Comparison of Spontaneous and Coherent Raman Spectroscopy for Biophysical Analysis

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Academic Year 2016 - 2017
In collaboration with

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Abstract

Coherent anti-Stokes Raman scattering (CARS) microscopy is a non-invasive label-free ultrafast spectroscopy method based on intrinsic vibrational contrast and is characterized by high sensitivity, high spectral resolution and 3D-sectioning capability. The present work is based on its extended implementation called multiplex or broadband CARS (MCARS) in which two synchronized laser pulse trains of narrow-bandwidth pump and a broad-bandwidth Stokes pulse are used to simultaneously excite all Raman frequencies from 500 up to 3500 cm$^{-1}$, of particular interest in biological applications. The stimulated frequencies are the same as in spontaneous Raman spectroscopy but, due to the creation of coherent light, they are emitted with an intensity which is several order of magnitude higher. The main disadvantage of the technique is due to the presence of a non-resonant background that alters the spectral shape and the position of the resonance peaks or even mask them, limiting the sensitivity of the system. A solution to the problem is represented by the Maximum Entropy Phase Retrieval (MEPR) algorithm, able to estimate the phase of third order electrical susceptibility and to re-construct Raman spectra. The method has been successfully applied for the characterization of polymers, human epithelial tissue and chemical solutions containing the triple bond C≡N, these latter of particular interest in the biological field. It has been proved that MEPR is not only able to retrieve qualitative Raman information but also to perform quantitative (concentration) measures. The study of rats brain tissue samples has shown that the MCARS setup is able to distinguish healthy tissue from tumorous tissue. The last part of experimental work, particularly innovative, involves the monitoring of an enzyme-inhibitor reaction whose velocity depends on the pH of the solution. It has been observed that if the reaction is sufficiently slow, Raman spontaneous spectroscopy is able to record the evolution of the system; if the pH level increases, the reaction is accelerated and only a higher performance setup, such as the MCARS system, can detect real-time data.
Abstract (ITA)

Questo lavoro nasce da una collaborazione con il Physikalisch-Chemische Institut (Istituto di Chimica-Fisica) della Ruprecht-Karls-Universität di Heidelberg. Lo studio è incentrato sullo sviluppo, la caratterizzazione e l’applicazione di un setup sperimentale per la spettroscopia Raman coerente anti-Stokes a larga banda (multiplex-coherent anti-Stokes Raman scattering, MCARS). Attraverso l’utilizzo di due treni di impulsi laser ultrabrevi, sincronizzati in spazio e tempo, è possibile stimolare l’emissione di luce alle stesse frequenze anti-Stokes della spettroscopia Raman spontanea ma, data la creazione di onde coerenti, l’intensità ottenuta risulta diversi ordini di grandezza maggiore. Pertanto la spettroscopia MCARS si sta affermando velocemente come tecnica di imaging non distruttiva, non legata all’uso di coloranti e con tempi di acquisizione più veloci della spettroscopia Raman spontanea. Lo svantaggio principale della tecnica è dovuto alla presenza di un background non risonante che altera la forma spettrale e la posizione dei picchi di risonanza e può, eventualmente, oscurarli del tutto, limitando la sensibilità del sistema. Una soluzione al problema è rappresentata dall’algoritmo di ricostruzione di fase a massima entropia (Maximum Entropy Phase Retrieval, MEPR), in grado di stimare la fase della suscettività elettrica del terzo ordine e di ricostruire spettri Raman. Il metodo è stato applicato con successo per la caratterizzazione di polimeri, tessuto epiteliale umano e soluzioni chimiche contenenti il triplo legame C≡N, queste ultime di particolare interesse in campo biologico. È stato dimostrato che la MEPR è non solo in grado di ricostruire spettri Raman in modo qualitativo ma anche di effettuare misure quantitative (di concentrazione). Lo studio di campioni di tessuto cerebrale di ratti ha dimostrato che il setup MCARS è in grado di distinguere il tessuto sano da quello tumorale. L’ultima parte del lavoro sperimentale, particolarmente innovativa, riguarda il monitoraggio di una reazione enzima-inibitore la cui velocità dipende dal pH della soluzione. Si è osservato che se la reazione è sufficientemente lenta, la spettroscopia Raman spontanea è in grado di registrare l’evoluzione del sistema; se il pH aumenta, la reazione è accelerata e solo un setup con prestazioni più elevate, quale è il sistema MCARS, può rilevare dati in tempo reale.
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5.9 Time evolution of: (a) C≡N bond in CN-I1, (b) S-H bond in L-Cysteine, (c) C=N bond in the products. First measure take after 20 min since the beginning of the reaction. Vertical scale: intensity in arbitrary units.

A.1 Scheme of a grating spectrometer: ES = Entrance Slit; M1, M2 = mirrors; G = gratings; D = detector; in the dashed box: 1 and 2 = incoming rays; \(\alpha\) and \(\beta\) = angles respectively at the left (positive) side and at the right (negative) side of the normal to the grating surface; \(d\) = distance between two consecutive grooves.
Chapter 1

Fundamentals of spontaneous and coherent Raman spectroscopy

1.1 Introduction and motivation

It was 1928 when the physicist C.V. Raman announced the discovery of physical effect that carries his name with the brief article 'A New Type of Secondary Radiation' \[2\] for which he was awarded the Physics Nobel prize in 1930. Raman spectroscopy is a branch of vibrational spectroscopy that employs electromagnetic radiation ranging from near-infrared up to near-ultraviolet to study vibrational modes and it is based on a two-photon scattering effect. A fundamental characteristic of this method is that the measured signal from a sample, either gaseous, liquid or solid, is strictly connected to the specific physical and chemical structure of the molecule under investigation providing a unique spectrum (comparable to a fingerprint). In particular, Raman spectroscopy provides information about frequencies and linewidth of vibrational modes which are coupled to the ground state, and therefore it is classified as vibrational spectroscopy in stationary regime. The performances of this popular and versatile technique have been improved in the last 40-50 years, especially after the invention of the laser in the early 1960s.

The importance of Raman spectroscopy goes beyond the mere chemical identification. It provides a non-destructive non-invasive label-free investigation tool together with high three-dimensional spatial resolution achieved by coupling the laser source with a well-known confocal geometry. It is clearly advantageous with respect to fluorescence microscopy, especially in biological application where staining is not tolerated or proper dyes are not available. The resolution of Raman microscopy is higher than infrared (IR) spectroscopy which employs higher wavelengths; nevertheless this two investigation methods are based on different and mutual exclusive selection rules that can extract complementary information, so they are both cur-
ently studied. The success of Raman, and of vibrational spectroscopy in general, is also due to the possibility of using commercial devices for lots of practical applications without a deep understanding of the physical effect they are based on. However detailed studies of these effects not only lead to better data analysis but are also responsible for huge improvement in the techniques and the creation of new sub-related ones.

The simplest implementation of Raman spectroscopy, based on spontaneous Raman emission, has many applications in the fields of physics, chemistry and biology. Among them are: molecule identification (e.g.: in art and archaeology [3], proteins classification [4], forensic analysis [5], biological tissue [6, 7]), structure elucidation (e.g.: graphene and graphite [8], dentistry [9], carbon nanotubes [10]), reaction monitoring (e.g.: food analysis and inspection [11]), quality control (e.g.: water monitoring [12]) and others. Raman scattering has many advantages compared to other techniques: high spatial resolution, minimum sample preparation, reduced influence of water bands (especially compared to infrared spectroscopy), low damage if near-infrared radiation is used.

