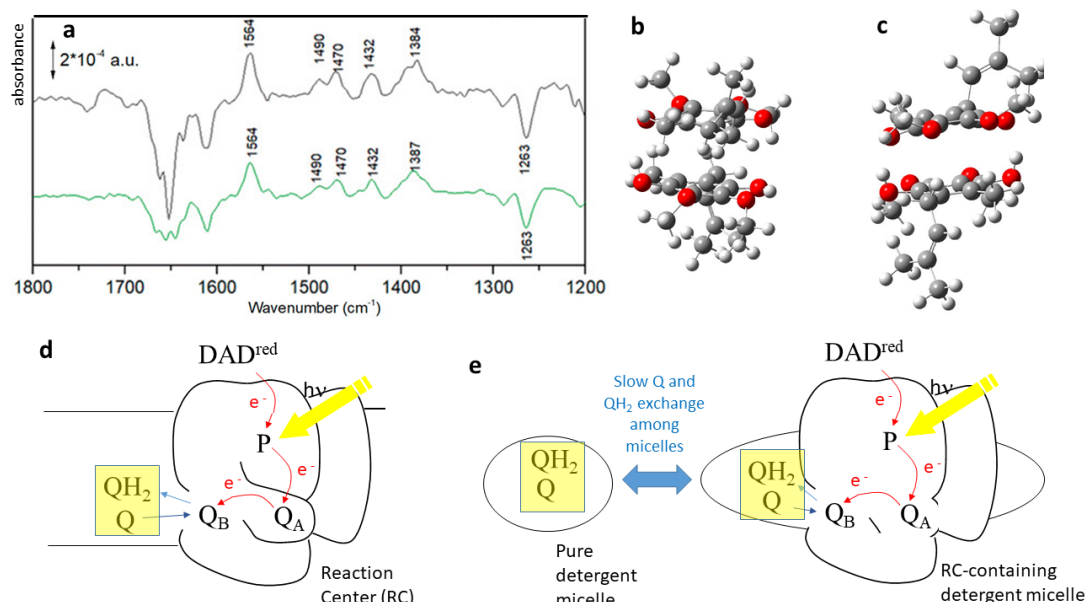


Figure 3. (a) FTIR difference spectra, reproduced from [32] showing $Q\cdots QH_2$ (quinhydrone charge transfer) complex formation in chromatophores (black trace) and isolated RCs (green trace) from *C. sphaeroides*. Spectra were obtained either directly (chromatophores) or after subtraction of Q_B^-/Q_B contributions (isolated RCs) but without any chemometric treatment of data. (b,c) Two possible proposed geometries for the $Q\cdots QH_2$ (quinhydrone charge transfer) complex as calculated in [32]. (d) Proposed scenario for quinhydrone formation on chromatophores from *C. sphaeroides*. (e) Proposed scenario for quinhydrone formation in isolated RCs from *C. sphaeroides*. Figure adapted from Mezzetti et al. *Int. J. Mol. Sci.* **2023**, *24*, 5233 (ref. [32]) published under CC BY 4.0 license. <http://creativecommons.org/licenses/by/4.0/>.

Relying on the availability of IR marker bands for different redox state of quinones (Q_A , Q_B , Q_A^- , Q_B^- , Q , QH_2 , $Q\cdots QH_2$), Mezzetti et al. recently investigated the process of photo-induced ubiquinone reduction in wild-type and PufX[−] chromatophores from another purple bacterium, *R. capsulatus* [36].

In PufX⁺ membranes the molecular events resemble those found in *C. sphaeroides* chromatophores. For time-resolved FTIR difference spectra under illumination, soon after beginning of illumination IR signals characteristic of a protein conformational change were observed. Progressive photoinduced Q reduction proceeded with slower kinetics.

In PufX[−] membrane the situation was more complicated. Spectra recorded during continuous illumination demonstrated a much smaller amount of photo-reduced ubiquinol. This is in agreement with previous articles reporting that the PufX protein is necessary for efficient QH_2/Q exchange at the Q_B site of the RC. Interestingly, Q consumption and QH_2 formation are associated with rapid formation of the semiquinone Q_A^- in the RC. This demonstrates that in PufX[−] membranes no efficient access to the Q_B site of the RC is provided to a large part of the membrane Q pool. The appearance of a positive band at 1560 cm^{-1} indicates also transient formation of a $Q\cdots QH_2$ quinhydrone complex.

It turned out to be difficult to apply time-resolved step-scan FTIR difference spectroscopy to purple bacterial RCs at room temperature, giving noisy spectra [26]. However, the technique was successfully applied to investigate the primary charge separation of a different kind of RC from *Chlorobium tepidum* at 100 K and allowed identification of IR marker band for the Bacteriochlorophyll dimer P_{840} in different redox and electronic states [40].

4.2. Photosynthetic Antennae

IR spectroscopy has been widely used to investigate light-harvesting complexes [41]. Time-resolved step-scan FTIR difference spectroscopy was also successfully applied to investigate the process on triplet formation in

water-soluble antennae complex called Peridinin-Chlorophyll *a* Complexes (PCPs) from the dinoflagellate *Amphidinium (A.) carterae* [42]. Triplet formation (localized of carotenoid Peridinin—Per—molecules) is an important photoprotective mechanism to avoid $^3\text{Chl-}a$ -triggered formation of $^1\text{O}_2$, a dangerous Reactive Oxygen Species (ROS).

This work inserted in a large debate on the nature of the triplet state on *A. carterae* PCP among different research groups using various spectroscopic techniques, including time-resolved FTIR spectroscopy (see [43] and refs therein). It demonstrated that no definite indication for a triplet state delocalization on Chl *a* molecules could be deduced, and that the triplet state is likely to be localized on a single Per molecule. Moreover, this and following research works were very important from a methodological point of view, as they demonstrated that:

- (1) Careful setting of actinic laser power and temperature control is necessary in time-resolved step-scan FTIR difference spectroscopy to avoid laser heating-induced artefacts that can be misinterpreted as an indication of $^3\text{Chl } a$ (this methodological conclusion is however valid for any step-scan measurement);
- (2) Single wavenumber precise kinetic analysis avoids kinetic misinterpretation arising from multiple band fitting-based software when similar (but not equal) kinetic behaviour of IR bands is present;
- (3) DFT calculations and comparison with vibrational spectra of isolated pigments can be extremely helpful in band assignment;
- (4) Raman and (even more) Resonance Raman spectra of Per in different solvents [44,45] are extremely useful in assessing how the C=O stretching frequency of Per is influenced by the surrounding environment (both in the protein and in prototypical solvents), through H-bond formation and dielectric-constant- induced Fermi resonance effects (see Figure 4). Also in this case, the synergy with calculations (notably Quantum Mechanics/ Molecular Mechanics—QM/MM—dynamics) turned out to be a key point [44,45].

