Ultrafast UV spectroscopy: from a local to a global view of dynamical processes in macromolecules†

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The aim of this Perspective article is to cover recent developments in the application of femtosecond UV spectroscopy to understand molecular dynamics, and outlining potential future directions in this area. With several examples from recent literature the added-value of these techniques and their capability to study in real time changes in structure, dynamics and electrostatic fields of macromolecules in a site-specific fashion, as well as to uncover concerted dynamics in biomolecules, will be shown and discussed. The emerging fields of UV pulse-shaping techniques and UV optical nonlinear spectroscopies will be discussed to outline their potential to generate a novel family of coherent nonlinear spectroscopies for spectroscopic and microscopic applications.

Introduction

In the last twenty years, ultrafast spectroscopy has dramatically changed our way of investigating matter. Now we can indeed follow in real time all the photo-inducible transformations occurring in states out of equilibrium with femtosecond resolution, and our comprehension of excited-state physics and chemistry has undergone a breathless leap.1–3 A less evident quality is that now we can access directly the times where thermal fluctuations occur, mainly by time resolving spectral diffusion processes. This has given us on the one hand the unique capability to disentangle homogenous and inhomogeneous line shape broadening, and on the other hand novel methodologies to track down collective motions and long-range interactions within a molecule, which are quickly covered or even destroyed by incoherent fluctuations.3–6

This novel capability to follow in real time the structural and energetic changes of electronic and vibrational excited states has on many occasions obliged researchers to reconsider well-established assumptions and develop new concepts. For instance, this is the case of the discovery of long-lasting coherencies in biomolecules7 and of ultrafast spin dynamics in metal complexes,8 or the comprehension of internal vibrational relaxation mechanisms9 and molecular dynamics close to avoided crossings and conical intersections.10 Above all, the investigation of biological systems and of coordination compounds has particularly benefited.

The last decade has seen an impressive improvement of ultrafast spectroscopic techniques and nowadays we have at our disposal the fs time-resolved versions of virtually all the steady state techniques (optical absorption, fluorescence emission, Raman, optical Kerr effect, THz spectroscopy, photoelectron spectroscopy, and even electron and X-ray diffraction) and completely new spectroscopic methodologies as coherent nonlinear optical spectroscopies and microscopy, which include multidimensional photon-echo, coherent anti-Stokes Raman, but also chemically sensitive techniques for all-optical recognition, etc.11–13 It is worth noting that a great leap forward has been made possible by the availability of single shot and broadband detection schemes, most of them employing charge coupled devices (CCDs) or fast photodiode arrays (PDAs), which have dramatically enhanced the investigative capabilities of these techniques. In addition, the development of
Femtosecond optical parametric amplifiers have provided relatively powerful and stable fs tunable visible (Vis) and ultraviolet (UV) pulses, allowing the extension of these techniques to the whole Vis and UV regions. A short description of the main techniques relevant to this perspective article can be found in Fig. 1.

Besides the general interest in investigating with real-time techniques the vast number of systems with transitions in the UV, one of the main drives to develop UV spectroscopies is to use as probes those UV chromophores, as for instance aromatic amino acids, that are constituents of bio-molecules, many molecular devices and supra-molecular structures.

Exploiting them as probes has the unique potential to open a new window on the dynamical properties of the macro-molecule edifice in a site-specific fashion.

The aim of this perspective article is to comment on the emerging field of ultrafast UV spectroscopy and its application to the understanding of molecular dynamics, as well as to give an outlook for future research with these methodologies.

In such a dynamical context, and so young when compared to the history of spectroscopy, the extension to the UV is rather limited and, as shown below, the first steps have been done only recently. It is important to remark that this extension concerns the capability of probing. Indeed the first works

Fig. 1 Schematic representation of the main spectroscopic techniques discussed in the text. (a) Pump–probe transient absorption (TA) spectroscopy: the sample is excited with a femtosecond pump pulse and the subsequent excited state dynamics are investigated by monitoring the changes in the sample absorption with a broadband fs probe pulse as a function of the relative delay time. (b) Time gated emission (TGE) spectroscopy: emission from the sample (represented by the green cone) is induced at time zero ($t_0$) by the excitation pulse and focused with a collection lens (CL) into a nonlinear optical shutter (NL-OS). This is a device that lets emission be transmitted only while illuminated by a fs pulse (gate), achieving a time gating of the emission signal from the sample at the desired time ($t_{gate}$). Different schemes exist to implement such a device, based on nonlinear phenomena as sum frequency, optical Kerr effect and parametric amplification. The gated emission is then analysed with a spectrograph. The two cartoons on the bottom show the pulse time order before and after the NL-OS (excitation and gate pulses in blue and red, respectively, while the emission signal is in green). (c) Four wave mixing (4WM), or photon echo (PE), spectroscopy: three beams are focused into the sample to generate in a phase-matched direction a fourth beam, called PE signal, which is proportional to the third order polarizability of the sample. In the homodyne detection scheme, only the intensity or the power spectrum of the PE signal are detected. In case of heterodyne detection a weak (but still 10 to 100 times stronger than the PE signal) auxiliary beam, called local oscillator (LO), is added collinearly to the PE signal to perform an interferometric detection. This approach gives access to the amplitude and phase of the PE electromagnetic field. The Figure shows the most popular non-collinear geometry but the collinear version is also possible, where all the beams follow the same path. Time ordering and nomenclature (pop. and coh. stand for population and coherence, respectively) are reported below the set-up schema. (d) Time resolved resonance Raman (RR) spectroscopy: in a similar manner as in TA spectroscopy the sample is excited at time zero by the pump pulse and the excited state dynamics are monitored with a second pulse, here called Raman probe, as a function of the relative time delay. The latter is used to generate Raman signals, which are collected usually in backscattering geometry to minimize scattering contributions from the pump and Raman pulses. The Raman probe has to be sufficiently spectrally narrow to be selectively resonant only with the transition under investigation and to not smear out Raman lines. Indeed it is chosen to have typically a width of 10 cm$^{-1}$ or less, which corresponds to a compressed time length of $\tau \sim 3$ ps time or longer (in the Figure this is schematically represented by a longer pulse). It should be noted that in all these techniques the positive time direction corresponds to a greater delay between the first pulse interacting with sample and the others, which is attained by lengthening the relative optical paths to the sample or to the NL-OS in case of TGE techniques. This is shown in the panels (a), (b) and (d) by the direction of the arrow labelled "time", and in the panels (b) and (c) by the leftward time axis of the graphs. Polychromatic beams in panels (a) and (c) indicate broadband pulses.
where UV excitation was exploited appeared already in the 70s and 80s but the detected spectral region was still the Vis and NIR. Despite being obvious, what makes a technique extended into the UV is the capability to probe with UV light, and in the following we will refer to UV spectroscopy with this meaning.

This article will be mostly concerned with the spectral region 250–400 nm (corresponding to the so called deep- and near-UV regions, or DUV and NUV, respectively). Several reasons limit the most relevant spectral interval to study molecular systems to $\lambda > 250$ nm, especially in the condensed phase. Indeed, most of the solvents absorb at shorter wavelengths, and the risk of photo-degradation and photo-ionization for most of the polyatomic molecules dramatically increases. As far as biomolecules are concerned, peptide-bond absorption starts at $\lambda < 230$ nm, making UV excitation and probing unselective. There are also several technical issues that make $\lambda < 250$ nm less accessible. First, as far as ultrafast applications are concerned, most of the commercially available optics suffers from a dramatic performance degradation approaching 200 nm, while single stack high reflection coatings, necessary for fs UV dielectric optics, cannot provide the same spectral broadness as in the Vis range. Second, most of the UV generation techniques are based on doubling Vis sources with crystals made out of $\beta$-BBO, which is the material of choice for Vis and UV application. However this crystal cannot be phase-matched for fundamental wavelengths $< 410$ nm and it experiences a dramatic drop in the nonlinear (NL) optical coefficients at $\lambda < 500$ nm. Alternative UV generation schemes will be shortly discussed in the following text.

The first part of the article is devoted to a short review of recent experimental developments and the improved capabilities of ultrafast UV spectroscopies. Different techniques will be discussed presenting their main features and their state-of-art. In the second section, the added-value of these methodologies will be illustrated with different exemplary studies from the recent literature. Each issue will be first framed in its general context and then the scientific contributions brought by UV extended techniques will be pointed-out. Last, some potential directions and perspectives for these new methodologies will be presented with a special attention to coherent spectroscopies.

State of the art: methodologies

The first pioneering works on ultrafast UV spectroscopies date to the early 90s. They employed low repetition rate (< 100 Hz) and high pulse energy (up to 1 mJ) laser systems based on dye laser technology. They reported the first UV probe and UV-vis pump transient absorption (TA) experiments (Fig. 1a) with a time resolution of several hundreds of fs to few ps. Very few articles reported studies with UV probing on photochemical processes, and all of them focused on photo-dissociation and formation of radical species and solvated electrons. These are indeed the ideal phenomena to be investigated with such sources, where strong pulses were required, while a high pumped versus unpumped signal contrast and long kinetics were able to compensate for the low repetition rate and signal to noise ratio. In these works probe and pump pulses were generated by doubling or tripling the fundamental frequency of the laser amplifier. Limited frequency tuning was achieved by changing the laser dye at the expense of a complicated and time consuming reoptimization. No fine tuning was possible and in practice all these works probed only few selected detection wavelengths.