However the technique is not lacking of drawbacks. The primary disadvantage of using the spontaneous Raman signal is its extremely small cross-section when compared to Rayleigh or fluorescence scattering (coming either from impurities or the sample itself). In the best case only one photon over $10^6$ is spontaneously Raman scattered [1]. Besides fluorescence signal and spontaneous Raman Stokes signal are spectrally overlapping. Therefore they cannot be separated using optical filters. Because of this, relevant peaks can be hidden or there can be, in general, a decreased sensitivity. Several fluorescence suppression methods have been developed to overcome this limitation and are based on the fact that the two contributions have different physical origins [13]. Other techniques have been implemented with the purpose of improving both signal and spatial resolution. In this regard it is worthy to mention surface-enhanced Raman scattering (SERS) which has gained popularity during the last years as the cross-section enhancement factor can be up to $10^{14} - 10^{15}$ [14]. This method, however, requires a correct and non-trivial choice of the nanostructured substrate to be deposited on the sample; besides this extra layer lacks of reproducibility and so does the technique. Tip-enhanced Raman spectroscopy (TERS), which unites SERS with scanning probe microscopy (SPM), achieves very high spatial resolution, even below the diffraction limit, but is subject to several tip-related issues [15].

An innovative approach to chemical selective imaging based on vibrational contrast has been developed in the field of ultrafast spectroscopy by coherent Raman
scattering (CRS) techniques which can produce much stronger signals. In particular, the work of this thesis is based on the development and the application of a broadband or multiplex-CARS (Coherent anti-Stokes Raman Scattering) setup, in which three incoming femtosecond light pulses undergo a nonlinear optical interaction and anti-Stokes Raman frequencies will be emitted from the sample. The fundamental difference is that the creation of a coherent Raman radiation results in increased signal levels. An M-CARS microscope collects potentially the same information that can be obtained with a spontaneous Raman microscope but with a signal order of magnitude that can be up to five times higher \cite{16}, leading to a faster scanning capability. Photobleaching and damage are suppressed as there is no population in the excited molecular states (the process involves only virtual states). M-CARS is more efficient than second or third harmonic generation (SHG, THG) in which the signal is non-resonant so it cannot provide both contrast and chemical selectivity at the same time. Sum-frequency generation (SFG) provides vibrational contrast but is only interface-sensitive. Besides CARS signal generation needs a very high excitation intensity which can only be achieved in the focal volume of a tightly focused beam provided by today state-of-the-art Ti:Sapphire laser. This implies that the 3D-sectioning capability, on a sub-micrometer scale, is intrinsic and there is no need for a confocal geometry. Finally the blue-shifted CARS signal lies on a spectral interval that can be easily isolated from other coexisting effects and doesn’t overlap with any fluorescent signal. Single-beam CARS (probing only one vibrational mode) was first reported in 1965 by Maker and Terhune \cite{17}. The availability of broad-bandwidth pulses lead to the possibility of exciting multiple Raman resonant frequencies at once and was first observed in 1974 \cite{18}. MCARS is nowadays a well-known experimental technique used in many biological \cite{19} and non-biological applications.

There is anyway an important issue to be solved: the output signal is nonlinearly mixed with a so-called non-resonant background (NRB), which doesn’t carry any relevant chemical information and arises from the electronic contribution. The NRB not only causes a small shift in the anti-Stokes emitted frequencies but can sometimes overwhelm relevant peaks. Consequently, several techniques have been developed to overcome this problem. Some of them exploit a post-acquisition reconstruction algorithm which is capable of retrieving the theoretical Raman signal \cite{20,21}. This is the main approach followed in the present work. Others are based on a modification of the optical setup itself so that the background is significantly reduced (a good overview is given in \cite{16}). An important method from this second group is known under the name of time-resolved CARS (tr-CARS) and exploits different temporal
behaviour of resonant and non-resonant signal components. This implementation has also been proposed as capable of chemically selective imaging exploiting Raman free induction decay [22]. This approach, not faced in detail in this thesis, has a huge potential because it gives not only faster but also higher chemically selective information respect to a spontaneous Raman microscope, especially when applied to distinguish very similar molecules [23].

After introducing the theoretical basis necessary to understand spontaneous and coherent Raman scattering (Chapter 1) a description of the setups will be provided (Chapter 2) with a particular focus on the experimentally implemented MCARS. Chapter 3 deals with the problem of retrieving chemically selective information from MCARS spectra. This means, basically, finding a way to retrieve the Raman spectrum. A first method, the maximum entropy phase retrieval (MEPR) algorithm (MEPR) has been used for this purpose and compared with time-resolved CARS. The MEM has been also proved to be able to extract quantitative concentration from a nonlinear optical signal. Thereafter two main applications of M-CARS have been investigated. The first is the chemically selective imaging of a mouse brain tissue in which spectra of an healthy and tumour area have been identified (Chapter 4). This application is not new itself but the measured data, processed using a maximum entropy phase retrieval algorithm, show peculiar agreement with the data obtained from the same sample with a spontaneous Raman microscope. The second application is a study on a specific enzyme-inhibitor reaction in a pH-controlled environment (Chapter 5). With this study it has been possible to prove that MCARS is advantageous respect to spontaneous Raman spectroscopy when applied to fast chemical reactions.
1.2 Spontaneous Raman scattering

In a light scattering process an incoming photon, of energy $h\nu_0$ (with $\nu_0$ photon frequency) distorts the electron cloud of a molecule and promotes the entire system to a higher energy state called virtual state. This new state is not stable and the photon is immediately re-emitted. Scattering is very different from absorption. First of all, there is no promotion of electrons to the excited states. Besides the energy of the radiation doesn’t have to match the difference $\Delta E$ between two energy levels to be scattered ($h\nu_0 \neq \Delta E_{molecule}$). The energy of the virtual state is related to both the photon energy (frequency) and the structure of the molecule. The most intense scattering effect is Rayleigh scattering (fig. 1.1(a)) and it occurs when the electron clouds relaxes without involving any nuclear motion (elastic scattering). The photon energy $h\nu_0$ is unvaried. In Raman scattering, instead, a photon is inelastically scattered by a molecule, which is initially in its ground state, and exits the material with a lower energy. The emitted radiation is called Stokes radiation (fig. 1.1(b)). If the molecule is already in the first excited vibrational level then the radiation can be emitted with a higher energy (frequency) and is referred to as anti-Stokes (fig. 1.1(c)). The extra energy is transferred from the molecule. The quantum yield of these scattering processes is very low: only one photon in a million undergoes

![Figure 1.1: (a) Rayleigh scattering (b) Stokes Raman scattering (c) Anti-Stokes Raman scattering (d) Infrared absorption. Full horizontal lines represent vibrational levels, dashed horizontal lines represent virtual states (ground state and first excited state); $h\nu_0$ is the energy of the incoming photon, $|g\rangle$ and $|\nu\rangle$ are respectively the energy of the vibrational ground state and first excited level. The spontaneously emitted Raman photons are represented by dashed lines.](image-url)
Rayleigh scattering; spontaneous Stokes Raman process is observed in one photon over $10^{10}$ up to $10^{12}$ \cite{24}. Being the molecules preferably in the ground state at room temperature the anti-Stokes Raman intensity is lower than the Stokes one. It is of fundamental importance to understand that the emitted frequencies are, in any case, an intrinsic characteristic of a molecule and are strictly related to its vibrational modes. For this reason every molecule has its own and unique Raman spectrum. This experimental technique is different respect to infrared (IR) spectroscopy in which the missing frequencies of the transmitted radiation (that means the absorbed one) are analysed and correspond to precise bonds vibrations called modes or vibrational frequencies (fig. 1.1(d)). However, an infrared spectrum is intrinsically related to the molecule and chemically selective as well as a Raman one. The difference consists in the fact that Raman and infrared active modes respond to different and opposite selection rules. Now the Raman selection rule will be recalled according to a classical approach.