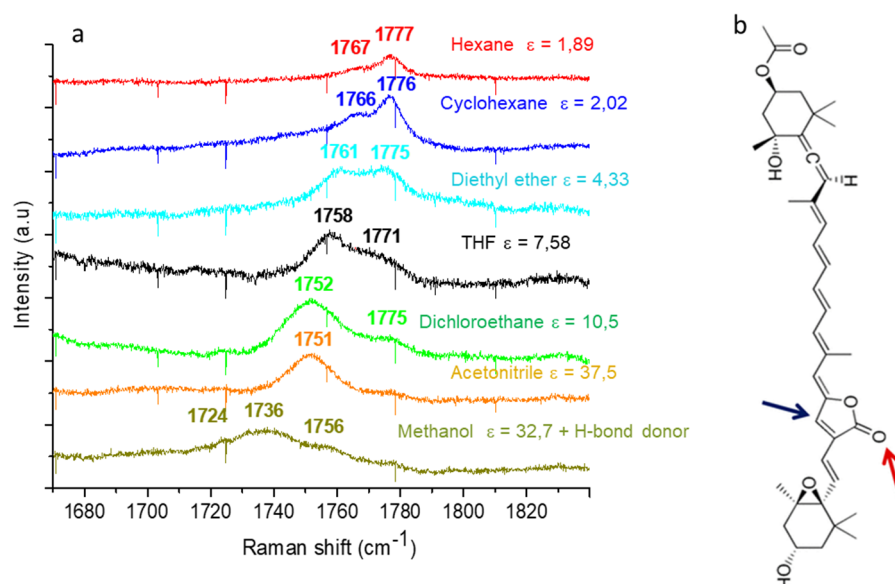


Figure 4. (a) Resonance Raman spectra of Peridinin (excitation wavelength of 514.5 nm, spectral window of 1670–1840 cm^{-1}) in different solvents (whose dielectric constant is indicated). The band(s) in the 1720–1780 cm^{-1} region are due to the stretching of the lactonic C=O and are influenced by two phenomena: first, Fermi resonance with a C-H vibration (leading to a splitting of the C=O stretching band) [45] and secondly, only in methanol, to different H-bonding status of the lactonic C=O (which can accept 0, 1, or 2 H-bonds from solvent molecules [44]). In all solvents but methanol, it is important to notice that the position of the C=O stretching frequency is shifted by the polarity of the solvent (whereas the C-H wagging vibration interacting through Fermi resonance is insensitive to solvent polarity). As a consequence, the phenomenon of Fermi resonance is maximum when the position of the C=O stretching frequency lies exactly at twice the value of the frequency of the wagging of the C–H in the lactonic ring. This situation takes place in the case of diethyl ether. The shape of the Fermi resonance doublet emerges as a probe of the polarity of the environment surrounding the lactonic C=O. The authors proposed that such an effect could also be exploited in Peridinins inside light-harvesting systems, like PCP [43–45]. (b) Structural formula of Peridinin. The red arrow indicated the lactonic C=O, the blue arrow the C-H in Fermi resonance with it.

A final time-resolved step-scan FTIR spectroscopy study was carried out in order to identify multiple IR marker bands for Per and ^3Per in *A. carterae* PCP [46].

For further information about infrared studies on PCP and other antennae complexes, the interested reader is referred to specific reviews [41,43].

4.3. Cyanobacterial Photoreceptors

Photoreceptors are light-sensitive proteins that upon absorption of a photon initiates a series of biochemical events.

Orange Carotenoid Protein (OCP) is a simple blue-green photoreceptor involved in the photoprotection of cyanobacterial photosynthetic membranes [47]. It is made up of a single, ~300 amino acid polypeptide chain (organized in two domains, a N-terminal domain NTD and a C-terminal domain CTD) and one keto-carotenoid. In naturally-occurring OCPs this carotenoid is 3'-hydroxyechinenone. However, photoactive OCPs expressed in *E. coli* containing either echinenone or canthaxanthin are commonly studied and used for specific applications, like e.g., synthesis of photochromic hybrid inorganic-protein materials [48], optical/fluorescent pH and temperature sensors ([49] and references therein), development of OCP with redox-controllable structural dynamics and photoprotective function [50]; for other possible applications of OCP and related proteins, see [51].

The photoactivation mechanism of OCP is under investigation since 2008, when its photoinduced conversion from an inactive, orange form to an active red form capable of binding specific light-harvesting complexes named Phycobilisomes (PBs) was reported [52].

In 2012 Wilson et al. constructed several OCP mutants, in order to investigate the influence of specific mutations on photoactivity, stability, and binding to PBs [53]. The disruption of the salt bridge between Arg155 and Glu244, which stabilizes the interaction between NTD and CTD, increased the rate of photoactivity and the stability of the photoactivated OCP. Static light-minus dark FTIR difference spectroscopy was used to detect possible structural changes induced by intense blue light in the mutated OCPs. FTIR difference spectra showed almost identical spectral features in the amide I region for wild-type and mutant OCPs, indicating a very similar structural response to absorption of light.

Some years later, static and time-resolved FTIR difference spectroscopy under continuous illumination at different temperatures was applied to investigate OCP photoactivation mechanism [54].

Time-resolved FTIR difference spectra at 277 K showed that light-induced conformational change proceeds through at least two different steps, both on the timescale of seconds (see Figure 5). Each step involves a partial reorganization of one or more α -helix domains. At early times after switching on the light, the disappearance of a non-water-exposed α -helix is observed. At longer times, spectra indicate the disappearance of a water-exposed α -helix (most likely either the N-terminal extension and/or the C-terminal tail). A detailed kinetic analysis confirms that these two events are deeply asynchronous [54].

Comparison of static FTIR difference spectra recorded in H₂O and D₂O suggests that some internal water molecules may play a role in the photoactivation mechanism [54].

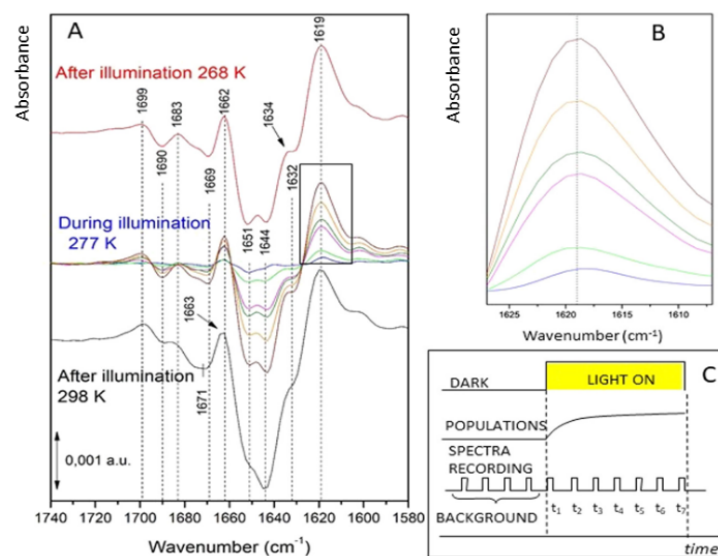


Figure 5. (A) Time-resolved FTIR difference spectra recorded on Orange Carotenoid Protein (OCP) from *Synechocystis* during illumination at 277 K, following the scheme shown in the lower right panel (panel C). For comparison, static light-minus-dark FTIR difference spectra recorded on OCP after illumination at 268 K and 298 K are shown at the top and at the bottom of the Figure. Spectra were recorded at the following times after onset of light: blue, 7 s; light green, 33 s; magenta, 101 s; dark green, 129 s; yellow, 189 s; brown, 270 s. (B). Enlarged

time-resolved spectra at 277 K showing the evolution of the 1619 (+) cm^{-1} band. Same timing as in (A). (C) Scheme of the experiment under illumination. Background spectra are recorded in the dark and, after onset of light, at increasing times. FTIR difference spectra are obtained subtracting the background from spectra recorded at different times. Reprinted with permission from Mezzetti et al. *J. Phys. Chem. B* **2019**, 123, 3259–3266 (ref. [54]). Copyright 2019 American Chemical Society.

More recently, several works have strongly suggested that the specific ketocarotenoid used as a chromophore can lead to different photoactivation mechanisms. In this framework, it becomes important to compare static FTIR difference spectra in OCP binding different ketocarotenoids (3'-hydroxyechinenone, echinenone, canthaxanthin). The final protein response looked very similar, and therefore it became important to identify marker bands for the different carotenoids in both the orange resting state and the red activated state [55]. This is a necessary step for time-resolved IR spectroscopy experiments with high time resolution (ps-ms) in order to follow the initial events of ketocarotenoid photophysics and its movement from the original binding site into the NTD (where it is located in the final red state).

4.4. Flavonoid Photochemistry and Photophysics

Flavonoids are natural organic compounds found in fruits, vegetables, and berries, with interesting biological properties. Their antioxidant role is the object of intense study. Photochemistry of flavonoid is also a widely explored field, especially for their relevance in several fields, from photoprotection to fading of flavonoid-based artistic pigments, from fluorescent-based protocols in analytical chemistry to applications in optics and optoelectronics.

Among flavonoids, flavonols (which are characterized by the 3-hydroxyflavone backbone) have a specific photophysics (see Figure 6a). In particular, upon excitation in the UV, flavonols (especially those devoid of the 5-OH group) are characterized by an Excited State Intramolecular Proton transfer (ESIPT) entailing formation of a fluorescent tautomer that emits in the green. In presence of factors that hamper ESIPT, also fluorescence from the neutral form (in the violet region) is possible. In addition, in some conditions, ground-state deprotonation of 3-OH moiety can also take place, leading to the formation of a fluorescent anion (emitting in the blue) [56]. Fluorescence of anionic form of flavonols is an important phenomenon in biophysics and analytical chemistry (see for instance [57]). Thanks to this interesting and multifaceted photophysical behavior, flavonols have found large applications in fluorescence spectroscopy and microscopy as environment-sensitive fluorophors [58]. Many turn-on flavonol-based fluorophors have also been developed [59] and applications in photolithography, radiation detection, CO-photorelease and photo-driven organic synthesis have been reported [60,61].