A milestone towards the development of more versatile and advanced UV spectroscopies is represented by the implementation of visible collinear and non collinear optical parametric amplifiers (in short OPA and NOPA, respectively) based on fs Ti:sapphire laser systems (see ref. 18 for an extensive review on the issue). They indeed generate tuneable pulses that can be finely varied over the entire visible region and straightforwardly extended into the UV by frequency doubling or upconverting with a powerful IR pulse. These arrangements result in 30–50 fs pulses with typically 0.1–1 $\mu$J energy and tuning from 250 to 350 nm.

Ultrafast transient absorption

One of the first works where the NOPA’s flexibility was exploited to investigate a chemical process was carried out by Schenkl et al. in 2005. In this experiment Chergui and co-workers developed a Vis-pump/UV-probe setup, based on two independent Vis NOPAs. They time-resolved the earliest steps of retinal photo-isomerisation in bacteriorhodopsin by measuring the UV transient absorption of proximal tryptophans (Fig. 2). Indeed tryptophan (Trp), as well as the other two aromatic amino acids, tyrosine (Tyr) and phenylalanine (Phe), are the only amino acids with distinctive absorption and emission activities, which are centred at 260–290 nm and 280–350 nm, respectively (histidine, another UV absorbing aromatic amino acid, has the first absorption band overlapping with the one stemming from the peptide bond and it is thus unsuitable as a UV probe). This experiment can be considered in many respects the first example that illustrates probably the main added-value of UV spectroscopies: the excited and probed chromophores are not at the same site, the probed unit is indeed used to monitor the functional process occurring on the cofactor and the effect of the relative electronic and structural dynamics on the retinal pocket. In the context of this perspective this is indeed one of the most striking aspects of this work: as is nicely illustrated with the next examples, the extension of the detection into the UV gives access to well localized transitions on molecular moieties different from the ones where the photochemical reaction is triggered. This allows us a revolutionary change of the point of view, where the physicochemical process is observed from “outside” the active site and more interest is paid to the host edifice response. This global approach is particularly relevant when we move to proteins. Another credit of this work was the proof of principle that Trp, and in general aromatic amino acids, are suitable naturally occurring probes for real-time studies on protein dynamics. It is a matter of fact that, as clearly shown by the examples below, the field of research where UV spectroscopies most proliferate is the study of biological processes.

Concerning detection schemes for TA measurements, despite that in the late 90s a TA set-up with broadband detection...
was already implemented,24 all the experiments were in practice based on the collection of single wavelength detected kinetics at different probe frequencies. Spectral profiles were then reconstructed during the data analysis. The intrinsic spectral resolution of this approach is 5–10 nm (corresponding to the width of a 100 fs probe pulse) and, despite the versatility of (N)OPAs, a spectrally resolved experiment would easily take several days. This severely limits the level of detail that could be extracted, because of a so coarse resolution and the unavoidable fluctuation of the experimental conditions (sample preparation, laser stability, alignment, etc.) over such a long time. This makes the comparison of different spectral regions or different samples much less reliable. To overcome these limitations, and driven by the continuous improvement of detectors, different UV pump probe set-ups for single-shot broadband-detections have been recently proposed.25,26 Indeed these detection schemes make the overall acquisition time much shorter and provide a much more reliable spectral profile. On the one hand, they make feasible investigations of less photostable molecules or when a limited amount of sample is available. On the other hand, it allows us to apply advanced global data analysis methods (such as for instance spectral moments and multivariate analysis),27 which can uncover new important dynamic features, such as frequency-time or frequency–frequency correlations, intermediate transient states, coherent dynamics, etc., which can easily be lost with reconstructed, coarsely resolved spectra.

**Time resolved resonance Raman**

The widening of the point of view on molecular processes and the intimate link between UV ultrafast spectroscopy and biomolecules is beautifully represented by a series of works by Mizutani and collaborators. They used time resolved UV resonance Raman (RR) measurements (Fig. 1d) on Trp and Tyr residues to uncover the primary protein response after the CO ligand photodissociation from carboxyhemoglobin (MbCO).28,29 RR techniques have the advantage that signals from many vibrational transitions in a wide frequency range can be simultaneously generated by a single probe pulse with a relatively narrow spectrum, while, thanks to the resonance enhancement effect, the vibrational spectra of the solute can be measured without any severe solvent interference.

In these experiments ps UV Raman pulses are generated by frequency doubling the output of a Raman shifter pumped with 400 nm light. Indeed, on the contrary to TA measurements, RR measurements do not need a fine tunable and short probe pulse. A suitable Raman pulse for (pre-)resonant measurement is less demanding in terms of spectral tunability (10s of nm steps is usually enough) but has to be strong (typically 1 μJ/pulse or more) and narrow enough to resolve vibrational shifts (few tens of cm⁻¹ width at most or 1 ps compressed time lengths at least). Because of these peculiar requirements, specific ps UV and Vis pulse generation schemes for Raman applications have been implemented.30

**Femtosecond time-gated emission**

Time-gated emission (TGE) spectroscopy is another key method that has been recently extended into the UV.31–34 Regardless of the specific detection interval, it consists of exploiting a nonlinear phenomenon (optical Kerr effect, sum frequency or optical parametric amplification) to gate with fs resolution the emission from the sample after a fs excitation pulse (Fig. 1b). It is able to access the earliest electronic dynamics in any molecular system and to follow with time the emission Stokes shift subsequent to the excitation.

The former has made it the technique of choice to determine photocycles and phenomena where extremely short lived (even <10 fs) excited and intermediate electronic states are involved, as for instance charge transfers in coordination complexes35–38 and biomolecules39,40 exciton relaxation in nano-systems,41 ultrafast photo-isomerization and transits through conical intersections in photo-switches.42,43 On the other hand, time resolved fluorescence Stokes shift (TRSS) and polarization anisotropy measurements have demonstrated to be tremendously insightful tools to investigate solvation dynamics of the
environment around a chromophore and mapping local fluctuations and conformational changes in macromolecules. It is worth mentioning the capability of ultrafast TGE techniques to estimate Förster Resonance Energy Transfer (FRET) rates with fs uncertainty, by measuring the decay (rise) of the donor (acceptor) signal. Since FRET efficiency dramatically depends on geometrical factors, as the relative distance and orientation of the donor–acceptor pair, this makes FRET quantum yield measurements a molecular ruler with sub-nm resolution.

The first studies in the UV were carried out by Zewail and coworkers with single wavelength detection techniques. They exploited the sensitivity of Trp residues to local electric fields to investigate the link between the hydration shell and dynamics as well as the functionality of biomolecules. In a similar manner, naturally occurring Trp and Tyr were exploited to map intra-protein local rigidity and hydrophobicity as well as to get an insight on phenomena of biological relevance, such as site recognition and energy transfer in several proteins.

UV-TGE has been also revealed as an ideal technique to look into DNA and RNA photophysics. In particular, several authors investigated the mechanisms of energy disposal and deactivation in DNA single and double strands, which is biologically relevant to the stability of DNA and protection against UV photo damage.

The first femtosecond fluorescence time gated set-ups with broadband detection in the ultraviolet were proposed very recently. They are based on the optical Kerr effect and on the sum frequency. The latter employs a 100 to 500 μm β-BBO as a NL crystal and it can achieve a temporal resolution as good as 100 fs, with an intrinsic limitation to the detection range given by the sum-frequency process (with an 800 nm gate pulse the shortest wavelength that can be up-converted in air is 270 nm). The former uses as a Kerr medium a 1 mm thickness fused silica plate, which gives an easier access to shorter wavelengths at the cost of a worse instrumental time response (~300 fs).

The great detail provided by broadband detection is clearly shown in a recent review of aqueous Trp relaxation dynamics and in a study on vibrational relaxation and cooling dynamics of nonpolar UV dyes in polar and nonpolar solvents. The capability to spectrally resolve the whole band allowed the authors to follow not only the energy stabilization due to the solvent response but also the transition from a non-statistical to a statistical distribution of the excess of vibrational energy, and so determine the internal temperature of the molecule as a function of time.

Coherent spectroscopies: photon-echo and 2D spectroscopies

One of the main limitations of solvation studies based on TGE appears when the excited state lifetime is too short to report on the full solvation dynamics or the solute–solvent interaction is too weak (e.g., in case of nonpolar solvation) to have a detectable Stokes shift. A more fundamental aspect concerns to what extent this experimental methodology, but also TA and RR techniques, provide information on the fluctuation occurring in the unperturbed system. This is indeed the common situation in nature and most of the chemistry occurs in the ground state rather than in the excited ones. In these approaches, the chromophore has to be excited and part of the excitation energy is unavoidably transferred to the surroundings. In addition, excited and ground states could have a different interaction with the solvent (different permanent electric dipoles, specific chemical interaction, different conformations, etc.). Most studies assume the validity of the linear response approximation (LRA), which predicts that the Stokes shift is related to the energy-difference autocorrelation function calculated either in the ground state or in the excited state. However one could question how much the observed dynamics of the solvent and of the solute are fully representative of the unperturbed ones and testing the validity of the LRA is indeed a matter of debate.