As the electrons are bond to the nuclei, the presence of nuclear modes is always perturbing the electron motion and consequently the electronic polarizability $\alpha$ which quantifies the ability of a molecule to form instantaneous dipoles and depends on the instantaneous position of the electrons. When a linearly polarized electromagnetic radiation is interacting with a sample, under the assumption of a driving frequency far away from any electronic resonance, an induced dipole moment will be created:

$$\mu(t) = \alpha(t)E(t) = \alpha(t)E_0\cos(2\pi\nu_0 t) \quad (1.1)$$

where $\alpha$ is the molecule polarizability and $E(t)$ is the intensity of the time-varying electromagnetic field of frequency $\nu_0$. For arbitrarily polarized light $\alpha$ becomes a tensor. If we consider, hypothetically, an absence of nuclear modes and nonlinearities, the polarizability could be approximated with a constant $\alpha_0$. Introducing the nuclear modes we can then expand it in a Taylor series up to the first order:

$$\alpha(t) = \alpha_0 + \left(\frac{\partial \alpha}{\partial Q}\right)_0 dQ(t) \quad (1.2)$$

where $dQ$ is the nuclear displacement of atoms from their equilibrium position, which can be written exploiting the model of the harmonic oscillator, obtaining:

$$dQ(t) = Q_0\cos(2\pi\nu_r t + \phi) \quad (1.3)$$

where $Q_0$ is the amplitude of the nuclear motion, $\nu_r$ is the nuclear resonance frequency and $\phi$ is the phase of the nuclear mode vibration. Uniting the previous equations
and exploiting the fact that the electric field can be written as:

$$E(t) = A \exp(-i\omega_0 t) + A \exp(i\omega_0 t)$$

the dipole moment can be written as:

$$\mu(t) = \alpha_0 A e^{-i\omega_0 t} + A \left( \frac{\partial \alpha}{\partial Q} \right)_0 Q_0 \{ e^{-i(\omega_0 - \omega_r) t + i\phi} + e^{-i(\omega_0 + \omega_r) t - i\phi} \}$$

If we look at the right-hand side of eq. 1.5, the first term represents Rayleigh scattering. It is interesting to note that the phase term does not appear in this term: Rayleigh scattered light is coherent. The second term describes the Stokes ($\omega_0 - \omega_r$) and anti-Stokes ($\omega_0 + \omega_r$) contribution and involves phase terms. This phase $\phi$ is dependend on the nuclear motion. At equilibrium nuclear motions are uncorrelated and so are their phases. Consequently the Raman scattered radiation is incoherent. From eq. 1.5 it is evident that, for Raman scattering to be able to occur, the radiation shall induce a change in the polarizability of the molecule:

$$\frac{\partial \alpha}{\partial Q} \neq 0$$

To be infrared active, instead, a vibrational mode should provoke a change in the dipole moment. It is possible to properly describe all possible complex molecule vibrations, and predict which one is Raman active and which is infrared active. It is not in scope of this text to explore this into details. In this regard it is worthy to mention the mutual exclusion rule, which states that, in a molecule with a center of symmetry, vibrations that are Raman active will not be infrared active and vice versa. Nonetheless, in general, symmetric vibrations show intense Raman spectral line and asymmetric vibrations are infrared active. It is now clear how this two investigation tools exploit different and mutually exclusive selection rules and provide complementary information about molecular structure. That is the reason why the development of both techniques is subject of current research. The intensity of the Stokes Raman radiation can be written:

$$I(\omega_s) = \frac{\omega_s^4}{12\pi\epsilon_0 c^3} Q_0^2 I_0 \left| \frac{\partial \alpha}{\partial Q} \right|^2$$

where $I_0$ is the intensity of the incoming radiation, $c$ is the speed of light and $\epsilon_0$ is the vacuum permittivity. The signal can be also explicitly written depending on the

\footnote{This is done by means of the so called group theory, studying the symmetry properties of the molecules and classifying them into point group, see for example [25].}
cross-section:

\[ I(\omega_s) = Nz\sigma(\omega_s)I_0 \]  

Comparing eq. 1.7 and 1.8 it is evident that the material cross-section is proportional to the variation of the polarizability.

1.2.1 Raman spectroscopy

Raman spectroscopy is a well-known and widely used technique. It can be used with solid, liquid and gaseous samples. Commercial Raman microscopes are nowadays available on the market and produced by several companies. Previous eq. 1.7 and 1.8 show that spontaneous Raman is a linear spectroscopy technique, as the relationship between the output intensity and the input signal is linear. The signal is also linear with respect to the density \( N \) of the scatterers, which means Raman signal can be a way to quantify this density. Due to the low quantum yield of the exploited effect, the invention of the laser in the 70s played a fundamental role in the development of this technique making the measurements possible in better conditions. A fast improvement followed: better signals, faster acquisition time per spectra, compact devices. The two main parameters that can be chosen in a Raman microscope are the laser frequency and the laser power. To obtain Raman excitation it is possible to use UV radiation (down to 200nm) up to infrared radiation (Nd:YAG laser line at 1.06\( \mu \text{m} \)). The main disadvantage of using visible light for excitation is the presence of a sometimes overwhelming fluorescent signal which comes partially from the sample itself and partially from external contaminations. Since the scattering depends on the fourth power of the frequency (see eq. 1.7) an ultraviolet (UV) source would give the highest sensitivity the least fluorescence and the least sample degradation. However, many compounds absorb UV radiation and this means that there is a high risk of sample degradation simply through burning. Besides, the quality of the optics required to handle UV radiation is very high. Therefore the most common choice is still to use visible radiation. A typical Raman Stokes spectrum, taken from a sample of polyethylene terephthalate (PET), is shown in fig. 1.2. Raman peaks are sharp and shown as a shift respect to the incoming radiation energy, expressed in wavenumbers:

\[ \tilde{\lambda}_{\text{shift}} = \frac{1}{\lambda_{\text{shift}}} = \frac{1}{\lambda_{\text{laser}}} - \frac{1}{\lambda_{\text{Stokes}}} \quad [\text{cm}^{-1}] \]  

\(^2\)However the classical model does not explain how \( \alpha \) is connected to the resonance behaviour. In other words: why we only see strong vibrations only if the difference between the emitted signal and the incident one is matching a vibrational frequency mode? A detailed answer is provided only by the semi-classical and full-quantum approach (see for example [26 ]

\[ \tilde{\lambda}_{\text{shift}} = \frac{1}{\lambda_{\text{shift}}} = \frac{1}{\lambda_{\text{laser}}} - \frac{1}{\lambda_{\text{Stokes}}} \quad [\text{cm}^{-1}] \]
which means the better the quality of the monochromatic light the better the resolution. The interpretation of Raman spectra is not straightforward. There is plenty of scientific literature (databases) which shows and classifies thousands of Raman spectra of known compounds or helps the interpretation of new ones. Several simulation software help to predict the molecule vibrations and the theoretical wavenumbers at which it shall be observed; other software interpretation tools can compare the acquired spectrum with an internal database to identify the substance. Moreover there are some general criteria that can be followed to identify different vibrations in different spectral area. In particular the area ranging from 400 up to 1500 cm$^{-1}$ is called fingerprint area (fig.1.2 (a)). Inorganic molecules have usually strong vibrations in this part of the spectrum. The vibrations in this spectral interval are mainly due to the molecule backbone. In the shown example it mainly consists of aromatic ring vibrations. Double and triple bonds are usually localized respectively in in the intervals 1500-1800 cm$^{-1}$ and 1800-2500 cm$^{-1}$ (fig.1.2 (b), (c)). For PET we have C=O and C=C double bonds vibrations. The H-stretching vibrations appear in the interval after 2500 in case of aqueous solutions this interval is practically overwhelmed from the water signal.