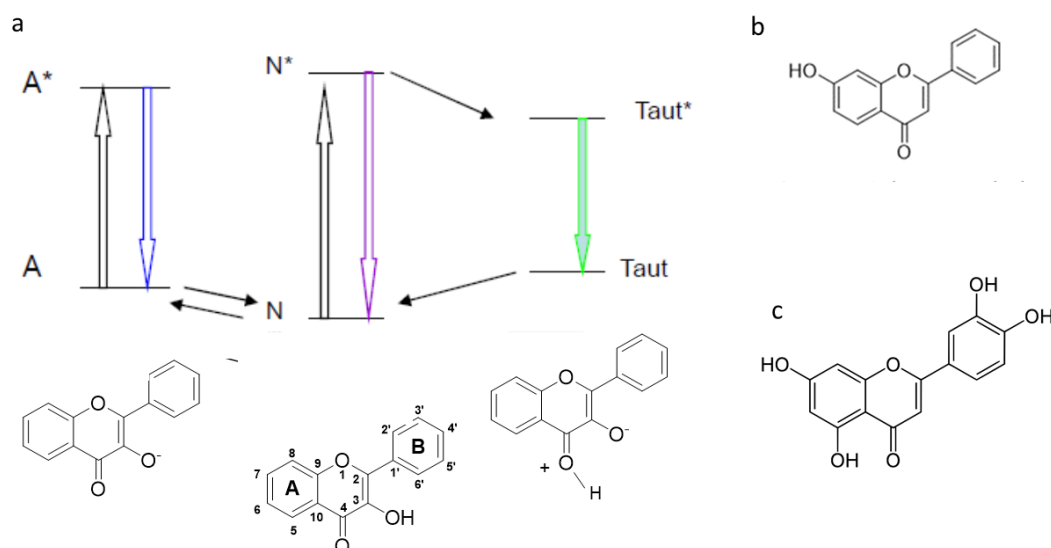


Figure 6. (a) Structural formula of 3-hydroxyflavone 3HF (Normal form N) with its numbering scheme, of the 3-hydroxyflavone anion (A), and of the photo-produced tautomer (T), along with the energy level for electronic transition in absorption and fluorescence. (b) Structural formula of 7-hydroxyflavone (7HF). (c) Structural formula of luteolin.

Protti et al. [62] investigated the photochemistry of 3-hydroxyflavone (3HF) and its complexes with Al(III), Zn(II) and Pb(II) in methanol and methanol/water mixtures by a multi-disciplinary approach, associating electronic, FT-Raman and FTIR spectroscopies with DFT/time-dependent (TD) DFT calculations and with a

traditional organic photochemistry strategy (illumination, chromatographic separation, characterization—MS and NMR—of isolated photoproducts). The presence of Pb (II) slows down photodegradation of 3HF, whereas the Al(III) complex is very photostable. The Zn(II) complex has a photo-reactivity comparable to free 3HF. In all cases (except the Al(III) complex) the photo-product is 3-hydroxy-3-phenyl-1,2-indandione, in agreement with the literature for 3HF photochemistry in methanol [63]. The cations therefore do not change the photoreaction. Interestingly, for Zn(II) and Pb(II) complexes, the photo-product 3-hydroxy-3-phenyl-1,2-indandione remained in its free form (no metal complexation). The effect of the presence of O₂ was studied. Opposite effects were observed for free 3HF and Zn(II) and Pb(II) complexes.

From a methodological point of view, on the one hand, FT-Raman made it possible to study in detail the structure of complexes and the effect of solvent on vibrational bands, whose exact frequency reflects specific solute-solvent interactions. On the other hand, time-resolved FTIR spectra under illumination were used to better understand the photodecomposition mechanism of metal complexes (see Figure 7).

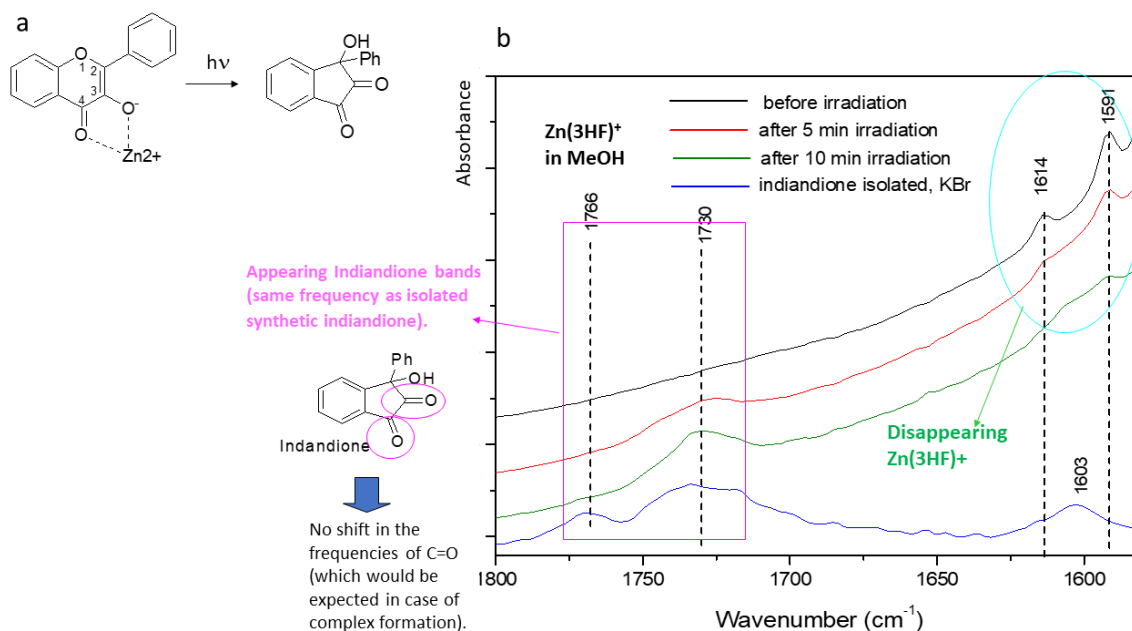


Figure 7. (a) Photodecomposition scheme of 3HF-Zn²⁺ complex. (b) FTIR spectra recorded at different times under illumination (black, red, green traces), and FTIR spectrum of isolated indandione (blue trace). Time-resolved FTIR spectra under illumination show progressive disappearance of the 3HF-Zn²⁺ complex and concomitant formation of indandione, characterized by two C=O stretching bands. The position of these bands is the same as in isolated indandione, assuring that the photoproduct does not interact with Zn²⁺ to form a complex. Note that in methanol solution, whereas the band at 1730 cm⁻¹ looks intense, the band at 1766 cm⁻¹ appear just like a weak shoulder, possibly because of specific solvent-solute interaction.

The photochemistry/photophysics of 3HF and its Al(III) complex was also studied in SiO₂ xerogels [64]. Interestingly, the extreme photostability of the complex seems to be lost inside these matrices, even though its photodecomposition is slower compared to free 3HF. This is surprising, as the strong fluorescence of the complex (observed also in solution) is maintained. Micro-Raman spectra suggest that a small fraction of 3HF remains free inside the pores. The photodecomposition of the complex may be somehow related to the presence of free 3HF. It should be added that the position of 3HF Raman peaks suggests that the molecule is located in an apolar environment.

A similar study was carried out by Landstrom et al. [65,66] on 3HF and 7-hydroxyflavone (7HF) in MCM-41 mesoporous silica nanoparticles. Both 3HF and 7HF-doped nanoparticles showed high fluorescence, stable after two months of air exposure. UV irradiation resulted in a moderate decrease in fluorescence. FTIR spectra (coupled with DFT calculations) were used to better understand the interaction of flavonoids with the surrounding matrix. FTIR showed that acetonitrile (the solvent used for the post-doping procedure) is partially retained within the pores in both 3HF-doped and 7HF-doped nanoparticles. This might have a role in stabilizing the two flavonoids, both to air exposure and to UV irradiation.