Coherent spectroscopies have the unique capability to overcome these limits by directly accessing ground state fluctuations. In addition they have the ability to disentangle homogeneous (dynamic processes from inhomogeneous, due to static effects, e.g. distribution of sites) contributions to line broadening, whereas conventional, linear techniques cannot. This enables isolation of the optical line of a chromophore out of an overcrowded spectrum and quantification of the extent of the environment rigidity and the fluctuations between the conformational substates. Currently several groups are developing photon echo (four wave mixing, or 4WM) spectroscopies (Fig. 1c) in the mid-IR and Vis domain.

Different photon-echo (PE) techniques exist, including, among others, the classical two-pulse echo, time-integrated and time resolved three pulse stimulated photon echo, photon-echo peak-shift (PEPS), and multi-dimensional heterodyne-detected photon echo (2D-PE or FT-4WM). They vary in the wave vector geometry, pulse energies, pulse timing, and detection schemes (see Fig. 1c and 3). For sake of completeness Fig. 3 shows also higher orders of wave mixing techniques.

As far as homodyne detected versions are concerned (see caption of Fig. 1 for a discussion on heterodyne and homodyne detection schemes), among which is the three pulse integrated PEPS technique, they allow us to trace the transition frequency correlation function and nowadays they are widely used to investigate fluctuation dynamics of the environment around a chromophore. Contrary to emission, the nature of PE is intimately coherent, since PE signals are generated by the interaction of the last of the three pulses with the space-frequency hologram written into the sample by the first two pulses. Similarly to hole-burning techniques, they perturb the inhomogeneous distribution of ground state transition frequencies and measure the recovery time of the statistically equilibrated distribution. As a consequence they can also track down solvation dynamics in absence of a strong solvent–solute coupling. In addition they can access ground state dynamics, revealing if the solvation dynamics would depend on the specific molecular electronic state and so a possible failure of the LRA.

Moving to the UV, there are several aspects that make an UV-PE experiment a particularly difficult task (see for instance ref. 70 and 71 for an extensive discussion), which have
prevented the implementation in this spectral range. Indeed there are very few related works and all of them appeared in the last few years.59,64,70,71 They focused on the question of polar versus nonpolar solvation, which is a topic of much interest72 but not suitable for techniques monitoring excited state dynamics such as TGE. This is indeed a notable case where these techniques show their limitations (i) because of the weakness of the solvent–solute coupling and (ii) in case of a nonpolar ground state and a polar excited state, because of a substantially different interaction in the ground and the excited state, both in terms of strength and range.

The Fourier transform (FT), heterodyne detected, versions (also called 2D spectroscopies) are the newest arrival in the family of modern optical spectroscopy. They have a strong analogy to multidimensional nuclear magnetic resonance techniques,65 where electronic dipoles, instead of magnetic ones, are detected. Exploiting the optical response of transitions localized at different sites within the same molecule, these techniques are able to uncover intramolecular concerted motions and long range interactions. The capability to spread the optical response of the system under investigation on several frequency dimensions (in principle one for each interaction pulse) allows us to isolate the homogenous line shape from the inhomogeneous distribution of transition frequencies and to determine directly the fluctuation time scale of the environment around the chromophores. The current state-of-the-art deals with mid- and near-IR and Vis range, while the first schemes with UV detection have been presented only in last two years.73–75 These articles report mainly technical aspects rather than physicochemical applications. For this reason they will be discussed later, in the section devoted to perspectives and future directions.

Different ultrafast UV spectroscopies and their typical field of applicability have been shown and commented above. After these seminal works, several studies with similar approaches appeared, where diverse molecular processes were investigated, such as electron transfer in photosynthetic systems76 and metal complexes,26,77,78 energy transfer and relaxation mechanisms after ligand photo-dissociation in different haemproteins,40,79,80,81 the primary events in vision and in photosensors;43,52,82,83 or photo-oxidative stress in DNA.84 The most illustrative studies among these will be discussed in the next section.

UV probes: a novel family of chromophores to study dynamical processes in macromolecules

As already pointed out in the previous section, exploiting UV probes to investigate structural and energy dynamics in biomolecules, has been one of the major drives to develop UV femtosecond spectroscopies. This includes all the nucleotides and aromatic amino acids, as well as biocompatible labels like (hetero-)cyclic compounds (in particular imines and diimines as pyridine and phenanthroline analogues, but also imidazole, etc.). Concerning aromatic amino acids, they are the only amino acids with a distinctive optical response and the concept to exploit them as naturally present local probes is not new. In particular Trp is routinely used with ns to steady-state techniques as a fluorophore or RR probe to follow folding/unfolding processes, as well as slow conformational changes as a function of temperature, pH, substrate concentration, etc.23

The lowest Trp absorption band is due to two transitions from the ground state to the two close-lying lowest excited states (usually labelled Lα and Lβ) of the indole moiety. The transition to the Lα state implies a remarkable difference of 6 D in the permanent dipole moment with respect to

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**Fig. 3** Schematic representation of the different possible nonlinear spectroscopies realized through different beam combinations with respect to phase matching and time/wavelength multiplexing. (a) Pump–probe spectroscopy in the four wave mixing (4WM) representation. Panels (b) and (c) non-collinear 4WM for coherent spectroscopies, and general pulse shaping or coherent control 2D spectroscopy to select initial state preparations, respectively (the local oscillator field in case of heterodyne detection is not shown. See for instance Fig. 1c and 10a). Energy level diagrams (b’ and (b”) depict the 4WM processes for coherent anti-Stokes Raman spectroscopy (CARS) and electron–vibrational–vibrational spectroscopy (EVV) respectively. The shown six-wave-mixing (6WM) experiments are 2D Raman (d) and resonant 6WM (e) spectroscopies. Adapted with permission from ref. 65. Copyright (2009) American Chemical Society.
the ground state. Trp indeed shows a strong solvato- and electro-chromic behaviour, being both dependent on the change in the permanent dipole moment between the excited and the ground state. Trp residues are therefore particularly well suited molecular-level sensors of electric field changes around and within the protein. Because of the non-degenerate and relatively strong transition moments it is also suitable to follow structural changes by monitoring emission and absorption anisotropy.

Hence, a big effort is going on to make these naturally occurring probes able to uncover, with real-time measurements, all the aspects related to protein-solvent interplay, and to map changes in the local electric field within a protein during a functional event.

To clarify the added-value derived by a direct probing of UV chromophores, few exemplary studies will be discussed below, describing first the general context and then dealing with studies based on ultrafast UV techniques.

**Intra-protein electrostatics: following functional electric field changes in photoexcited retinal proteins**

Retinal proteins are among the most popular examples of biophotosensors, where light-induced redistributions of charge on the chromophore drive the structural changes needed for the biological function. Franck–Condon excitation of all-trans retinal results in an immediate change of a permanent dipole moment by more than 12 D, causing a sudden polarization of retinal. Note indeed that a ~10 D dipole on retinal creates a giant field of ~10 MVcm⁻¹ on the nearby amino acids of the retinal pocket.

Transient absorption studies show that in the first 200 fs no isomerization occurs and suggest that a skeletal change takes place, which, in case of the well-studied bacteriorhodopsin (bR), corresponds to a large twisting of the C13–C14 bond. Isomerization occurs later (~500 fs) and is followed by a slower vibrational relaxation of the isomerized retinal (the so-called J to K transition). The time scale of charge translocation and its interplay with the initial twisting, and subsequent isomerization, have been a topic of great debate. To address this issue, Cherghi and co-workers used natural tryptophan residues to probe the evolution of the electric field within bR. They excited the retinal with 560 nm photons and monitored Trp absorption changes with tunable UV pulses, achieving a time resolution of 80 to 90 fs. Absorption spectrum of bR (Fig. 2b) indeed exhibits a band in the visible, due to retinal, and a band at 280 nm, due to the retinal itself. The ground state. Trp indeed shows a clear time-dependent signal. Authors ascribed it to a significant (Stark) red shift of its L absorption band in response to the light-induced charge translocation along the retinal. Trp thus appears as a local ultrafast “voltmeter” probing the local charge reorganization during the isomerization process.

As a consequence the authors proved that: (i) the Stark shift of the Trp absorption band and therefore the underlying retinal charge translocation occur on a sub-50-fs time constant; and (ii) on the timescale of the isomerisation process (500 fs), the Stark shift reaches a long-lived plateau, indicating a different electrostatic environment compared with that of ground state bR. This points to a long-lived change in the electrostatic environment of Trp, which lends credence to an “electrostatic conflict” arising already in the early J intermediate of the photocycle of the protein because of the isomerisation.