![Raman spectrum of a polyethylene terephthalate (PET) sample with excitation wavelength of 532nm, dwell time: 1s, average over 20 acquisitions](image)

Figure 1.2: Raman spectrum of a polyethylene terephthalate (PET) sample with excitation wavelength of 532nm, dwell time: 1s, average over 20 acquisitions (a) Fingerprint area, (b) Localization of double bonds vibrations, (c) localization of triple bonds vibrations, (d) H-stretch area, (e) PET molecular structure.

A spectrum of the quality like the one shown in fig. 1.2 is obtained averaging 20 spectra with an acquisition time per single spectrum of 1 s. This means that the
total acquisition time is 20 s. Other kind of samples can require even higher global acquisition time due to several reasons, for example a lower density of scatterers or a high light dispersion due to a very inhomogeneous sample surface. For certain applications it can happen that a Raman microscope is not able to detect the desired vibration because either it is masked by a strong fluorescent signal or the cross-section is too small. It is clear then that a further evolution in Raman vibrational spectroscopy would benefit from both generation of higher signal levels and fluorescence suppression. This is precisely the purpose of coherent Raman scattering techniques, described in section 1.4.

1.3 Ultrashort pulses and nonlinear spectroscopy

It was 1991 when the discovery of self-mode-locked lasers (Sibbett et al. [27]) made available few-cycle-duration optical pulses, called ultrashort pulses. Since then it has been possible to develop time domain vibrational spectroscopy techniques that are able to induce and detect events that have a duration of pico and femtoseconds. Coherent Raman techniques represents a sort of evolution of classical Raman spectroscopy and are based on the use of this ultrashort pulses. The following paragraph describes the main characteristics and parameter of this pulses. Paragraph 1.3.2 gives an introduction about nonlinear optics with a focus on four-wave mixing effect, which is the base to understand MCARS.

1.3.1 Ultrashort pulses description and propagation

A pure monochromatic electromagnetic wave can be written, using complex notation, as:

$$E(t) = E_0 e^{i\omega_0 t}$$  \hspace{1cm} (1.10)

which represents an unlimited cosine function, whose angular frequency is $\omega_0$. A single light pulse can be obtained multiplying the cosine by some shaping function, called envelope. From a computational point of view it is easier to show the effects that an ultrashort pulse encounters during propagation considering a pulse with a Gaussian profile:

$$E(t) = E_0 e^{(-\Gamma t^2+i\omega_0 t)}$$  \hspace{1cm} (1.11)

where $\Gamma$ is the shape factor of the Gaussian envelope. When referring to the pulse duration the full width at half maximum (FWHM) is used, which in this case is $\Delta\tau = s\sqrt{\ln 2(\Gamma)^{-1/2}}$. The pulse instantaneous frequency $\omega_i(t)$ is defined as the
time-derivative of its temporal phase \( \varphi \), which is:

\[
\omega_i(t) = \frac{\partial \varphi}{\partial t} = \omega_0
\]  

(1.12)

It is possible to obtain the pulse spectrum taking the Fourier transform of eq. 1.11:

\[
E(\omega) = \int_{-\infty}^{+\infty} E(t) \exp(-i\omega t) dt = \exp\left[\frac{-(\omega - \omega_0)^2}{4\Gamma}\right]
\]  

(1.13)

where the spectrum has again a Gaussian shape and it is centred at \( \omega_0 \). The pulse angular frequency bandwidth, expressed again as FWHM, is now: \( \Delta \omega = 4\sqrt{\Gamma \ln 2} \). It can be shown that pulse duration and its spectral bandwidth are, in general, related and satisfy the following inequality:

\[
\Delta \tau \Delta \omega \geq \frac{1}{2}
\]  

(1.14)

which has very important consequences when working with ultrashort pulses. The shorter the required pulse is the broader the necessary bandwidth. The equality in eq. 1.14 holds only for a Gaussian shaped pulse, called transform limited (TL) pulse. For a given symmetrical spectrum there exist only one pulse that can be TL. Experimentally, \( \Delta \nu \) is preferred over \( \Delta \omega \) and the relationship eq. 1.14 becomes:

\[
\Delta \tau \Delta \nu \geq K
\]  

(1.15)

where \( K \) is a constant depending on the pulse shape. In particular, for TL Gaussian pulses \( \Delta \tau \Delta \nu = 0.441 \). If a TL Gaussian pulse crosses a transparent medium, after a propagation of \( L \) in the z direction, its spectrum becomes:

\[
E(\omega, z) = \exp\left(\frac{-(\omega - \omega_0)^2}{4\Gamma}\right) \exp[-ik(\omega)L]
\]  

(1.16)

where \( k(\omega) = \frac{n(\omega)\omega}{c} \) and the refractive index is as well frequency dependent. The quantity \( k(\omega)L = \phi(\omega) \) is called spectral phase. It represents the phase of each frequency of the pulse and it is zero for TL Gaussian pulses. To calculate analytically the effects of the propagation it is useful to Taylor expand the spectral phase around the carrier frequency at \( \omega_0 \):

\[
\phi(\omega) = \phi'(\omega_0) + \frac{1}{2}\phi''(\omega_0)(\omega - \omega_0)^2 + \frac{1}{6}\phi'''(\omega_0)(\omega - \omega_0)^3 + ...
\]  

(1.17)

an expansion up to the third order is usually sufficient (unless very broadband pulses are studied).

In eq. 1.17
• $d\phi/d\omega = \tau_g(\omega)$ is called group delay and it represents the arrival time of a very narrowband pulse (quasi-monochromatic wavepacket) at frequency $\omega$.

• $D_2 = \phi''(\omega_0) = k''(\omega)L$, where $L$ is the propagation length, is called second-order dispersion or group delay dispersion (GDD), measured in $fs^2$.

• $D_3 = \phi'''(\omega_0)$ is called third-order dispersion (TOD).

Exploiting its definition it is possible to write the group delay as:

$$\tau_g(\omega) = \phi'(\omega_0) + D_2(\omega - \omega_0) + \frac{1}{2} D_3(\omega - \omega_0)^2 + O(\omega^3) \quad (1.18)$$

In an ideal non dispersive medium, i.e. a medium in which the refractive index does not depend on the wavelength ($n(\omega) = \text{const.}$), being $\phi(\omega) = k(\omega)L = \frac{n(\omega)}{c}\omega L$, it will be: $D_2 = D_3 = 0$. In this case the pulse is called transform limited (TL) and it is the pulse with the shortest duration which is compatible with a specific frequency spectrum (fig. 1.3 (a)).

If one of the dispersion terms is nonzero, then different spectral components will arrive at different times. The pulse is said to be temporally-chirped. In particular if $D_2 > 0$ the pulse is positively-chirped (blue component will arrive after the red ones); if $D_2 < 0$ the pulse is negatively-chirped (red component will arrive after the blue ones (fig. 1.3 (c),(d))). In the visible frequency range second order dispersion is negative in all materials. Only in the near-infrared $D_2$ starts to be negative [28].