The effect of solvent on 3HF vibrational bands was also investigated. Seitsonen et al. [67] reported a detailed study associating IR and Raman spectroscopy with detailed calculations. They studied the vibrational properties of 3HF in gas phase and in CCl₄, CHCl₃ and acetonitrile, performing dynamic DFT simulations using the

QuickStep module of the CP2K package, and associated them to static DFT calculations (without and with implicit solvent). Nearly all 3HF vibrational bands in the 1550–1660 cm^{-1} range turned out to be very sensitive to the surrounding solvent. In the case of acetonitrile solutions, specific solute-solvent interactions (notably hydrogen bonds) have been proposed to be particularly critical. Interestingly, the same conclusion was also obtained by TD-DFT calculations with explicit acetonitrile molecules to simulate UV-Vis absorption spectra [68], and by a physical organic approach using Hydrogen bonding basicity and acidity of solvents [69].

In another study, Salomon et al. [70] adsorbed 3HF on the surface of nanoparticles of TiO_2 , with and without a conformal Al_2O_3 layer. The kinetics of uptake—along with XPS characterization—suggested formation of a monolayer. FTIR spectroscopy showed that 3HF molecules use the coordinatively unsaturated metal ions on the surface to form metal complexes (see Figure 8). Upon light irradiation, the photoluminescence of the surface complexes can be modulated according to the binding metal ion. Solid-state emitters in a high quantum yield (~15%) are thus obtained. In addition, photoinduced charge injection can be promoted or inhibited (according to the thickness of the insulator layer) as demonstrated by pump-probe experiments. A multifunctional hybrid system based on coordination chemistry 3HF monolayers was therefore developed.

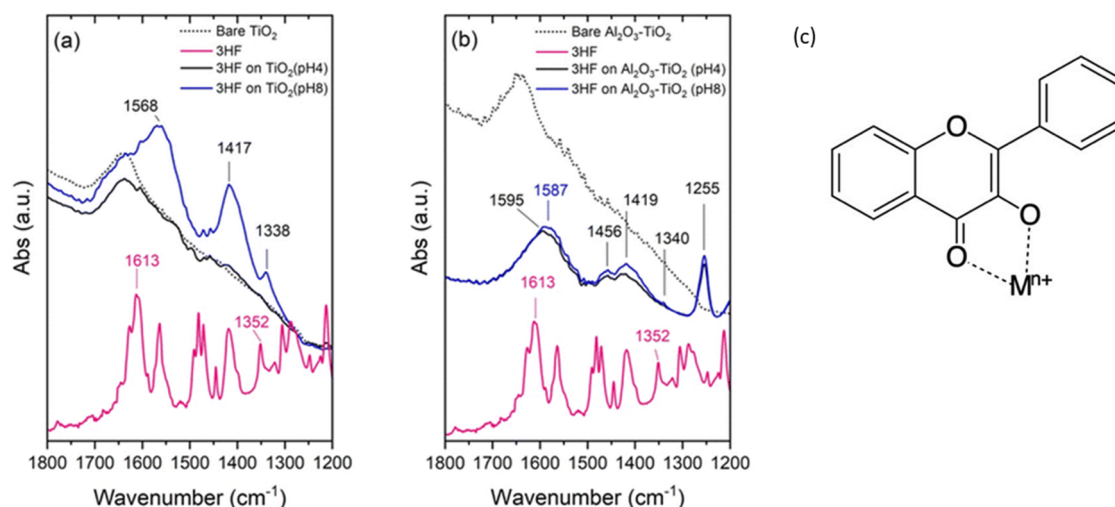


Figure 8. (a) FTIR spectra of a film of TiO_2 nanoparticles (NPs) before (dotted gray lines) and after (solid black and blue lines) the anchoring of 3HF. Solid blue and black lines refer to the uptake at pH 8 and pH 4, respectively. The FTIR spectrum of 3HF (pink line) is reported for comparison purposes. (b) FTIR spectra of a film of TiO_2 nanoparticles (NPs) covered by a 100 Å-thick layer of Al_2O_3 . FTIR spectra were recorded before (dotted gray lines) and after (solid black and blue lines) the anchoring of 3HF. Solid blue and black lines refer to the uptake at pH 8 and pH 4, respectively. The FTIR spectrum of 3HF (pink line) is reported for comparison purposes. (c) The proposed anchoring mechanism of 3HF on surface atoms. Reproduced from Solomon et al. “Tunable physics through coordination chemistry: formation on oxide surface of Ti and Al chelates with 3-hydroxyflavone capable of electron injection and light emission”, *Dalton Trans.* **2022**, 51, 18489–18501 (ref. [70]), published under CC BY 3.0 Unported license.

Marrassini et al. [71] studied the interactions of luteolin (a flavonoid with interesting biological properties, but also largely employed in art as component of yellow pigments, for instance yellow lakes) with three alcohols (methanol, 1-propanol, 1-butanol) and DMSO. FT-Raman, Vis-Raman and UV-Raman spectroscopies were employed, associated to DFT calculations. Interestingly, the vibrational bands in the 1700–1550 cm^{-1} region do not show relevant solvent-induced shifts. This is likely to be related to the presence of the intramolecular H-bond between the 5-OH groups (donor) and the carbonyl, as suggested by literature data [72]. DFT calculations are in agreement with this hypothesis. In addition, the same DFT calculations suggest that the C=O stretching of luteolin is implicated in many vibrational modes. Interestingly, these calculations suggest that the 1650–1660 cm^{-1} band (the most upshifted band in the explored 1550–1700 cm^{-1} region) should be interpreted as mainly due to the 5-OH bending.

Whereas the present study is particularly helpful in the interpretation of vibrational spectra of luteolin in different systems, probably the main point suggested by this present work is the strength of the intramolecular hydrogen bond interaction $5\text{OH}\cdots\text{O}=\text{C}$, which apparently is not perturbed by protic solvents, in agreement with NMR data. This peculiar H-bond interaction is believed to be the molecular basis for the high photostability of luteolin [73,74].

4.5. DNA-Protein Interactions

Serum Response Factor (SRF) is a transcription factor member of the so-called MADS-box family. This protein binds to a specific DNA sequence (called Serum Response Element, SRE) in the promoter region of target genes. SRF is involved in orchestrating disparate programs of gene expression linked to muscle differentiation and cellular growth [75]. A core domain of ~90 amino acids is sufficient for the activities of DNA binding, dimerization and interaction with accessory factors [76]. Within the SRF core is a DNA-binding region, called MADS-box, which shows extensive sequence homology with many eukaryotic regulatory proteins.

SRF binds specifically to the SRE as a homodimer. SRF binding to SRE requires a consensus sequence CC(AT)₆GG, the so-called CARG box. X-ray structures of core-SRF co-crystallized with SREs bearing transcriptional active CARG boxes of c-fos and α -actin genes are available [77,78].

Raman spectroscopy has been widely applied to investigate DNA-protein complexes [79].

A non-resonant Raman difference spectroscopy approach was used to study the molecular mechanism of SRF-SRE molecular recognition, by recording a spectrum of the [SRF core—SRE] complex and subtracting from it the spectra of the individual, isolated component (SRF core and SRE) [80]. In the difference spectrum the vibrational signature of the molecular recognition process appears.

First, a Raman spectrum of the isolated SRF core was recorded. Both amide I and amide III bands revealed a distribution of secondary structures. Using specific software, the α -helix contribution was estimated to be in the 29–34% range, and the β -sheet contribution in the 37–39% range. The tyrosine doublet (825 and 853 cm⁻¹) was clearly visible and from the intensity ratio $I_{853}/I_{825} = 2$ it was possible to deduce that two of the three Tyr residues (Tyr158, Tyr173, and Tyr195) are involved as an acceptor in a strong hydrogen bond from a positive donor as Lys and/or Arg.

Then, Raman spectra of core-SRF with SRE were recorded. Looking at the difference spectra the first striking feature of the {[core-SRF)-SRE^{fos}] complex minus [(core-SRF) & (SRE^{fos})] difference spectrum is the negative band at 1660 cm⁻¹, indicative of a reduction of β sheets content. Another clear feature of {[core-SRF)-SRE^{fos}] complex minus [(core-SRF) & (SRE^{fos})] difference spectrum, which is however evident also in the absolute spectrum of the [(core-SRF)-SRE^{fos}] complex, is the modification of the intensities of the Fermi doublet of tyrosine. In the (core-SRF)-SRE^{fos} complex we have $I_{853}/I_{825} = 1.6$. This suggests that upon complex formation two Tyr residues are exposed to water solvent (whereas in isolated core SRF only one Tyr residue was solvent exposed) [80].