This experimental approach has two peculiar advantages when compared to Vis and IR experiments that directly probe the retinal response: (i) the charge displacements become detectable with high sensitivity through an electric dipole interaction in the UV; and (ii) the local electric field changes are followed in real time from femtoseconds to picoseconds or more.

More recently Mizuno et al. reported a ps UV-RR study on the structural changes triggered by the retinal photosomerisation in the Sensory Rhodopsin II (SRII) protein. They focused on the response of Trp and Tyr residues to time-resolved signal transduction process. Due to a poorer time resolution of 3.4 ps, the change in the retinal pocket electrostatic field manifested as a pulse limited signal bleach, but they succeeded to directly observe structural changes on the ps-to-ns domain. The same authors exploited Tyr UV-RR bands to investigate changes in the hydrogen-bond network, rather than local electrostatics, around the chromophore of the photoactive yellow protein, a putative blue-light sensor. They elucidated the structure of the hydrogen bonding network around the chromophore, a p-coumaric acid (pCA), in the ground and excited state, arguing that the change of the hydrogen bonding of the excited pCA could facilitate the isomerisation of the chromophore.

These examples show how UV femtosecond TA and RR spectroscopies on aromatic amino acids can provide subtle information as local charge reorganizations and changes in hydrogen bonding networks during a protein functional activity.

**Solvation and local fluctuation in biomolecules**

Uncovering those fluctuations in a protein which play a functional role is one of the main challenges of the modern science. There is an almost countless literature on this topic and more discussion can be found elsewhere. Strictly related to local and global fluctuations, the mechanisms of redistribution and dissipation of excess of vibrational and
electronic energy are at the centre of intensive studies in particular aimed at understanding the role they play in intra-protein signalling and protection from heat- and photo-induced damage.

It is clear that the capability of Trp residues to report on the dynamics around a specific site that is different and distant from the cofactor widens the angle of view, framing local dynamics in the context of a global process. In the case of photo-triggerable processes it could also reveal the presence of vibrational or electronic coherences.7,89

One of the first examples where ultrafast fluorescence TRSS of Trp was exploited to map local fluctuations in and around a protein, is the series of works by Zewail and collaborators on hydration dynamics at the protein surface2,46,54,56,90 and on intra-protein local solvation47,48 in several biological systems. They probed in real time the hydration dynamics of bulk water near the protein and at the protein–water interface (the so-called biological water),7,54,56,90,91 revealing that the biological water has a completely different behaviour with respect to the bulk water (Fig. 4a and b). They found that strong (electrostatic) interactions with the charge residues at the protein surface decrease the disorder of water at the interface, defining a rigid water structure with a much longer solvation time (10–100 ps, typically biphasic) than the bulk one (~1 ps). The former is a direct measure of surface hydration assisted by fluctuations of the protein: indeed on these timescales proteins are not rigid and their fluctuations modulate water solvation by allowing for restructuring in the new non-equilibrium state.46

These experiments stimulated a rich computational activity, which shifted original interpretation based on water exchange towards an interplay between protein and solvent dynamics.50

In a study on the enzyme protein subtilisin Carlsberg Pal et al. compared the hydration dynamics at the protein surface with the one 7 Å far from the protein, by measuring the TRSS of the single Trp residue at the protein surface and of a fluorophore covalently bonded and sticking ~7 Å out of the protein (Fig. 4c and d). While in the former case they found the same previously described pattern, in the latter case they observed a dominant solvation, bulk-type, time of 1.5 ps and the near disappearance of the long ps component (Fig. 4e and f). This is to my knowledge the first experimental determination of the extent of the biological water layer around the protein in physiological conditions.

The same authors reported innovative studies on the hydration dynamics in the DNA grooves and the influence of drug binding on DNA hydration.2,92,93 As observed in proteins, water molecules at the surface of DNA are dynamically ordered and authors reckon a crucial role of such a water in interfacial recognition, not only of drugs but also between macromolecules. This study was carried out by following the time evolution of the Stokes shift and polarization anisotropy of the visible fluorescence of the drug, but not the natural UV emission from nucleotide bases, since their fluorescence lifetimes are too short to give the full temporal evolution of water solvation. Indeed, regardless the specific nucleotide, their excited-state lifetimes fall in the sub-picosecond time scale,32,94 and an excess of electronic energy is dispersed in the water in less than 1 ps.92 This confirms the occurrence of ultrafast internal conversion channels, which were rationalized by invoking the presence of conical intersections.33,92,94 These articles also report that the excited-state deactivation and energy mechanisms are more efficient in water than in other solvents. Since a so-fast deactivation is supposed to have a key role in the stability of DNA and protection against UV photo damage, these studies confirm a specificity for water, as far as dynamical processes in biomolecules are concerned.

These studies have also the merit to clarify for the first time the role of biological water in site-selected recognition with a real-time technique, not only in the DNA grooves but also in the binding sites of several proteins.2,48,95

Unfortunately, as discussed in the previous section, solvation studies of DNA strains are intrinsically limited by the very short excited state lifetime of the nucleotide bases and affected by the important amount of electronic energy converted into local heat (almost 100 kcal mol⁻¹) in less than 1 ps. For these
reasons the investigation of DNA photophysics with coherent techniques is highly desirable.73,75

Driven by these considerations and by the interest to prove the feasibility of UV PE experiments, Ajdarzadeh Oskouei et al. developed an UV 3 pulse PEPS set-up to exploit nonpolar UV dyes as a probe and to benefit from the unique capability to access ground state dynamics and higher sensitivity of PE techniques.59,64,70,71 In addition, common visible dyes are usually large and very complex while standard UV dyes typically are smaller and, often, rather simple molecules and thus very appropriate for thorough theoretical and experimental characterization. They found that solvation dynamics depends on the electronic state of the solute and the electronic dephasing is unexpectedly sub-100 fs also in nonpolar solvents. This result contradicts models proposing that only the interactions between the solute and the first solvent shell play a major role in electronic dephasing and predicting dephasing times as long as 1 ps in the case of nonpolar solvation. Authors suggest that the electronic dephasing originates primarily from intramolecular dynamics and, in particular, internal conversion (IC) and intramolecular vibrational redistribution (IVR). In this respect, they uncoupled electronic dephasing from the environmental dynamics. PE techniques have also been successfully employed in the Vis domain to quantify the immunological evolution of protein flexibility in antigen-antibody recognition but, to my knowledge no similar study on proteins have been carried so far with UV-PE techniques. Remarkably the first UV 2D-PE experiments were carried out on single nucleotides, as discussed below.73,75

After these studies, TGE and coherent spectroscopies, when extended into UV domain, have emerged as an invaluable tool to clarify the intimate link between the biological function of a protein and the fluctuations in and around the protein.

Dissipation and redistribution of energy excess in proteins: proteinquakes or inelastic dissipation?

Mapping local fluctuations and identifying the functionally relevant ones are one side of the same coin, where the other is understanding how a localized excess of energy, generated during a biological function, is redistributed amongst the protein modes. An excess of mechanical and thermal energy is generated not only upon light absorption, but also during a chemical reaction at the active site of an enzyme. Energy dissipation and redistribution are also relevant both to understand protein stability and as a possible long-range interaction mechanism.88,99 When looking for real-time studies of such processes, there are very few works. They appeared very recently and are based on UV spectroscopy of Trp and Tyr residues in haemoproteins.28,40,51,80,100

Haemoproteins indeed are ideal systems to study the vibrational energy flow in proteins. The haem group is relatively isolated from the rest of the protein and intra-haem IVR is faster than the dissipation channels toward the protein edifice.80,101,102 Furthermore it is characterized by extremely short-lived excited states (< 150 fs) because of very efficient nonradiative channels.40,80,103 This makes possible to optically deposit in a well localized site up to 40 000 cm−1 (or 100 kcal mol−1) of excess of vibrational energy (EVE) in few hundreds of fs (corresponding to an almost instantaneous rise of the haem temperature of 500 K80,101,102), allowing time-resolved studies of the energy redistribution processes (Fig. 5a–c). In addition to this thermal jump, a photo-excitation of the haem cofactor can induce an ultrafast axial ligand dissociation from the central iron atom, depending on the protein and the oxidation state of the haem group. This triggers an out-of-plane dislocation of the metal and a doming of the haem that, right after the dissociation, leaves the system in an out-of-equilibrium state. A long-lasting debate concerns the question, whether the redistribution of this impulsively released strain energy and heat occurs as a mechanical (deformation) coherent wave propagating from the center to the protein periphery or in an incoherent fashion, where the protein edifice behaves as an anharmonic, inelastic reservoir.81,99,104,105 It is worth mentioning that these structural changes at the haem site are...
supposed to trigger in haem- and myoglobin a global conformational change from the so-called oxy to deoxy structure.99,106

UV spectroscopy of Trp and Tyr is ideal to uncover these mechanisms, because these amino acids can act as intra-protein site-specific probes to report the arrival of the perturbation.