Substituting eq. 1.17 into eq. 1.16 and trasforming back to time domain it can be calculated [29] that the output temporal shape, if observed in the temporal frame of reference moving with the group velocity of the carrier wave, is:

$$E_{out}(t) = \frac{2\pi A_0 \tau_p}{(\tau_p^4 + D_2^2)^{1/2}} \exp\left(-\frac{t^2}{2\tau_{out}^2}\right) \exp[i\phi_{out}(t)] \quad (1.19)$$

where $A_0$ is the pulse envelope at $t=0$ and:

$$\tau_p = \frac{\Delta \tau}{2\sqrt{\ln 2}} \quad (1.20)$$

$$\tau_{out} = \tau_p \left[ 1 + \left( \frac{D_2}{\tau_p^4} \right)^2 \right] \quad (1.21)$$

and

$$\phi_{out}(t) = \omega_0 t - \phi(\omega_0) + \frac{D_2 t^2}{2(D_2^2 + \tau_p^2)} \quad (1.22)$$
eq. 1.21 and 1.22 show that, after propagating in a dispersive medium, the pulse experiences a temporal broadening (which is not dependent on the sign of $D^2$) caused by a a time-varying temporal phase((fig. 1.3, (b)). The instantaneous frequency is, by definition:

$$\omega_i = \frac{d\phi_{out}(t)}{dt} = \omega_0 + \frac{D_2t}{D_2^2 + \tau_p^2}$$

(1.23)

in which it is evident how the frequency increases or decreases with the time, depending on the sign of $D_2$. Because the time dependence of the instantaneous frequency is linear with the time, the acquired chirp is as well linear.

The possibility of controlling, and in particular of reducing, pulse dispersion is fundamental for short pulses application and a nontrivial problem. There are many
techniques developed to achieve this control, a good compendium of which is given in [28].

1.3.2 Ultrafast nonlinear optics

The effects described in the previous section are generated by the linear response of the medium to the incoming radiation. In low intensity source regime the light-matter interaction is linear, which means that we can describe the optical properties of the material with parameters (e.g. refractive index) which depend only on the medium itself. When the radiation intensity is higher, for example in the case of laser emitted light, the interaction with the electromagnetic field, becomes nonlinear so the main optical properties of the medium depend on the radiation intensity. It shall be clarified that when the expression linear medium is used, we mean that we are in the first situation but potentially the same medium can show nonlinear effects: the intensity is just too low to observe them. When, instead, we use the term nonlinear medium we mean a medium in which the light intensity is so high that nonlinear effect can be observed, but they still coexist with linear ones.

When electromagnetic radiation interacts with a medium, its electric field component interacts with the charged particles of the material. Because visible and near-infrared light oscillates at frequencies on the order of $10^3$ THz the driving frequencies are too fast for the nuclei to move, therefore only the electron will answer to the rapid radiation. As a result of the external driving field the electrons are displaced from their equilibrium position and a dipole moment will be generated: $\mu(t) = -e r(t)$ where $e$ is the electronic charge and $r(t)$ the charge separation distance. The material is globally described by a macroscopic polarization $P$, obtained by multiplying the dipole moment by the molecule density, obtaining $P(t) = N\mu(t)$. This polarization can be decomposed in the sum of a linear (L) and a nonlinear (NL) components:

$$P = P_L + P_{NL}$$ (1.24)

Considering only the linear part, starting from Maxwell equations in the case of a non-magnetic medium, in absence of free currents and charges we can write, for a linearly polarized pulse in planar wave approximation the following equation:

$$\frac{\partial^2 E}{\partial z^2} - \frac{1}{c^2} \frac{\partial^2 E}{\partial t^2} = \mu_0 \frac{\partial^2 P_L}{\partial t^2}$$ (1.25)

where $c = \sqrt{\mu_0 \varepsilon_0}$ is the speed of light in vacuum, $z$ is the propagation direction and $E(z,t)$ is the electric field. It is evident how $P(z,t)$ acts as a driving term for the
electric field which, generalizing the expression of equation 1.11, can be written as:

\[ E(z,t) = A(z,t)\exp[i(\omega_0 t - k_0 z)] \]  

(1.26)

where \( A(z,t) \) is the slowly varying pulse amplitude (envelope), \( \omega_0 \) is the carrier frequency, \( \varphi(t) \) is the temporal phase and \( k_0 \) the wave vector. The linear polarization can be written in a similar way as:

\[ P(z,t)_L = p(z,t)\exp[i(\omega_0 t - k_p z)] \]  

(1.27)

where \( k_p \) is the wave vector of the polarization. In case of linear polarization \( k_p = k_0 \).

In the limit of weak applied electric field the polarization \( P(t) \) is proportional to \( E(t) \) and can be written:

\[ P(t) = \epsilon_0 \chi^{(1)} E(t) \]  

(1.28)

where \( \chi^{(1)} \) is the first order (linear) dielectric susceptibility, related to the linear refractive index according to:

\[ n_L(\omega) = \sqrt{1 + \chi^{(1)}} \]  

(1.29)

In a medium with no free charges or currents and no magnetization, under slowly varying envelope approximation (SVEA)\(^3\) it can be shown that the pulse envelope \( A(z,t) \) of a linearly polarized pulses satisfies the parabolic equation:

\[ \frac{\partial A}{\partial z} - \frac{i}{2} k_0^2 \frac{\partial^2 A}{\partial t^2} = 0 \]  

(1.30)

the pulse properties are modified after propagation as discussed in section 1.3.1.

To analyse nonlinear effects and enter the nonlinear optics regime, both components of the polarization must be taken into account and the starting equation, instead of eq. 1.25, is:

\[ \frac{\partial^2 E}{\partial z^2} - \frac{1}{c^2} \frac{\partial^2 E}{\partial t^2} = \mu_0 \frac{\partial^2 P_L}{\partial t^2} + \mu_0 \frac{\partial^2 P_{NL}}{\partial t^2} \]  

(1.31)

where the expression for \( P_{NL} \) is:

\[ P_{NL}(z,t) = p_{NL}(t)\exp[i(\omega_0 t - k_p z)] \]  

(1.32)

where \( p_{NL} \) is the polarization envelope. It is important to underline that in the expression of the nonlinear polarization the wave-vector \( k_p \) is, in general, different respect to the one of the electric field. Applying again a SVEA on \( p_{NL}(t) \), it is possible to find (see e.g. [30]) an equation describing the spatial and temporal evolution.

\(^3\)Neglecting variations of the envelope over propagation lengths of the order of the wavelength.
of the electric field envelope $A(z,t)$ in nonlinear regime:

$$\frac{\partial A}{\partial z} + \frac{1}{v_{g0}} \frac{\partial A}{\partial t} - \frac{i}{2} k_0^2 \frac{\partial^2 A}{\partial t^2} = -i \frac{\mu_0 \omega_0 c_0}{2 \epsilon_0} p_{NL} \exp[-i \Delta k z]$$

(1.33)

the quantity $\Delta k = k_p - k_0$ is called wave-vector mismatch between the nonlinear polarization and the electric field. In case of nonlinear effects the displacement of the electron has a nonlinear dependence of the electric field so the total polarization is better described as a power series expansion:

$$P(t) = \epsilon_0 \left[ \chi^{(1)} E(t) + \chi^{(2)} E(t)^2 + \chi^{(3)} E(t)^3 + \ldots \right] =$$

$$P^{(1)}(t) + P^{(2)}(t) + P^{(3)}(t) + \ldots$$

(1.34)

where $\chi^{(2)}$ is the second-order nonlinear susceptibility, responsible for second-order nonlinear effects, like second harmonic generation (SHG), sum frequency generation (SFG), difference frequency generation (DFG). It exists only in non-centrosymmetric crystals (as every even order effect); $\chi^{(3)}$ is the third-order nonlinear susceptibility and it is responsible for third-order nonlinear effects, which are observable in all transparent materials. Considering that both $E$ and $P$ are vectors, the $n$-order susceptibility is actually an $n$-1 order tensor. Third order nonlinear susceptibility is responsible for several interactions; for the scope of this thesis only the so-called four-wave mixing effect, involving three incoming electric field, will be analyzed.