The study was completed by Raman studies on complexes between core-SRF and two DNA sequences similar to SRE^{fos}, but with a single or double bases variation. The above-mentioned pattern of peaks did not appear in the corresponding Raman difference spectra, confirming the specificity of the molecular recognition between SRF and SRE [80].

4.6. Prebiotic Chemistry Studies

The interaction between amino acids and silica surfaces is a topic relevant for several research fields such as bio-nanotechnology [81], green chemistry [82], biomedical sensors [83] or peptide-based pharmaceuticals [84].

In addition, interactions between amino acids and silica are highly significant in prebiotic chemistry. SiO₂ surfaces have long been proposed to facilitate peptide-bond formation through the condensation of two amino acids, a critical step in producing the first peptides on the early Earth during the origin of life [85].

El Samrout et al. [86] studied the factors governing the formation of linear peptides and cyclic dimers (diketopiperazine, DKP) on silica surfaces—with varying surface areas and differing populations of silanol and siloxane groups controlled through thermal treatments. They focused on glycine deposited from both gas and liquid phases. The resulting products were characterized by FTIR and Raman spectroscopy, X-ray diffraction, and thermogravimetric analysis. The results highlighted the key role of “nearly free” silanols in generating ester intermediates that promote the formation of linear peptides rather than DKP on surfaces with medium silanol densities (1.4–2.7 nm⁻²). In contrast, surfaces with isolated silanols (≈ 0.7 nm⁻²) show quenched reactivity, whereas hydrogen-bonded silanols (≈ 4.5 nm⁻²) interact only weakly with glycine, favoring its cyclization to DKP. Glycine deposition from the liquid phase can also yield both DKP and linear polymers, depending on glycine loading and surface properties. Overall, these findings underscore the high complexity of glycine surface chemistry in polymerization/cyclization reactions and emphasize the value of surface-science approaches for evaluating geochemical scenarios relevant to prebiotic chemistry.

The investigation on peptide formation on silica surfaces was extended to mixtures of amino acids by Sakhno et al. [87]. They presented evidence for the formation of linear oligopeptides with nonrandom sequences from couples of amino acids co-adsorbed on silica and subjected to simple thermal activation. Mixtures of (glutamic acid + leucine) and (aspartic acid + valine) were deposited from aqueous solutions onto fumed silica and exposed to a single heating step at moderate temperature. The evolution of these systems was monitored using X-ray

diffraction, FTIR spectroscopy, thermogravimetric analysis, HPLC, and electrospray ionization mass spectrometry (ESI-MS). In all cases, clear signatures of amide-bond formation were observed. FTIR data are particularly interesting as FTIR was used as in situ technique. Whereas activation of individual amino acids on silica typically leads to products that can be considered evolutionary dead ends, the mixed systems—particularly (glutamic acid + leucine), and to a lesser extent (aspartic acid + valine)—yielded linear peptides with high efficiency, reaching lengths of up to hexamers. Peptides of this length had not previously been reported in surface-mediated polymerization scenarios, except in cases where amino acids were deposited via chemical vapor deposition, a process unrealistic under prebiotic conditions. Moreover, products did not contain all possible sequences of amino acids, indicating sequence selectivity in the polymerization reaction. These findings are relevant for prebiotic chemistry research, as they suggest that simple conditions may lead to the emergence of nonrandom peptides.

The results obtained for Leu + Glu mixture prompted a detailed study on leucine adsorbed on silica. In addition, Leu is also a valuable model for testing methods of characterizing biomolecules on mineral surfaces, combining a small, peptide-like organic molecule with one of the most widely used inorganic materials.

Abadian et al. [88] deposited L-leucine in the sub-monolayer regime onto high-surface-area fumed silica and examined it using a multipronged approach involving macroscopic techniques (thermogravimetry, X-ray diffraction XRD), in situ spectroscopies (FTIR, NIR and multinuclear solid-state NMR), and DFT modeling with calculated NMR parameters. These methods provide detailed insights into the adsorption and interaction mechanisms (see Figure 9). Leucine was found to be strongly adsorbed through a network of specific hydrogen bonds—often involving co-adsorbed water molecules—without forming covalent bonds to the surface. Its adsorption state depends sensitively on the drying procedure: insufficient drying creates liquid-like environments around the functional groups, whereas vacuum drying leaves only a few water molecules (approximately five per leucine). The most stable structural models feature zwitterionic leucine interacting directly with surface silanols via its ammonium group, while its carboxylate group interacts through bridging water molecules. The experimentally measured NMR chemical shifts are well reproduced by these models, supporting a description of leucine as a molecular probe for specific silica surface sites known as silanol nests.

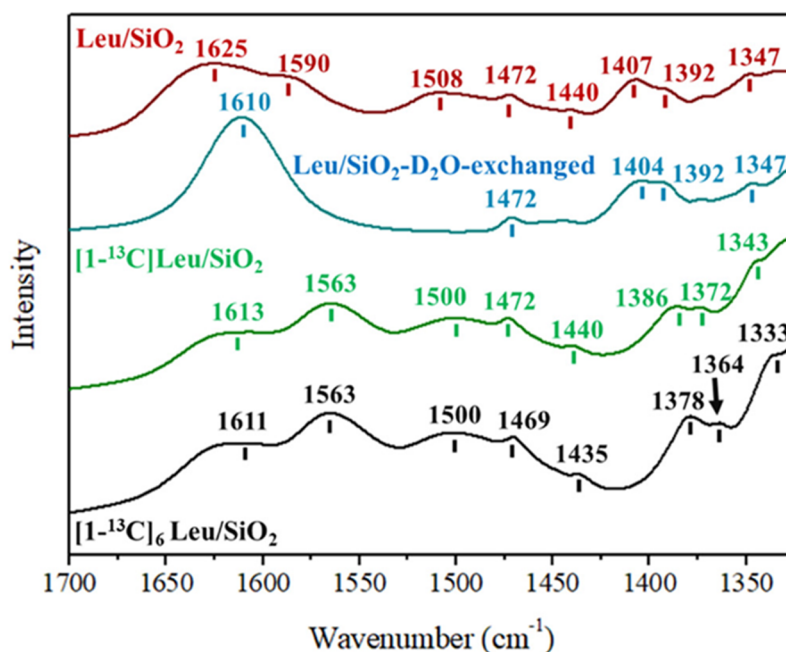


Figure 9. FTIR spectra of Leu deposited on SiO₂ surfaces. The use of isotopically-labelled Leu makes it possible to assign vibrational bands and to understand the interaction between Leu and the surface. Reprinted with permission from Abadian et al. *Langmuir* **2022**, 38, 8038–8053 (ref. [88]) Copyright 2022 American Chemical Society.

FTIR and NMR spectroscopies were also used to study other aspects in origin-of-life research, notably the origin of nucleotides. Ter-Ovanessian et al. [89] investigated the first step of pyrimidine biosynthesis via the orotate pathway. This study provides a useful model for testing the hypothesis of geochemical continuity in protometabolic pathways at the origin of life. In modern biochemistry, carbamoyl phosphate (CP) is the initial high-energy intermediate required for constructing the uracil ring of uracil monophosphate. Accordingly, this study investigates the plausibility of CP formation and persistence under prebiotic conditions. The behaviour of

CP in water and in aqueous ammonia, in the absence of enzymatic catalysis, was characterized using ATR-FTIR (see Figure 10), ^{31}P , and ^{13}C NMR spectroscopies. In pure water under ambient conditions, CP exhibits a short-lived period of apparent stability before decomposing within hours into cyanate and carbamate/hydrogen carbonate species. Cyanate, which is less labile than CP, could nevertheless act as a viable prebiotic carbamoylating agent. In ammonia-containing solutions, CP decays more rapidly and produces urea. These observations indicate that CP itself is unlikely to have been a prebiotic reagent. By contrast, cyanate and urea may represent more plausible substitutes, as both are “energy-rich” (possessing high free enthalpy in aqueous solution) yet comparatively resistant to hydrolysis. Additional high-energy inorganic molecules—including trimetaphosphate and phosphoramidates—were evaluated as potential CP precursors. Although they did not yield CP or alternative carbamoylating agents, they did participate in energy-transfer processes, particularly through the formation of high-energy P–N bonds. Current work focuses on assessing the ability of such carbamoylating agents to promote aspartate carbamoylation, the subsequent step in the orotate pathway.