One of the first studies was carried out by Mizutani and co-workers on myoglobin with UV-RR spectroscopy (Fig. 6).28,29 In these studies, changes of Trp and Tyr UV-RR bands allowed authors to track down picosecond conformational changes of the side chains and environmental changes around those residues in a site-specific fashion. Based on these spectral changes, authors proposed a detailed microscopic description of the concerted movement of protein helices, impulsively driven by changes in the haem, in the first picoseconds, and then by rearrangements toward a deoxy structure, in hundreds of ps to nanoseconds. Notably, they did not observe any vibrational energy propagation, excluding that the observed dynamics may be due to a transient thermal effect.

However, in the case of myoglobin, the haem cofactor is almost in contact with the solvent and the lack of intraprotein energy dissipation could be in part due to an efficient dissipation toward the external environment. To avoid such channels, several research groups moved to horse-heart cytochrome c (Cyt c), a relatively small membrane-bound haem protein which plays an important role in many electron transfer processes (Fig. 5a).40,80,81,100 Indeed, in contrast to myoglobin, the 6-coordinated haem of Cyt c is buried well inside the protein and, accordingly to molecular dynamics simulations,102 a very weak influence of the solvent on the energy relaxation is expected. The thermal bath of the cofactor is therefore the protein itself.

Very recently, several studies appeared where this class of proteins has been investigated with different UV spectroscopies (tunable single-wavelength UV- and Vis-TA upon 400 nm excitation,81 broadband UV- and Vis-TA and TGE upon excitation at 530 nm, 400 nm and 288 nm,40,80 anti-Stokes UV-RR on Trp residue upon 537 nm and 405 nm excitation100) The haem centre was excited in order to study the interaction between the haem moiety of ferric and ferrous Cyt c and its single Trp residue. The latter is located at 9 Å from the haem and its fluorescence is strongly quenched by FRET to the haem group (Fig. 5b and c).40 In agreement with Vis studies they found that for the ferrous state the haem undergoes ultrafast photolysis of one of the axial ligand (a methionine labeled Met80) in less than 100 fs and, after vibrational cooling within several picoseconds, it recombines back to the original 6-fold coordination with a biphasic decay of 5 and 16 ps (Fig. 5d).80,101 Conversely, ferric haem does not undergo any photo-dissociation, maintaining its 6-fold coordination. Relaxation and cooling show a biphasic decay with a fast, 1–3 ps, and a slow, ~10 ps, component (Fig. 7), slightly dependent on the oxidation state (compare with Fig. 5).80 The former component describes the heat transfer from the

Fig. 6 Picosecond conformational changes after ligand photo-dissociation in carbonmonoxy myoglobin (MbCO). Pump and Raman probes are at 408 nm and 232 nm, respectively. (a) Picosecond UV-RR difference spectra of photo-dissociated horse skeletal MbCO in the range 650–1800 cm\(^{-1}\). The top trace is a probe-only spectrum corresponding to the UV-RR spectrum of MbCO divided by a factor of 30. The bottom trace is a deoxyMb-minus-MbCO difference spectrum. Spectra have been offset for clarity. (b–c) Crystallographic structure of horse Mb (PDB ID code 1DWR). (b) Close-up of the region near the two Tyr residues. (c) Close-up of the region near the two Trp residues. The orange arrows indicate the directions of motions of the F helix and FG corner after CO dissociation, expected from crystallographic studies. From ref. 28. Reprinted with permission from the National Academy of Sciences of the USA.

Fig. 7 Energy flow through cytochrome c. (a) Temporal intensity changes of the anti-Stokes Raman bands of Trp\(_{59}\) in the range of 5 to 100 ps, upon Soret band excitation (405 nm) and with Raman probe at 230 nm. Intensity kinetics of the anti-Stokes W18 and W16 lines (759 and 1015 cm\(^{-1}\) modes of Trp, respectively) are shown in panels b and c, and compared with the respective Stokes traces (inverted in sign). Stokes intensity changes instantaneously upon photo-excitation, while anti-Stokes intensity exhibits a 1–3 ps delayed response. Increase and decrease of the anti-Stokes band intensities is assigned to the flow of excess energy from the haem to the Trp residue and the release from the Trp residue to the surroundings, respectively. Changes in the Stokes UV-RR intensities are explained as a change in the hydrogen bonding between the haem and Trp\(_{59}\). Reprinted with permission from ref. 100. Copyright (2011) American Chemical Society.
haem to the Trp,\textsuperscript{80,100} while the latter is due to the statistical redistribution of the EVE among the low frequency modes of the protein and the heat transfer to the solvent.

The pure geminate rebinding process on a ps timescale, the unequivocal assignment of the Trp response and the weak influence of the solvent make this protein a promising model system to investigate energy redistribution.

To explore the effect of the amount of energy deposited in the haem on the energy redistribution process, Consani \textit{et al.} varied the excitation from 530 nm to 400 nm and to 288 nm, corresponding to an impulsively deposed EVE up to 100 kcal mol\textsuperscript{-1} (or a haem temperature increase of up to 500 K).\textsuperscript{80} In agreement with the results reported by Fujii \textit{et al.},\textsuperscript{100} they found that the kinetics of the energy flow is not affected by the amount of excess energy deposited, being an intrinsic feature of the protein. Accordingly, authors asserted that the protein behaves as a linear, harmonic system that, as a cage, protects the haem and its functionality. They also followed the Trp-haem resonance energy transfer process by directly exciting the Trp with a UV pump. They observed that this process induces the Met80 detachment with almost unity quantum yield, just as under direct laser excitation. However, despite practically identical photocycles, the two excitation routes are significantly different in the way how the EVE is redistributed: with the excitation via Trp, part of the total EVE (∼20 kcal mol\textsuperscript{-1}) is left on the amino acid generating a excited haem colder than upon direct excitation. This points out that in energy transfer processes the number of amino acids involved in the energy transfer path could play a role in facilitating the dissipation of the excess of energy.

Zang \textit{et al.} also reported a distinctive 40 ps component in the decay of Trp signal, present only in the ferrous version.\textsuperscript{83} They argued that this was evidence of a deformation coherently propagating through the molecule (a so called protein-quake\textsuperscript{105,107}), triggered by the impulsive bond breaking. As observed by the authors, a propagation time on the time scale of hundreds of ps was expected.\textsuperscript{78,100} However neither Fujii \textit{et al.} nor Consani \textit{et al.} observed in the ferric and ferrous forms, respectively, any similar long component (Fig. 5d and 7). This supports the absence both of a thermal protein-quake (i.e. a shock wave due to the haem thermal jump) and of a mechanical one (a deformation wave propagating from the haem group due to the sudden iron-ligand bond breaking). Accordingly the excess of vibrational energy is dissipated in few ps in an incoherent fashion from the haem to the bath, constituted of the low frequency modes of the protein first and by solvent later.

The localization of the vibrational energy during the flow across the protein has been related to the presence of anharmonic couplings amongst the protein vibrational modes.\textsuperscript{108} It is tempting to relate the lack of localization to the aforementioned harmonic behaviour. Moving to the lack of signature of a “mechanical protein quake”, since recombination occurs in ∼10 ps while, as also pointed out by Zang \textit{et al.}, conformational changes likely propagate in sub-ns, the haem and its pocket could get back to the stable, ligated configuration prior to any important structural change. This would prevent the released strain energy from leaving the haem pocket. It also agrees with the lack of any long-lasting change in the protein configuration.

Other alternative explanations exist as an unfavorable position of the Trp residue to report on a global structural change.\textsuperscript{80} Consani \textit{et al.} reported also a nonlinear dependence of the Trp response on the pump power, not present in the Vis interval, which could be also responsible for the 40 ps component reported by Zang \textit{et al.} These aspects deserve still more experimental and theoretical investigation.

A few methodological notes deserve to be added: (i) the article by Bram \textit{et al.} on the electronic relaxation in cytochrome c upon Vis and UV excitation\textsuperscript{40} exemplifies the unique capability of TGE techniques to characterize time scales as short as 40 fs with an uncertainty of 10 fs or less, in contrast with TA measurements where the shortest detectable time constant is typically 150 fs (compare Fig. 5c and d). This allowed the characterisation of IC dynamics and branching ratios from the highest excited states. (ii) The sub-picosecond energy transfer from the Trp to the haem is strongly dependent on the protein oxidation state, revealing a different geometry, at least of the haem pocket, in the ferrous and ferric forms. This capability of the FRET Trp–haem pair to play the role of a molecular ruler has been recently used to study local structure flexibility and conformation fluctuations in myoglobin.\textsuperscript{51} (iii) Consani \textit{et al.} report the first broadband TA spectrum extending from 270 to 700 nm (Fig. 5d), which is, to my knowledge, the broadest ultrafast absorption spectrum shown so far.\textsuperscript{80} It allowed the authors to cover the first four electronic transitions of the haem and revealed, for instance, that the spectral range below 360 nm contains a feature typical of photolysis. Contrary to the usually monitored Soret band, it is scarcely influenced by cooling-related spectral signatures and it appears to be an ideal fingerprint to disentangle recombination processes from other relaxation mechanisms. (iv) Fujii \textit{et al.} showed for the first time anti-Stokes UV-RR spectra of aromatic amino acids with a time resolution of picoseconds (Fig. 7).\textsuperscript{100} This is a very promising tool to get information on the time evolution of the vibrational energy deposited on these residues. Indeed the intensity of anti-Stokes lines is directly related to the population of hot vibrational states and in this respect they are a unique signature of the temperature of the probe and, in case of high frequency modes, they are background free.