The quantities $P^{(2)}(t)$ and $P^{(3)}(t)$ are called respectively second and third order polarization. It must be noticed that writing equations like 1.34 and 1.28 implies that the polarization at time $t$ depends on the value of the electric field at the exact same time. This assumes an instantaneous response by the medium and implies that it must be also dispersionless and lossless (see e.g. [31]).

As already explained at the beginning of this section, nonlinear effect require high field intensities to be observed. To be specific we are interested in coherent Raman scattering, which is a third-order nonlinear effect so it is based on the magnitude of $\chi^{(3)}$. We would expect that applying an electric field on the order of the atomic field implies $P^{(3)}(t) \sim P^{(1)}(t)$, which is true for intensities on the order of $\sim 10^{14} W cm^{-2}$. Nevertheless it is already possible to detect third-order response with input intensities only around $\sim 10^{10} W cm^{-2}$. Therefore even if orders of magnitude lower than the first order, they will be detectable and even suitable for applications in microscopy (especially biology).
1.3.3 Four-wave mixing

The third-order nonlinear polarization can be written as (from eq. 1.34):

\[ P_{NL}^{(3)}(t) = \epsilon_0 \chi^{(3)} E(t)^3 \] (1.35)

This polarization is induced by an electromagnetic field composed by up to three different frequencies:

\[ E(t) = E_1 e^{i(\omega_1 t - k_1 z)} + E_2 e^{i(\omega_2 t - k_2 z)} + E_3 e^{i(\omega_3 t - k_3 z)} + c.c. \] (1.36)

and the phenomenon is called four-wave mixing. By plugging in eq. 1.36 into eq. 1.35 the explicit form of \( E^3(t) \) contains 22 different frequencies (44 if considering positive and negative frequencies separates). The generated frequencies are:

\[ \omega_1, \omega_2, \omega_3, 3\omega_1, 3\omega_2, 3\omega_3, \]

\[ \omega_1 + \omega_2 + \omega_3, \omega_1 + \omega_2 - \omega_3, \omega_1 + \omega_3 - \omega_2, \omega_2 + \omega_3 - \omega_1, \]

\[ 2\omega_1 \pm \omega_2, 2\omega_1 \pm \omega_3, 2\omega_2 \pm \omega_1, 2\omega_2 \pm \omega_3, 2\omega_3 \pm \omega_1, 2\omega_3 \pm \omega_2 \] (1.37)

we can gather this in one formula and say that the nonlinear polarization occurs at frequencies: \( \omega_{FWM} = \pm \omega_i \pm \omega_j \pm \omega_k \) for \( i, j, k = 1, 2, 3 \) and FWM stands for four-wave mixing. In particular, the mechanism in which \( \omega_{FWM} = 3\omega \) is called third harmonic generation (THG). If the frequency difference \( \omega_1 - \omega_2 \) of two of the incoming laser fields is tuned to a resonance with a Raman active mode at \( \Omega \) the process \( \omega_{FWM} = \omega_1 - \omega_2 + \omega_3 = \Omega + \omega_3 \) is called coherent anti-Stokes Raman scattering (CARS). We can express eq. 1.35 indicating the variable each quantity depends on:

\[ P_{FWM}^{(3)} = \chi^{(3)} (\omega_{FWM}, \omega_1, \omega_2, \omega_3) E(\omega_1) E(\omega_2) E(\omega_3) \] (1.38)

With no depletion approximation of the pump (input) fields, it is possible to solve a SVEA equations system (basically exploiting eq. 1.33) to obtain the explicit expression for the envelope of the i-th Cartesian component of the FWM field (see e.g. [32]):

\[
[A_{FWM}(z)]_i = -\frac{2\pi \omega^2_{FWM}}{k_{FWM} \cdot c^2} \chi^{(3)} \cdot A_{1j} A_{2k} A_{3l} \exp\left(\frac{i\Delta k z}{2}\right) \times \\
\exp(-\alpha_{FWM} z) \cdot \frac{\sin\left(\frac{\Delta k z}{2}\right)}{\Delta k} z
\] (1.39)

where \( c \) is the speed of light, \( A_{1j}, A_{2k}, A_{3l} \) are the \( j,k,l \)-th Cartesian components of the input fields, \( z \) is the propagation length inside the material, and the wave-vector
has been expressed as a complex quantity to take wave damping into account, so it is globally written as:

\[ k_{FWM} = k_{FWM}' + i\alpha_{FWM} \]  

(1.40)

the \( \Delta k \) is:

\[ \Delta k = k_1' + k_2' + k_3' - k_{FWM}' + i(\alpha_1 + \alpha_2 + \alpha_3 - \alpha_{FWM}) \]  

(1.41)

and it is called wave-vector mismatch (in the z-component). If \( \Delta k = 0 \) we have the so-called phase matching condition. In phase matching there is the highest possible efficiency for the generation of \( \omega_{FWM} \).

### 1.4 Coherent Raman Scattering (CRS)

The spontaneous Raman signal, described in section 1.2, is an incoherent signal. The phase of the wave emitted by a single molecule is not correlated with the others in the sample. This is due to the fact that the system input is given by a single pumping frequency \( \omega_p \) and a second frequency, \( \omega_s \), is spontaneously emitted (fig.1.4 (a)).

Coherent Raman scattering is a parametric four-wave mixing process. It exploits a type of light-matter interaction in which two incoming synchronized train of pulses at \( \omega_p \) and \( \omega_s \) (\( \omega_p > \omega_s \)) are incident on the sample and the material response depends on their difference \( \Omega = \omega_p - \omega_s \). In particular, \( \Omega \) can be tuned to the vibrational modes, \( \omega_v \), of the material under study. If so, stimulated excitation of vibrational transition occurs. Both spontaneous and coherent Raman interactions allows vibrational spectroscopy, but in CRS the molecule bonds oscillate in phase (constructively) and the signal can be several order of magnitude higher. The advantages of vibrational imaging through coherent Raman scattering are many: (i) it does not require molecule staining; (ii) there is no population transfer to the electronic states so there is no sample damage; (iii) the sensitivity is higher than spontaneous Raman; (iv) the signal generation mechanism intrinsically allows 3D sectioning capability; (v) near-IR excitation avoids sample heating because of water absorption, and allows a deep penetration depth; (vi) being based on ultrashort pulses CRS allows to time resolve Raman free induction decay which can be the base for an additional chemical contrast mechanism. All the mentioned characteristics make CRS techniques particularly suitable for biological applications.

Among all CRS techniques coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) are the most used. In the most common and simple
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Figure 1.4: Difference between (a) spontaneous Stokes Raman scattering, (b) Stimulated Raman Scattering (SRS), (c) Coherent anti-Stokes Raman Scattering (CARS)

CARS configuration the spatially and temporally overlapping pump and Stokes beam are used to generate an anti-Stokes blue-shifted beam at $\omega_{AS} = 2\omega_p - \omega_s$ (fig.1.4 (c)). In stimulated Raman scattering (SRS) we have a situation in which $\omega_1 = \omega_2 = \omega_p$ is the pump frequency and $\omega_3 = \omega_4 = \omega_s$ is the Stokes frequency (fig.1.4 (b)). This effect actually encompasses two effects, stimulated Raman gain (SRG), concerning the intensity of the Stokes pulse, and stimulated Raman loss (SRL), concerning the intensity of the pump beam. This technique will not be studied in details in the present work. It is enough to say that SRS is based on the quantification of these intensity gain and loss, and presents advantages and disadvantages compared to CARS (see further sections). It is important to underline that all this third-order nonlinear effects are happening at the same time in the $\chi^{(3)}$ sample.