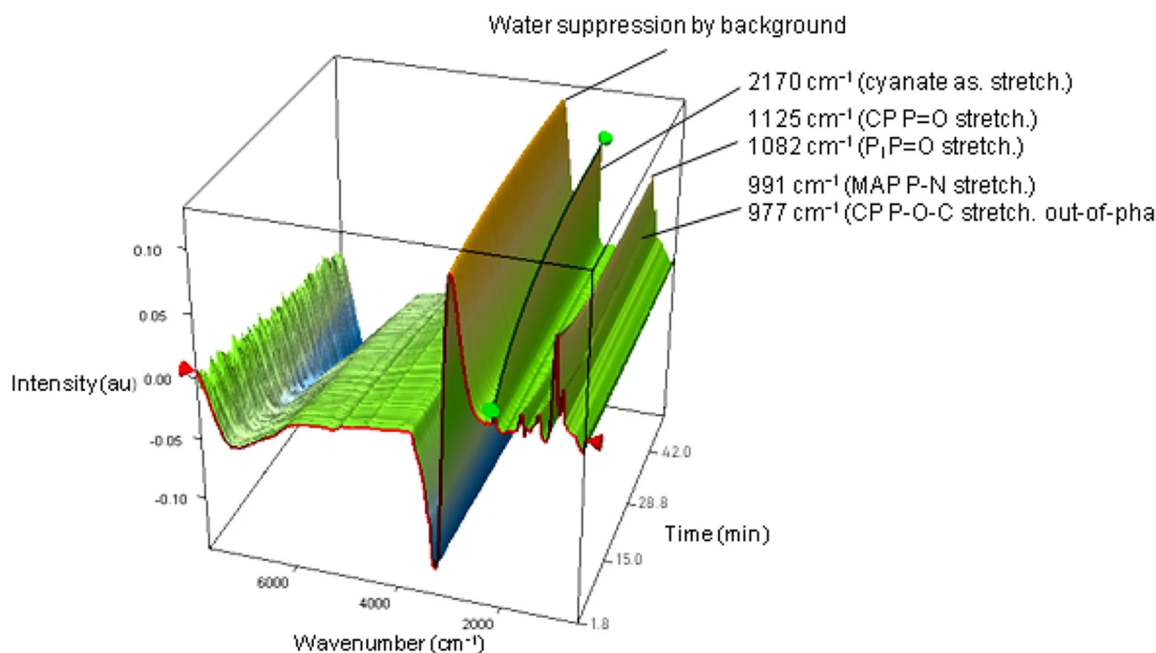


Figure 10. Stacked ATR-IR spectra of Carbamoyl Phosphate kinetics in ammonia. The green highlighted curve corresponds to the evolution of the cyanate band. Figure reproduced from Ter-Ovanessian et al. *Scientific Reports*, 2021, 11, 19356 (ref. [89]), published under CC BY 4.0 license <http://creativecommons.org/licenses/by/4.0/>.

4.7. Reactions at the Iron Oxide-Water Interface

This research line addresses the issue of soil/water decontamination by exploring the mechanisms of environmentally friendly strategies that involve the chemical degradation of organic contaminants. Attention was first focused on molecular-scale adsorption mechanisms of the antibiotic nalidixic acid (NAL) on ferrihydrite and maghemite surfaces.

Time-resolved ATR-FTIR spectroscopy under continuous flux conditions allowed monitoring of the adsorption process directly on the iron oxide surfaces (in situ) under realistic conditions [90]. The technique provided information on molecular interactions of the organic molecule with the iron oxide surfaces by observing characteristic vibrational bands of NAL (see Figure 11).

NAL was found to primarily adsorb on both ferrihydrite and maghemite via a bidentate mode.

In addition, thanks to DFT calculations, the study provided a complete FTIR band assignment for both protonated and deprotonated NAL, which helped clarify the adsorption process at a molecular level.

In following works ([91]; Robinson et al. submitted) NAL degradation by heterogeneous Fenton-like reactions taking place at the solid-liquid interface was studied. The aim was to explore the ability of O_2 to act as the sole oxidant, by comparison with strong oxidants as H_2O_2 . In-situ ATR-FTIR spectroscopy made it possible to monitor in situ the progressive degradation of NAL, suggesting details on the oxidation mechanism, which starts with a ring-opening in the first degradation steps.

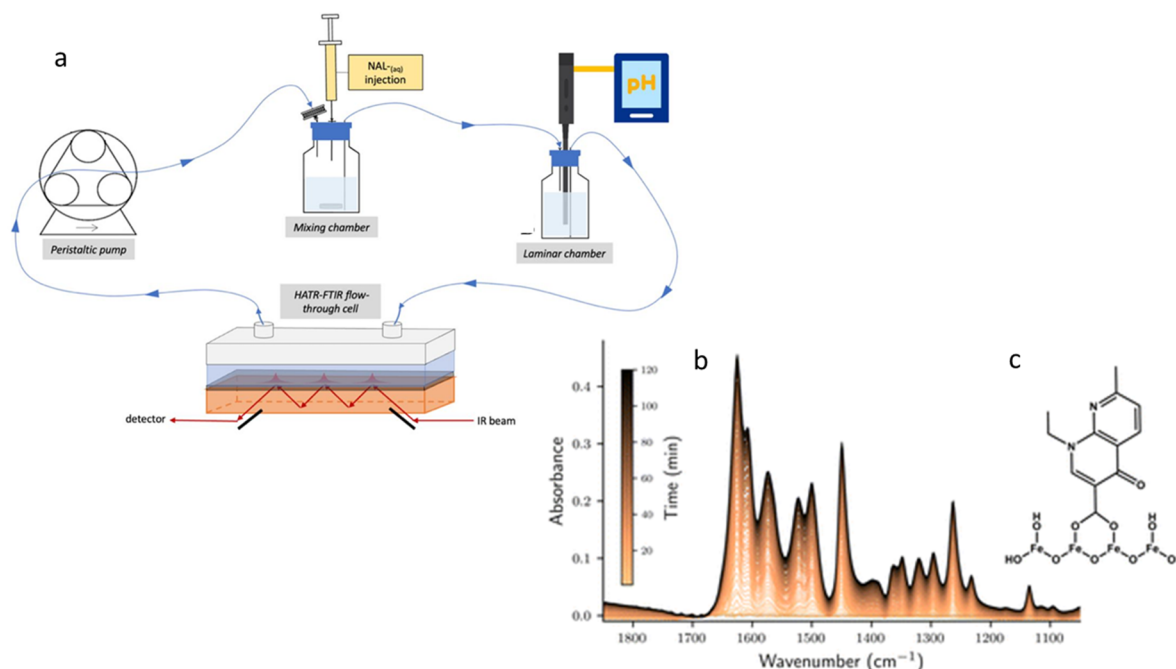


Figure 11. (a) Instrumental set-up for adsorption studies of Nalidixic Acid (NAL) on Maghemite and Ferrihydrite surfaces. (b) ATR-FTIR spectra showing progressive adsorption of NAL on ferrihydrite. (c) Proposed binding modes of NAL of Ferrihydrite surfaces, through a bidentate bridging mode. Reproduced from Schuh Frantz et al. *New J. Chem.* **2024**, 48, 19403–19417 (Ref. [90]) with permission from the Royal Society of Chemistry.

4.8. Biomineralization Processes

The last field shown as an example of the power of vibrational spectroscopy in elucidating reaction mechanisms is the study of processes of biomineralization.

Calcium phosphate biomineralization [92]—though often simulated by simple precipitation—is actually a highly regulated biochemical process. A key regulator is alkaline phosphatase (ALP), which controls the local concentration of orthophosphate ions available for mineral formation.

Foley et al. [93] investigated enzyme-assisted homogeneous mineralization as a biomimetic approach, focusing specifically on how ionic substitutions affect the resulting calcium phosphate minerals. Mineralization reactions were carried out at physiological conditions (pH 7.4, 37 °C) while varying ALP substrate concentration and Fluoride concentration.

Several techniques (X-ray Diffraction—XRD, X-ray photoelectron spectroscopy—XPS, FTIR spectroscopy) were used synergically. The authors found that Hydroxyapatite (HAP) forms after 48 h under all tested conditions.