Following the flow and the dissipation of excesses of electronic and vibrational energy through a complex molecule is a challenging task. Only the synergic use of different spectroscopies can report on the various involved processes (electron–phonon coupling, vibrational energy localization, structural changes, etc.) and, as discussed above, UV techniques have the peculiarity to report on the response of the molecular edifice and on the propagation through it.

**Charge transfer in metal complexes**

Understanding the structure and dynamics of electronic excited states of coordination and organometallic compounds is an important goal of current, photophysical, photochemical and theoretical research, from the point of view both of fundamental science and applications. Indeed, metal-based compounds have been used as sensitizers for solar-energy conversion, organic light-emitting diodes (OLED), photocatalysts,
non-linear optical materials, opto-magnetic data storage, luminescence-based sensors, and active components of electron- or energy-transfer assemblies (see for instance two recent special issues on Coordination Chemistry Reviews edited by M. Haga, O. Ishitani and N. Kitamura in 2010109 and by A. Vlček in 2011110). The main reason is that in most of these systems the lowest electronic transition corresponds to a singlet metal-to-ligand charge transfer (MLCT) state, which can be easily tuned in the visible with the suitable choice of the central metal atoms and the ligands. It is believed that upon excitation an electron density is immediately transferred to the ligand, giving rise to an intense change in the permanent dipole, typically of 4–5 D. Then, very efficient intersystem-crossing (ISC) processes (typically <100 fs, but faster than 30 fs have been observed in metal tris-bipyridine complexes8,37) bring the molecular system to a long-lasting triplet or quintet state with an almost unitary yield, before any important solvent rearrangement occurs (see ref. 35 and references therein). This provides a well localized electron, ready to be used for a variety of photophysical and photochemical processes. In addition, by varying the ligands around the metal centre, it is possible to fine tune the energy structure to match it to that of the electron acceptor. However, the formation of the charge separated state (the electron and energy redistribution mechanisms and the ensuing structural response of both the excited molecule and the surrounding environment to stabilize the charge on the ligand) are not yet fully understood and despite nearly 20 years of active research the underlying mechanism is still under debate.

As recently pointed out by Vlček and collaborators,77,111 Vis-TA spectra of polypyridine complexes do not contain spectral features diagnostic of the presence of a reduced ligand in the excited state of such complexes. They mostly originate from ligand-to-metal charge transfer (LMCT) transitions of the lowest excited state, which are more sensitive to the electronic state they originate from, rather than to the fractional charge on the bipyridine (bpy) or phenanthroline (phen).78,111 Prior to this study, based on similarities between spectra of excited state absorption and of one-electron reduced bpy,30,112 it was the opinion that Vis-TA spectra would contain weak but detectable spectral markers of the reduction state of the ligand. Conversely, they found that the only spectral feature that is really diagnostic of the presence of a reduced ligand in the excited state of such complexes is an intense intra-ligand (IL) UV band at ~370 nm and ~300 nm for bpy and phen ligands, respectively.

In analogy to the studies exploiting the Trp response discussed above, here we can benefit from UV spectroscopy by accessing these transitions, to discover the other half of the story: the charge transfer process from the point of view of the accepting ligand.

The first systematic study where the arrival and the subsequent stabilization of the transferred electron were monitored by detecting IL states was recently reported by El Nahhas et al.77,78 They used UV-Vis pump-probe spectroscopy and Vis TGE to study a series of rhenium carbonyl-diimine complexes ([ReL(CO)3(N,N)]; L = axial ligand, (N,N) diimine ligand). (a) Molecular structure and the orbitals involved in the transition responsible for the UV (373 nm) excited state absorption (ESA) band in case of (N,N) = bpy. The dominant intra-ligand (IL) nature of this transition should be noted. (b) Time resolved absorption spectra of [ReBr(CO)3(bpy)] in dimethylformamide measured at selected time delays after 400 nm, ~100 fs excitation at magic angle. (c) Kinetic traces from data in panel (b) at different wavelengths. They are vertically displaced for clarity. Note in panels (b) and (c) the lack of dynamics after 5 ps in the visible region, where ESA band have mainly a ligand-to-metal charge-transfer nature. Conversely, the UV region shows a longer component of 20 ps (which becomes 1 ns in ultra slow solvents, such as ionic liquids). As shown in panel a, this is an IL transition reporting on the status of charge localization on the ligand only. Reprinted with permission from ref. 77. Copyright (2010) American Chemical Society.

Fig. 8 Solvent-slaved formation of charge separated states in rhenium carbonyl-diimine complexes ([ReL(CO)3(N,N)]; L = axial ligand, (N,N) diimine ligand). (a) Molecular structure and the orbitals involved in the transition responsible for the UV (373 nm) excited state absorption (ESA) band in case of (N,N) = bpy. The dominant intra-ligand (IL) nature of this transition should be noted. (b) Time resolved absorption spectra of [ReBr(CO)3(bpy)] in dimethylformamide measured at selected time delays after 400 nm, ~100 fs excitation at magic angle. (c) Kinetic traces from data in panel (b) at different wavelengths. They are vertically displaced for clarity. Note in panels (b) and (c) the lack of dynamics after 5 ps in the visible region, where ESA band have mainly a ligand-to-metal charge-transfer nature. Conversely, the UV region shows a longer component of 20 ps (which becomes 1 ns in ultra slow solvents, such as ionic liquids). As shown in panel a, this is an IL transition reporting on the status of charge localization on the ligand only. Reprinted with permission from ref. 77. Copyright (2010) American Chemical Society.

with other diimine metal complexes, they found that the singlet MLCT relaxes in 100–140 fs toward a multiplicity of hot triplet states that in ~1 ps populate the lowest triplet MLCT. Longer dynamics were not observed in the Vis range (except rotational diffusion ones) and this would lead to the (erroneous) conclusion that the charge separation is achieved in <1 ps and no other dynamical processes are going on afterwards. Conversely, when detection is moved to UV, the IL band (which is a ππ* ← π transition in case of reduced bpy*) shows an increase of up to 20%, in 15 ps in the fast-relaxing dipolar solvents (dimethylformamide and acetonitrile) and in 1 ns
in slow ionic liquids (Fig. 8b and c). These time constants scale with the solvation (dielectric) relaxation times of the solvent (typically ~1 ps in fast dipolar solvents and up to ~100 ps in ionic liquids), being however ~10 times slower. This behaviour is fully confirmed by time-resolved infrared (TRIR) measurements on the carbonyl ligands, used as IR markers of electron density redistribution on the Re atom upon excitation.113 Based on these evidences, the authors derived a scenario rather different from that previously inferred from Vis measurements: after a 100–140 fs ISC followed by a IC in ~1 ps, the full equilibration of the lowest excited state takes place in several tens of picoseconds, involving electronic and vibrational relaxation on the units-of-picoseconds time scale, as well as slower restructuring of the local solvent that takes place in ~15 ps in dipolar solvents, extending to ~1 ns in ionic liquids. To rationalize the correlation of the reorganization times with the solvent response times but on a time scale 10 times slower, authors proposed that relaxation is due to reorganization within a supramolecular cluster, consisting of the chromophore and several strongly interacting local solvent molecules, probably lying between the ligands. In this respect, ligand intercalation in tris-bipyridine complexes has been recently predicted by QM/MM simulations.114

This explanation provides a unifying description of the results in different polypyrindine systems that do not fit with the picture arising from the Vis measurements, such as unusually slow anisotropy kinetics,115 or vibrational spectral evolution,126 and to frame in a more general context former ps time-resolved studies at λ > 350 nm.116

More remarkably, because MLCT states are doorway states to charge separation in metal-based supramolecular complexes, the present results are of importance as they show that the solvent molecules are not just spectators of the process but modulate it by a retarded reorientation dynamics due to their presence between the ligands of the complex.

Before concluding, a study by Consani et al. on the relaxation mechanisms in high-spin state of aqueous iron trisbipyridine complexes should be mentioned.8,26 They found an astonishingly fast double spin-flip, with the formation of a vibrationally hot quintet state in 130 fs. Due to a so-far ISC from a bonding to an anti-bonding state, they observed the generation of a coherent wave-packet in the quintet state. This is the first evidence of vibrational coherence in high-spin states of such complexes. Remarkably, they discovered this behaviour by exploiting the lowest quintet state absorption band in the UV region near 300 nm.