1.4.1 Coherent Anti-Stokes Raman Scattering (CARS) spectroscopy

Coherent anti-Stokes Raman scattering (CARS) is a nonlinear Raman spectroscopy technique which involves three laser pulses: the pump, the Stokes and the probe beam, whose frequencies are $\omega_p, \omega_s, \omega_{pr}$. They interact with a sample to generate a blue-shifted anti-Stokes field at $\omega_{as} = \omega_p - \omega_s + \omega_{pr}$ which resonantly enhances when the Raman shift $\omega_{pr} - \omega_s$ coincides with the frequency of a Raman active molecular vibration. The general case in which $\omega_p \neq \omega_{pr}$ is referred to as three-color CARS (fig. 1.5 (a)); two-color CARS uses only two input frequencies as $\omega_p = \omega_{pr}$ (fig. 1.5 (b)) and is the one preferred in this work and to which all equations will refer to from now on. The main advantage of CARS is that it is an anti-Stokes process and, as a result, fluorescent free spectra can be obtained. Using eq. 1.35, the nonlinear
polarization $P^{(3)}$ at the anti-Stokes frequency is given by:

$$P^{(3)} = \chi^{(3)} E_p^2 E_s^*$$  \hspace{1cm} (1.42)

where the symbol $*$ stands for complex conjugate; $\chi^{(3)}$ can be explicitly written as (see e.g. [33]):

$$\chi^{(3)} = \frac{A_R}{\Omega - (\omega_p - \omega_s) - i\Gamma_R} + \chi^{(3)}_{NR}$$  \hspace{1cm} (1.43)

in which $\Omega$ is, as usual, the vibrational frequency, $\Gamma_R$ is the HWHM$^4$ of the Raman line and $A_R$ is the Raman scattering cross-section. The first term in eq. 1.43 represents a resonant contribution which reaches its maximum when $\Omega = \omega_p - \omega_s$. The second term represents the non-resonant contribution, due to the electronic contribution, which is independent on the Raman shift and real (a constant) [16]. An example of a possible combination giving non-resonant contribution is given in fig. 1.5 (c). The CARS signal amplitude is easily derived from eq. 1.39 and analytically results in (see e.g [33]):

$$I_{CARS} \approx |\chi^{(3)}|^2 I_p^2 I_s \left[ \frac{\sin(|\Delta k| \cdot L/2)}{|\Delta k|/2} \right]^2$$  \hspace{1cm} (1.44)

where $I_s$ and $I_p$ are the Stokes and pump beam under non depletion approximation.

---

$^4$Half Width at Half Maximum
and \( L \) in the propagation length inside the material. The quantity \( \Delta k = k_{as} - (2k_p - k_s) \) is the wavevector mismatch, therefore the CARS signal depends on the propagation directions of all involved beams. It follows that the intensity of eq. 1.44 is maximized when the wave vectors satisfy the phase matching condition:

\[
|\Delta k| \cdot L = |k_{as} - (2k_p - k_s)| \cdot L < \pi
\]

(1.45)

this condition is depicted in fig. 1.6.

Figure 1.6: Phase-matching condition for two-color CARS.

To study the intensity behaviour in more detail, we can write equation 1.43 as:

\[
\chi^{(3)} = \chi^{(3)NR} + \text{Re}\{\chi_R^{(3)}\} + i\text{Im}\{\chi_R^{(3)}\}
\]

(1.46)

where the susceptibility has just been written as the sum of a resonant (R) and a non-resonant part (NR), and the resonant part is the sum of its real and imaginary component. It is easy to visualize CARS signal shape explicitly writing \( \chi^{(3)} \) in real and imaginary parts. We already know that the resonant part \( \chi_R^{(3)} \) is a complex quantity that depends on the so-called detuning \( \Delta r = \Omega_r - (\omega_{pu} - \omega_s) \) and can be written as:

\[
\chi_R^{(3)} = \sum_r \frac{A_r}{\Delta_r - i\Gamma_r}
\]

(1.47)

in which the sum is over all the \( r \)-th Raman active modes. For monochromatic and linearly polarized input fields, the third order susceptibility is, actually, a tensor that can be written as:

\[
\chi^{(3)}_{ijkl} = \chi^{(3)NR}_{ijkl} + \sum_k \chi^{(3)R}_{ijkl}
\]

(1.48)

where \( i,j,k,l \) are the cartesian polarization components of CRS, pump, probe and Stokes. In case of two-color CARS the two relevant independent tensor components are (see e.g. [16]):

\[
\chi^{(3)R}_{1111} = \frac{A_{r,1111}}{\Delta_r - i\Gamma_r} = CNT_r \frac{\alpha^2 + \frac{4}{15} \gamma_s^2}{\Delta_r - i\Gamma_r}
\]

(1.49)
\( \chi_{1221}^{(3)R} = \frac{A_{r,1221}}{\Delta r - i \Gamma r} = C N \Gamma r \frac{1}{\Delta r - i \Gamma r} \)  

(1.50)

Both of this component show that \( \chi_R^{(3)} \) is directly proportional to the density number of the active Raman scatterers \( N \); \( \alpha^2 \) and \( \gamma_s^2 \) are the terms of isotropy and symmetry anisotropy invariants of the spontaneous Raman scattering tensor. It follows that the CARS signal is proportional to the square of the scatterers density. From eq. 1.49) and 1.50 we can write in a simpler way:

\[
\chi^{(3)} \propto \sum_r \frac{N}{\Delta r - i \Gamma r}
\]

(1.51)

so we can express the real and imaginary part for one resonant component as:

\[
\text{Re}\{\chi_R^{(3)}\} \propto \frac{N \Delta}{\Delta^2 + \Gamma^2}
\]

(1.52)

\[
\text{Im}\{\chi_R^{(3)}\} \propto \frac{N \Gamma}{\Delta^2 + \Gamma^2}
\]

(1.53)

The measured intensity is then proportional to the square of \( \chi_R^{(3)} \) so:

\[
I_{\text{CARS}} \propto |\chi^{(3)}|^2 = |\chi_R^{(3)}|^2 + |\chi_N^{(3)}|^2 = |\chi_R^{(3)}|^2 + |\chi_N^{(3)}|^2 + 2\text{Re}\{\chi_R^{(3)}\} \chi_N^{(3)}
\]

(1.54)

The behaviour of the different components together with their total sum is shown in fig. 1.7. The nonlinear mixing between the resonant and the non-resonant components leads to a red-shift of the peak frequency. The non-resonant contribution amplifies the real part of \( \chi_R^{(3)} \) at the expenses of the imaginary part. Because this non-resonant contribution is, in general, unknown, the introduced distortion makes the interpretation of CARS spectra difficult. This is the main drawback of this technique. The NR background represents a problem since: (i) it provokes spectral distortion; (ii) it can generate image artefacts (we see a peak where there is not); (iii) there is a nonlinear dependence between the signal strength and the number of scatterers \( N \); (iv) it limits the ultimate sensitivity of the technique. This clearly represents an disadvantage of CARS when compared to other techniques like two-photon microscopy, which doesn’t present NRB, and SRS, which is insensitive to the NRB. Several methods have been developed to get rid of the non-resonant background, or at least minimize its effect, either with a specific setup modification or with post acquisition signal processing with mathematical algorithms. This fundamental problem, which is not completely solved at the present date, will be faced in more detail in Chapter 3.
1.4.2 Multiplex-CARS (MCARS)