Upon increase of the enzyme substrate concentration the authors observed expansion of the HAP unit cell, but minimal change in particle morphology. This was attributed to increased incorporation of CO₃²⁻ (carbonate) into the lattice. Upon increase of fluoride concentration (with substrate fixed), contraction of the HAP unit cell was observed, along with formation of elongated, well-crystallized rod-like particles. This reflected substitution of hydroxyl groups by F⁻, consistent with fluoridated apatite formation.

FTIR spectroscopy was a key technique to monitor simultaneously proteins (through amide I and amide II bands), PO₄³⁻, and CO₃²⁻ (see Figure 12).

The authors could conclude that ionic substitutions induced by changes in biochemical conditions—specifically carbonate and fluoride incorporation—can significantly alter both the lattice dimensions and morphology of biomimetically mineralized hydroxyapatite. ALP-assisted mineralization proved to be a powerful model for studying such biomimetic and biologically relevant mineralization processes.

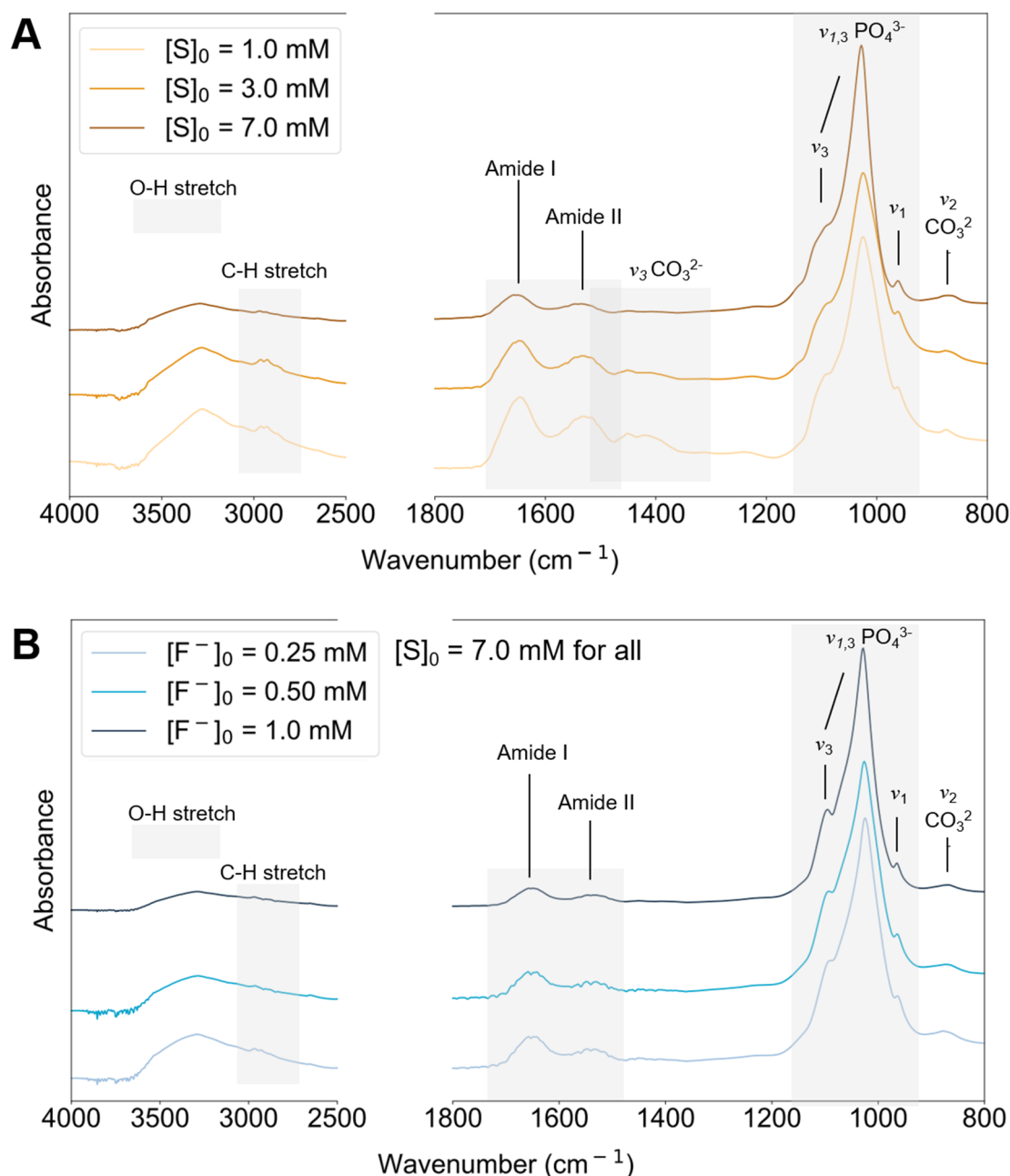


Figure 12. Selected ATR-FTIR spectra of substituted Hydroxyapatites synthesized by homogeneous enzymatic catalysis (**A**) at variable substrate concentrations, $[S]_0 = 1.0, 3.0$ and 7.0 mM , or (**B**) at $[S]_0 = 7.0 \text{ mM}$ and variable fluoride concentrations $[\text{F}^-]_0 = 0.25, 0.50$ and 1.0 mM . Figure taken from Foley et al. *Int. J. Mol. Sci.* **2023**, *24*, 43 (ref. [93]) published under CC BY 4.0 license. <http://creativecommons.org/licenses/by/4.0/>.

The synergic use of several spectroscopic techniques was then applied to chondrocyte hypertrophic differentiation [94] a process that triggers articular cartilage degradation through matrix remodeling and pathological mineralization. This is an applied research topic, as this process is closely tied to Osteoarthritis (OA) progression. However, the temporal dynamics of all events are poorly defined.

Using an *in vitro* model where immature murine articular chondrocytes (iMACs) differentiate sequentially into prehypertrophic (Prehyp) and hypertrophic (Hyp) states, Jaabar et al. tracked mineralization and matrix remodeling during the transition from Prehyp to Hyp chondrocytes [94].

In more detail, the authors used multiple complementary techniques to characterize differentiation, mineral deposition, and matrix changes: Immunofluorescence microscopy, Biochemical and molecular assays, Atomic Force Microscopy (AFM), Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM-EDS), ATR-FTIR spectroscopy (focusing on the evolution of the PO_4^{3-} band region), XRD, and specific assays to assess apoptosis.

Concerning Mineralization Dynamics, a mineral phase appeared 7 days after hypertrophic induction. By 14 days, this mineral spread through the extracellular matrix and developed into poorly crystalline, carbonate-

substituted hydroxyapatite (HAP). Over time, the proportion of crystalline material increases relative to amorphous content, indicating progressive mineral maturation.

The authors could follow also Matrix Remodeling. Significant matrix turnover occurred within the first 7 days of Hyp differentiation. More in details, Type II collagen and aggrecan decreased, and Type X collagen, a hallmark of hypertrophy, emerged. Furthermore, MMP-13, a key collagenase in OA, showed increased enzymatic activity, supporting active matrix breakdown.

The authors could follow also Chondrocyte Apoptosis: a slight but measurable increase in apoptotic cells occurred during Hyp differentiation. Phosphorus-rich structures detected by SEM-EDS likely correspond to apoptotic bodies, which may nucleate mineral deposition.

In conclusion, the study by Jaabar et al. [94] delineated a clear sequence of events during hypertrophic differentiation of articular chondrocytes: Early matrix degradation (0–7 days), Onset of mineral deposition (around day 7), Formation and maturation of carbonate-substituted hydroxyapatite (after day 14 and beyond).

These findings provided mechanistic insight into the pathological mineralization and matrix remodeling observed in osteoarthritis, emphasizing the role of hypertrophic differentiation, matrix turnover, and apoptosis in driving cartilage calcification.

A final study by Foley et al. [95] focused on biomimetic hydroxyapatites.

Biomimetic hydroxyapatites are in fact extensively investigated for applications in repairing mineralized tissues—most notably dental enamel, which is acellular and therefore cannot regenerate after damage. Natural enamel possesses a precisely organized hierarchical structure that is extremely challenging to reproduce at the macroscopic scale. Consequently, successful restoration strategies must imitate the fundamental biochemical principles that underlie in vivo biomineralization.