These studies exemplify how useful UV spectroscopies (and in particular UV-TA and UV-TGE) are to follow processes occurring in and around the ligands of a metal complex during the formation and stabilization of a charge separation state.

Coherent spectroscopies: new tools for tracing long distance interactions and chemically sensitive detection

In the previous section we saw, with several examples, the potential of ultrafast UV spectroscopies to provide information not only on the directly photo-excited moiety but on the hosting molecular edifice. Exploiting the new UV probes, we can finally move our focus from the “epicentre” to the “periphery”, with the aim to track down concerted motions, long-distance interactions, and modulation of local dynamics by changes in distant sites.

Coherent and multidimensional spectroscopies are a novel family of methodologies which have the potential to address these issues with an even higher degree of insight.

Optical coherent spectroscopy exploits the capability of coherent sources, namely lasers, to induce in-phase optical oscillations of microscopic electric dipoles in the sample, to generate a macroscopic optical signal, whose features (direction, polarization, spectrum, phase, time evolution) reveal a great variety of information about the quantum behavior of the material under study. When molecular systems are investigated, structural, chemical, dynamical properties can be unraveled.5,11,63,117,118

This approach has revolutionized modern spectroscopy and, when coupled with a heterodyne detection scheme, they give rise to time-resolved multidimensional nonlinear optical spectroscopies61,65,68,69 (also called pulsed Fourier transform, or heterodyne detected, photon echo, or four wave mixing, spectroscopy). Over the past few years, there has been a growing effort aimed at developing these techniques, which are the optical analogues of multidimensional Nuclear Magnetic Resonance (mD-NMR)119 ones (NOESY, COSY, etc.). Nowadays, a variety of 2D vibrational (2DIR) spectroscopies have been proposed theoretically and implemented experimentally120 in the infrared120 and, more recently, in the near-IR and visible ranges to probe electronic transitions.4

Their power lies in the fact that: (i) they can reveal without any a priori specific knowledge, couplings among different sites within the molecular system; (ii) they can easily separate inhomogeneous and homogeneous contributions to spectral line shapes; (iii) and lastly, they can disentangle overcrowded standard (1D) spectra (Fig. 9a). These techniques have been demonstrated to provide structural information and to track down several processes of biological interest,4,12,122,124 such as amyloid fibril formation,123 drug–enzyme interaction specificity,124 resonance energy transfer in photosynthetic systems,5,7 but also electron–hole dynamics in quantum structures125,126 and semiconductors.127 Very recently the superior structural information provided by 2D spectra was successfully used to perform all-optical protein recognition.13

Extending these techniques to the UV would offer an invaluable tool to look into dynamical phenomena in macro-molecules and supra-molecular systems. Concerning biomolecules, it would make possible to exploit the natural amino-acid residues present in all proteins, disentangling their typically over-crowded electronic spectra, and delivering a global picture of their dynamics and structure, via the time-dependent couplings between residues.21 Engineering of molecular devices inspired by nature (the so called bionanotechnology128) will greatly benefit from this global approach to protein dynamics. In particular, these techniques would be an insightful tool to design and optimize molecular devices powered by solar light, where UV labels could be placed in any site to monitor energy and charge transfers, as well as structural and thermodynamic changes in real time, without perturbing the photon-energy doorway centres.
This research field is attracting more and more attention from theoretical and experimental groups. The first computational studies appeared very recently.\textsuperscript{129–131} Authors predicted that UV-2D spectra contain characteristic signatures of different aromatic amino acids transitions and of the interactions between them.\textsuperscript{130} Transition couplings were found to be very sensitive to the changes of residue–residue interactions and induced by residue mutations. Simulating UV-2D signals of amyloid fibrils, they explored this selective response to keep track of formation kinetics of amyloid fibrils associated with Alzheimer’s disease, since UV-2D signals carry characteristic signatures of fibril size and geometry.\textsuperscript{129} They also showed that it could be possible to identify fibrillation propensity of a given protein.\textsuperscript{131}

Moving to implementing UV-2D spectrographs, the first steps have been taken only in the last years. There are two main technical obstacles that have prevented this development: the necessity of phase-stable pulses and of ultra-broadband UV sources. These are actually the two essential ingredients for a proper implementation of a FT-2D set-up in the UV.

These techniques, in analogy to mD-NMR, require phase stability of the pulses. They were indeed initially developed in the IR region where, due to longer wavelengths, phase stability can be achieved more easily and only recently the first experiment in the Vis range was performed.\textsuperscript{4} In this work, Fleming and co-workers exploited for the first time electronic dipoles to measure energy transfer processes and couplings in the Fenna–Matthews–Olson (FMO) light harvesting photosynthetic protein complex (Fig. 9b). They also nicely showed the great potential of this technique to disentangle over-crowded 1D electronic spectra. This seminal experiment was made possible thanks to the use of passive phase-stabilization by diffractive optics as good as $\lambda/100$ at 600 nm (Fig. 10a).\textsuperscript{68} The beams are pairwise manipulated in such a way that any phase shift introduced by wobbling optics elements generates a compensating phase shift on the conjugated fields.\textsuperscript{132} Nowadays, inherently phase-stabilized...
schemes based on conventional optics exist with comparable performances.\textsuperscript{133} Since a minimal phase stability of $\lambda/50 - \lambda/30$ is required for a proper FT analysis, these novel designs have paved the way to extending coherent nonlinear techniques into the UV domain. The first fully non-collinear coherent UV 2D spectroscopy was introduced only in 2010.\textsuperscript{74} The main credit is that they successfully proved the feasibility of multidimensional optical spectroscopy in the UV domain, between 250 and 375 nm. Since they used only reflective optics the concept can be transferred to all wavelength regimes. However, this set-up instead employed rather spectrally narrow UV pulses ($\sim$8 nm width at 287 nm), while much broader pulses are necessary to fully benefit from such a methodology.

In the FT approach, the sample has indeed to interact simultaneously with all the spectral components of interest. The spectral information will then be retrieved by Fourier-transforming the heterodyned signal with respect to the time delays of the relative pulses. Thus, to have a 2D spectrum it is necessary to generate pulses broad enough to cover all the spectral region of interest. Several groups are developing generation schemes for powerful (0.1–100 $\mu$J/pulse) ultrashort (sub-10 fs) NUV and DUV pulses, based on different generation processes as achromatic phase-matching frequency doubling\textsuperscript{134} and sum-frequency mixing,\textsuperscript{135} filamentation in rare gas-filled cells,\textsuperscript{136} or four-wave mixing in hollow fibers.\textsuperscript{137} The achromatic phase-matching frequency doubling scheme,\textsuperscript{134} developed by Riedle and co-workers, deserves a particular mention. It is currently the approach that delivers the broadest spectrum (275–375 nm) and matches most of the chemically relevant UV region (Fig. 10b). With a doubling efficiency of 20\% it can provide few $\mu$J per pulse, when pumped with a two-stage ultra-broadband NOPA.\textsuperscript{18} This is strong enough both for non-coherent spectroscopic applications (a typical pump pulse energy in an ultrafast experiment is $\sim$0.1 $\mu$J in 100 $\mu$m diameter spot\textsuperscript{80}) and four-wave-mixing experiments, where a few tens of nJ per pulse is required\textsuperscript{4} (in this respect, they can be considered “protein friendly” techniques).

Towards a new generation of versatile UV 2D spectrographs

Strictly connected to UV broadband pulse sources, a new generation of pulse-shapers for UV application have been implemented.\textsuperscript{138,139} This research field has been always developed in parallel with coherent spectroscopy for two main reasons. First, to keep the temporal properties of an ultra-broadband pulse under control, neither grating- nor prism- based compressors are suitable any more, because of the non-negligible contributions from higher group velocity dispersion (GVD) orders. This is particularly critical with UV pulses, where after an interaction with even a few millimetres of UV-grade silica, the time structure is dramatically affected.\textsuperscript{140} Only the greater flexibility of a pulse-shaper can, in principle, compensate for any order of GVD. Second, in 2003 Warren and collaborators already demonstrated that it is possible to implement a robust and phase-stable collinear 2D-PE set-up, creating, with an acousto-optic pulse-shaper, a collinear three-pulse sequence with well-controlled and variable inter-pulse delays and phases.\textsuperscript{117} More recently, a scheme based on a pump-probe like geometry was proposed for the Vis\textsuperscript{141} and UV range,\textsuperscript{73} where the first pulse pair on the sample is collinear, and the generated signal is then collinear with the third pulse, which, at the same time, acts as a local oscillator for the heterodyne detection (Fig. 11a). This greatly simplifies the phasing problem and permits rapid acquisition of 2D absorption spectra. While this has been demonstrated with narrowband UV pulses,\textsuperscript{73} it should perfectly be applicable to (ultra-)broadband UV pulses.

When compared to non-collinear 2D-PE set-ups based on

![Fig. 11 Pulse-shaping and 2D spectroscopy (a) An automated UV 2D-PE set-up based on a pump–probe set-up, with pulse-shaped pump beams. The UV-pump at $\sim$260 nm is generated from IR pulses by nonlinear crystals (NC) and afterwards separated by a dichroic mirror (DM). It goes into a pulse shaper (the area enclosed with a dashed line) and becomes a phase-locked pump–pump pulse pair. The IR passes through the second set of nonlinear crystals (NC2) to generate the UV probe. The UV pump–pump and probe pulses are focused into the sample by the curved mirror (CM). The probe spectrum is detected with a spectrometer as a function of the pump–pump delay $T$. (b) Non collinear 2D-PE set-up based on 2D pulse shaping. Multiple phase-stable output beams are created and overlapped at the sample in a phase-matched boxcars geometry with a 2D pulse-shaper based on a 2D liquid crystal spatial light modulator (SLM in figure, while L1 and L2 stand for lenses, FP for focal plan and LO for local oscillator). With this approach, pulse timing can be modified without mechanical delay lines and shape, phase, and spectral content of each beam can be independently specified. Pulse shaping easily provides excellent phase stability among all output beams. Panel (a), reprint with permission from ref. 73, copyrights (2009) Optical Society of America. Panel (b) reprinted with permission from ref. 126; copyright (2007) American Chemical Society.](image-url)
conventional components, the collinear generation of pulse-shaped pulse pairs makes the implementation of a robust phase-stable set-up definitely easier, but at the cost of renouncing the capability of the independent manipulation of the beam features (in particular polarization\textsuperscript{142}), of a background free detection and of an optimal signal heterodyning. To benefit fully from a non collinear geometry and coherent generation and manipulation of the interaction fields, Nelson’s group implemented a 2D-PE experiment at 800 nm based on a 2D liquid crystal (LC) spatial light modulator (SLM), allowing an automated 2D phase and amplitude pulse shaping of non-collinear, fully phase-coherent fields (Fig. 11b). It provides a fully independent control of wavevectors, pulse timings and phases, as well as spectral content of each field. This approach can generate waveforms of high complexity as multiple pulses or chirped pulses, with a superior flexibility, even when compared with conventional 2D-PE set-ups. Unfortunately the LC SLM technology cannot operate in the UV. Micromirror SLM based on micro electro-mechanical systems (MEMS) has been proposed for femtosecond pulse shaping in the UV,\textsuperscript{143} showing a potential to push the 2D SLM scheme to the UV. However, the current MEMS SLM are still too low performing\textsuperscript{144} but recent developments on MEMS linear mirror arrays are promising.\textsuperscript{138}

As, to the author’s knowledge, there are no UV-2D spectrographs yet employing ultra-broadband sources either with non collinear geometry or with collinear pulse-shaped pulses, but now both technical obstacles are overcome and the theoretical studies clarified the effective potentials of these methodologies. In addition, recent success in obtaining UV homodyne photon-echo signals from UV dyes in different solvents\textsuperscript{9,64,70,71} definitively proves the feasibility of UV-PE experiments. Thus it is easy to envisage in the next years a proliferation of such studies and set-ups.

**Advancing on all-optical chemical sensitive spectroscopies**

Another context where coherent spectroscopies have proved to have important implication is in identifying chemical species with an all-optical approach. Standard vibrational spectra can, in principle, unequivocally recognize a (organic) molecule detecting in the so-called fingerprint region of the spectrum (500–2000 cm\textsuperscript{-1}).\textsuperscript{11,145} Coming to biological samples, congestion of the vibrational spectra grows exponentially with the molecule size and sample heterogeneity and the chemical sensitivity is dramatically restricted, as in case of non-resonant coherent techniques (as Coherent Anti-Stokes Raman Spectroscopy, see Fig. 3b), to classes of molecules, as proteins, lipids, and DNA.

The capability to provide both homogeneous lines and mutual anharmonic coupling makes 2D spectra a unique and easily-readable fingerprint of a protein. Indeed, while vibrational lines can just reveal the presence of a given amino acid in the sample, the presence of anharmonic coupling reports on the nature of the amino acids nearest to it. The capability to provide such rich structural information has been recently proved, making 2DIR techniques able to perform all-optical protein recognition.\textsuperscript{12,13} Remarkably, since they are nonlinear spectroscopies, the signal, as for multiphoton excitation microscopy, is depth-selective for 3D-imaging, when coupled to a microscope.\textsuperscript{11}

As nicely shown by UV-RR experiments, the resonance effect with UV pulses gives the impressive selectivity to pick the signal from a given amino acid off hundreds other present amino acids. The development of multidimensional experiments where UV pulses are exploited to retrieve vibrational couplings is one of the next directions where coherent UV spectroscopy could have an unforeseeable impact. In this respect a promising scheme for extension into UV is the so-called electron–vibrational–vibrational (EVV) scheme (Fig. 3b’).\textsuperscript{12,13} Here the first two pump pulses are in the IR region and are used to excite resonantly two different vibrational modes. The third pulse probes the polarization generated by the two IR beams. It is usually a visible, non-resonant beam such that the coupling strength of the two vibrations can be “read-out” by the detection of visible photons. Relevant to UV extension, when resonant with an electronic transition, the detected signal is multiplicatively enhanced, becoming a triply resonant interaction. This methodology was shown to be able to discriminate among ten different proteins with a very high confidence (>90%), and to quantify their content, with an impressive sensitivity limit of few pmol. It also has been found that the intensity of combination bands is very sensitive to chemical modification of the protein as e.g. phosphorylation, paving the way to characterize post-translation modifications of a protein. These chemical modifications occur after the protein translation and are strongly dependent on the cell environment. In this methodology, the chemical sensitivity mainly relies on the resonance selectivity of the first two pulses, and in case of over-crowed IR spectra, degradation in chemical-recognition would be unavoidable. To what extent the chemical-sensitivity suffers from the sample heterogeneity and molecular complexity would be spectacularly pushed further when the probe pulse is tuned to the UV. In this mixed scheme, where the first two pulses are still in the IR, signals from a specific subgroup of amino acids would be selectively enhanced. The recent advances in UV pulse shaping make possible even more sophisticated detection schemes, where the first two direct IR interactions could be replaced by pairs of UV interactions, which would coherently populate vibrational modes via coherent Raman excitation (Fig. 3d and e). This would enhance the chemical selectivity even more, since the populated modes would be the only ones coupled to the resonant electronic transition. Thanks to the novel pulse-shaping techniques, such a complicated interaction scheme, with five pulses interacting and coherently controlled, can be easily achieved, as was nicely shown by Silberberg and collaborators in the case of single-pulse coherently controlled nonlinear Raman spectroscopy.\textsuperscript{11,145}

**Conclusions**

The aim of this perspective is to cover recent developments in applications of ultrafast UV spectroscopy to the understanding of molecular dynamics, and to give an outlook for tomorrow’s research with these methodologies. Over the last years, femtosecond UV spectroscopy has taken a giant leap forward and now transient absorption, time gated emission,
resonant Raman and homodyne four-wave mixing set-ups are available down to $\lambda = 250$ nm. Looking at the near future, the time is now mature for the novel family of UV FT coherent spectroscopies, among which multidimensional photo-echo dominates.

Innumerable systems with optical transitions in the near- and deep-UV regions can now be investigated with real-time techniques or manipulated with optical coherent methods. This is by itself an extraordinary breakthrough that heralds an increasing interest in these techniques. Nevertheless, as demonstrated with examples from recent literature, their unique added value stems from the possibility to use as probes a variety of UV chromophores, most of them containing aromatic mono- or oligo-cyclic organic groups, for example aromatic amino acids, nucleotide bases, imides and (di)-imines. These species are constituents of biomolecules, many molecular devices and supra-molecular structures. Exploiting them as a probe opens a new window on the (thermo)dynamical properties of the macromolecule edifice in a site-specific fashion. They provide dynamical information on intramolecular electrostatics, fluctuations and local rigidity, structural changes, anharmonic coupling and long-range interactions as well as vibrational and electronic coherences.

The advantage of this novel family of UV chromophores is emphasized when phenomena triggered by visible light are investigated. Ultrafast UV spectroscopies can provide information not only on the directly photo-excited moiety but also on the hosting molecular edifice. They allow us moving our focus from the “epicentre” to the “periphery”, with the aim to track down concerted motions, long distance interactions, and modulation of local dynamics by changes in distant sites, and in general on the host edifice reaction. The capability of 2D spectra to provide a “global” spectral signature of the molecule under investigation paves the way to all-optical chemical-recognition methods and chemical sensitive microscopy.

Currently most of the research activity is bio-oriented, probably because this is the community where concepts as collective motion, functional dynamics, and long-range interactions were born and are at the very base of the comprehension process. However both these concepts and the techniques herein presented can naturally be extended to any macromolecule and supra-molecular systems. The capability to exploit probes transparent to the VIS radiation and sensitive to changes in the electronic state and charge localization gives these novel methodologies a great potential to become new insightful tools to develop photo-powered nanodevices, molecular electronics, efficient solar-light harvesters, or to understand elementary steps in self-assembling processes.

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Notes and references