The approach employed by Foley et al. uses enzymes incorporated into polyelectrolyte multilayers to locally generate inorganic phosphate. This creates a microenvironment conducive to mineral nucleation and growth, effectively recapitulating key biochemical processes of natural enamel formation. Building on this, a method is introduced to create seriated (layer-by-layer) mineral architectures with scalable thickness, continuous mineral growth over time, and adjustable crystal morphology.

The study showed that the system supported the formation of cohesive, robust mineral layers. These layers grew into macroscopic standalone materials composed of fluoride- and/or carbonate-substituted HAP. The resulting materials exhibited crystal structures and compositions closely resembling native human mineralized tissues, including enamel.

The strategy allowed precise synthetic control across multiple length scales, from molecular to macroscopic.

FTIR spectroscopy was used to monitor phosphate and carbonate bands in $\sim 1460\text{--}850\text{ cm}^{-1}$ region during the mineralization process.

In conclusion, this enzymatically driven, multilayer-based mineralization platform offered a powerful pathway for generating enamel-like biomimetic materials. Its ability to regulate mineral chemistry, morphology, and hierarchical organization makes it particularly relevant for restorative dentistry and broader biomineral material design, bridging the gap between nanoscale biomineralization mechanisms and practical macroscopic applications [95].

5. Conclusions and Perspectives

The aim of this perspective article is to show, through a series of examples from the research I have carried out so far, how vibrational spectroscopy can be a key technique in the study of reaction mechanism. The variety of systems studied underline how mid-IR spectroscopy, NIR spectroscopy, non-resonant Raman spectroscopy, resonant Raman spectroscopy can be applied—often in synergy among them or with other spectroscopic techniques.

Some points clearly emerge.

- (1) Vibrational spectroscopy provides a way to follow evolution of chemical species even in complex systems through specific marker bands, often with no need of chromophores or peculiar molecular properties. This evolution is followed in situ with minimal procedure of sample preparation (in some cases no sample preparation step is necessary).
- (2) Vibrational bands are informative on the structure. In this way, in situ insights on structure of products or intermediates can be obtained. This, along with the availability of kinetics (through marker vibrational bands), are of extreme help in elucidating reaction mechanism.
- (3) Static vibrational spectroscopy studies on the system before the reaction can give precious pieces of information on specific issues, e.g., interaction with the solvent/surrounding matrix, or hydration state of the

system. These pieces of information are normally very important in the detailed study of reaction mechanism and might become very powerful when associated to kinetic spectroscopy studies.

- (4) Band assignment is a tedious but important task. It becomes mandatory in Infrared difference spectroscopy of complex systems like proteins, where spectral contributions in the difference spectrum (or spectra) from various molecular species are expected. This task (often called “from bands to bonds” step) does not follow a unique protocol, but the solidity of band attribution determines the solidity of a research work. Classical approaches are isotope labelling of reactants, DFT calculations, comparison of spectra of isolated products, intermediates (when possible) and in some cases of isolated reactants.
- (5) The time scale used in time-resolved vibrational spectroscopy (notably in time-resolved IR spectroscopy) depends deeply on the reaction under study. In several cases it is useful to slow down the reaction (by lowering the temperature, changing the pH or the solvent, exploiting kinetic isotope effects etc.) so that evolution of the system can be followed. In some cases, experiments at low temperature makes it possible to trap intermediates.
- (6) Extreme care should be taken in avoiding spectral artefacts due to inappropriate experimental conditions or signal processing, sample ageing, heating effects (especially in light-induced reactions), spectral contaminations (e.g., water vapour in IR spectroscopy, fluorescent impurities in Raman spectroscopy).
- (7) Data analysis techniques can be very useful, provided that a clear difference is kept in mind between strategies corresponding to a simple mathematical analysis (e.g., SVD, PCA, 2D-COS) and those entailing a real elaboration of data (MCR-ALS, kinetic model-based analysis). When using these approaches, parallel and simpler strategies of data analysis are strongly advised. In any case, when publishing raw data should always be provided to the reader.
- (8) Theoretical chemistry calculations are extremely helpful, but in the scientific literature sometimes improper use of quantum chemistry software can be found. It is strongly advised to ask for advice (or even collaboration) to experts in the field, especially for advanced calculations (e.g., QM/MM dynamics, Car-Parrinello methods etc.). Scientific collaboration with theoreticians is the approach I have always followed.

Vibrational spectroscopy applied to reaction mechanisms has a bright future ahead. In some fields explored in this perspective, like molecular mechanisms in photosynthesis ([96,97] and refs therein) and in photoreceptors ([97,98] and refs therein) vibrational spectroscopy is extremely active. Furthermore, several recently-developed approaches, like transient 2D-IR extended to the micro and millisecond scale [99], the coupling of time-resolved methods with vibrational microspectroscopy and nanoscopy (see for instance [100,101]) and the use of microfluidics in vibrational spectroscopy [102,103] are very promising.

A new and very interesting field is the use of vibrational spectroscopy in process research and development (see for instance [104]).

The most interesting trend is however in the approach used by scientists when studying a chemical reaction (or, more broadly speaking, a time-evolving system). In the last decades of XXth century, the attention paid to the reaction mechanism was somehow limited, in the sense that almost exclusively physical chemists interested in mechanistic studies employed vibrational spectroscopy (and, more broadly speaking, advanced spectroscopy methods and other physical tools) to investigate the reaction at the atomic scale. There were only some specific fields where vibrational spectroscopy was quite largely employed, like photobiology, and catalysis.

In the last 15–25 years the investigation of the reaction mechanism has become an almost mandatory step, and vibrational spectroscopy proved to be one of the most powerful tools. An example of this increasing interest is the success of recent conferences and schools devoted to vibrational spectroscopy applied to reaction mechanisms, see for instance [105–107].

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Conflicts of Interest

The author declares no conflict of interest.

Use of AI and AI-Assisted Technologies

During the preparation of this work, the author used chat GPT to rewrite sentences in improved English. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article

List of Abbreviations and Acronyms

2D-COS	Two-Dimensional Correlation Spectroscopy
3HF	3-hydroxyflavone
7HF	7-hydroxyflavone
ACN-d ₆	deuterated acetonitrile
ADC	Analog-to-Digital Convertor
AFM	Atomic Force Microscopy
ALP	alkaline phosphatase
ATR	Attenuated Total Reflectance
CP	Carbamoyl Phosphate
CP2K	an open source electronic structure and molecular dynamics software package
CTD	C-Terminal Domain
DAD	3,6-Diaminodurene (2,3,5,6-tetramethyl- <i>p</i> -phenylenediamine)
DFT	Density Functional Theory
DKP	Diketopiperazine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDS	Energy Dispersive Spectroscopy
ESI-MS	Electrospray ionization Mass spectrometry
ESIPT	Excited-State Intramolecular Proton Transfer
FT	Fourier Transform
FTIR	Fourier Transform Infrared
GC-MS	Gas chromatography-mass spectrometry
HAP	Hydroxyapatite
HPLC	High Performance Liquid Chromatography
Hyp	Hypertrophic
iMAC	immature murine articular chondrocytes
IR	Infrared
MADS box	The MADS box is a conserved DNA sequence motif that encodes the DNA-binding MADS domain. The MADS domain binds to DNA sequences of high similarity to the motif CC[A/T] ₆ GG (called the CArG-box).
MCM-41	Mobil Composition of Matter n. 41. It is a mesoporous material with a hierarchical structure from a family of silicate and aluminosilicate solids.
MCR-ALS	Multivariate Curve Resolution by Alternate Least Squares
MS	Mass Spectrometry
NAL	Nalidixic acid
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
NP	Nanoparticle
NTD	N-terminal domain
OA	Osteoarthritis
OCP	Orange Carotenoid Protein
PB	Phycobilisome
PCA	Principal Component Analysis
PCP	Peridinin-Chlorophyll- <i>a</i> -Protein
Per	Peridinin
PreHyp	Prehypertrophic
QM/MM	Quantum Mechanics/Molecular Mechanics
RC	Reaction Center

RH	Relative Humidity
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SRE	Serum Response Element
SRF	Serum Response Factor
SVD	Singular Value Decomposition
TD-DFT	Time-Dependent Density Functional Theory
TMPD	<i>N,N,N',N'</i> -Tetramethyl- <i>p</i> -phenylenediamine
UV-Vis	Ultraviolet-Visible
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

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