CARS microscopes can be tuned to detect a specific molecule vibration. This inefficient and, besides, dynamical changes in a whole spectrum are difficult to follow. This limitations have been overcome by broadband or multiplex CARS spectroscopy, first demonstrated by Akhmanov et al [18]. In MCARS spectroscopy a narrowband pump pulse and a broadband Stokes pulse are used to simultaneously excite multiple Raman resonance frequencies at the same time. This allows to probe a whole vibrational spectrum up to 3500 cm$^{-1}$ in a single acquisition which is usually realized on a time scale of tens or hundreds of milliseconds. The technique can be employed to perform hyperspectral imaging by recording the complete vibrational spectrum at every position in a sample with a resolution on the order of micrometers. The energy level diagram describing this technique is shown in fig. 1.8. The bandwidth of the pump pulse, which in the two-beam configuration is also the probe pulse, determines the inherent spectral resolution. The Stokes bandwidth instead determines the spectral width of the generated CARS spectrum and so of the measurable Raman shifts. MCARS spectroscopy is always combined with microscopy for imaging purposes. All the pulses overlap in time and space. The sample excitation scheme exploits a collinear geometry and the laser beam is focused on the sample with a
Oppositely to spontaneous Raman signal, CARS signal is highly directional due to the phase matching condition. This condition (see eq. 1.45) has been very limiting in the past. In the first CARS implementation, called BOXCARS, all beams were finely tuned to propagate in different directions to achieve the best phase matching condition. The configuration is very difficult to implement because it is difficult to calculate the phase matching condition and the angles between the beams are around 7°. The setup described in Chapter 2 uses a collinear geometry, which nowadays is very common. This excitation is realized under tightly focused conditions under which the phase matching becomes insensitive to the laser wavelength. This situation is optimal because it allows to acquire the whole spectrum without changing the laser alignment. The MCARS setup used in the present work has two detection modalities: forward-MCARS (f-MCARS) that measures in transmission, and epi-MCARS (e-MCARS) that measures the backscattered radiation. Typical acquisition times per spectrum are on the order of milliseconds. Consequently an MCARS microscope can be seen as an attempt to create a faster Raman microscope. The addition of a time-delay line in the setup allows to perform time-resolved MCARS (tr-MCARS) to probe Raman induction decays, which constitute another potential chemically selective imaging method. All these characteristics make MCARS a very promising investigation tool.
Chapter 2

Experimental setups description
and characterization

2.1 Raman experimental setup

Two different Raman microscopes have been employed carrying out this thesis, both in 180° configuration. The first one is from WITec GmbH and the second one from Horiba Scientific (Xplora Plus). A general scheme that can be used to describe these systems is shown in fig. 2.1. The light coming from a CW powered laser is focused on a sample plane (b). No special sample preparation is required. The excitation/detection system is coupled with a high resolution confocal microscope. Confocal microscopy requires a point source (the laser) which is focused onto the sample (point in plane (c)). The Raman scattered light is collected through the same objective as the excitation light has been delivered. The light is then focused through a pinhole (e) at the front of the detector. Only the light coming from the image focal plane can reach the detector. Consequently the confocal geometry allows chemical selective high resolution 3D imaging, with a resolution below the μm scale.

The choice of the pinhole size is fundamental as it implies a trade-off between signal intensity and highly confocal excitation spot. The collection lens is responsible for both focusing the light in the specific focal point and collecting the emitted radiation. A notch optical filter is used to filter out the intense Rayleigh scattered light. The light that has been focused on the pinhole is then undergoing diffraction so that different chromatic components (difference frequencies) can be spatially separated and measured. This is done by a monochromator based on a diffraction gratings system (see A). The detector consists of a CCD camera. A three axis piezoelectric controlled stage allows 2D and 3D imaging.

In the WiTec microscope the laser light is delivered through a single-mode optical fiber (fig.2.1, f). This fiber supports only a single transversal mode which, in weakly
guiding approximation, is the \( LP_{01} \) mode. The beam is focused to a diffraction-limited spot. After being collected, the scattered Raman signal is focused onto a multi-mode fiber (so, in this case, the detection system is not mounted like in the figure but can be far away). The collection efficiency is around 75\%. In this case the core of the multi-modal fiber is actually playing the role of the pinhole. Because of this, to achieve maximum detection efficiency, the fiber is mounted in the image plane of the microscope. The wavelength used in this system is 532 nm with a declared resolution down to 220 nm; the available gratings are 600 and 1800 gr/mm.

In the Horiba system both the excitation and the detection modules are mounted in the upper part of the microscope system (Olympus Microscope BX41). This results in a very compact device. The detection system is equipped with a four-grating turret (600, 1200, 1800 and 2400 gr/mm) and a CCD detector with 1024x256 pixels. The main characteristics of the two used laser sources of this system are shown in Table 2.1. The confocal resolution is below 500 nm at 532 nm.

The choice of the excitation wavelength is not always obvious. As noticed in the previous chapter, Raman spectra present fluorescence background. When a higher wavelength \( \lambda \) is chosen this background drops but the resolution is affected. An example on how a Raman spectra can be dramatically affected by fluorescence is
<table>
<thead>
<tr>
<th>Laser type</th>
<th>Wavelength (nm)</th>
<th>Power (max) (mW)</th>
<th>Resolution with 50 µm slit cm (with 2400 gr/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>solid state laser</td>
<td>532</td>
<td>40</td>
<td>≤ 1.4</td>
</tr>
<tr>
<td>laser diode</td>
<td>785</td>
<td>100</td>
<td>≤ 1.2</td>
</tr>
</tbody>
</table>

Table 2.1: Laser characteristics

Figure 2.2: (a) Raman spectrum of inhibitor CN-I4. Peaks after 2500 cm\(^{-1}\) are due to the solvent. The peak around 2200 cm\(^{-1}\) is due to the triple C-N bond which will be deeply investigated in Chapter 5, (b) Raman spectrum of polyethylene terephthalate (PET). The band at 1776 cm\(^{-1}\) is due to the carbonyl stretch \([1]\), (c) chemical structure of CN-I4, (d) chemical structure of PET. Acquisition parameters: excitation @532nm, dwell time 1 s, averages 20, 100% laser power.

shown in fig.2.2 In graph (a) the spectrum of a chemical inhibitor dissolved in DMSO (see Chapter 5) has been acquired (chemical structure shown in (c)). It is evident how the spectrum is affected by a very high fluorescent background. This could be already predicted as the sample appears naturally yellow. On the other hand the already encountered polyethylene terephthalate (PET) spectrum (chemical structure in (d)) has a flatter spectrum. The present work utilizes spontaneous Raman microscopes to (i) acquire reference spectra to verify MCARS signals and (ii) compare the performances of these two systems.
2.2 Multiplex-CARS experimental setup

2.2.1 Principles of white-light generation

One of the key features of an MCARS experiment is the availability of a very broad Stokes spectrum. This is obtained through the process of white-light (or supercontinuum) generation which consists in the formation of very broad and continuous spectra by propagating high power light pulses through $\chi^{(3)}$ nonlinear media. This is possible thanks to the phenomenon of self-phase modulation (SPM) which causes a variation of the refractive index through which the pulse propagates. After crossing the material, the spatial coherence of the temporal coherence can be lost, but the spatial coherence is still very high. A pulse with a bandwidth of few nanometers can be transformed in a supercontinuum spectra with a spectral extension of several hundreds nanometers. To understand how this physical interaction works it is necessary to model how a pulse propagates in a $\chi^{(3)}$ medium. This is done by specializing eq. 1.33 for the case in which the third order nonlinear polarization is created by identical pulses \(^{1}\) so that (see e.g. [31]):

$$p_{NL}(z, t) = \frac{3}{4} \chi^{(3)} \epsilon_0 |A|^2 A e^{i(\omega t - kz)}$$

(2.1)

substituting eq. 2.1 into eq. 1.33 and considering the equation in the reference frame of the pulse, it results:

$$\frac{\partial A}{\partial z} - i \frac{k' \epsilon_0}{2} \frac{\partial^2 A}{\partial t^2} = -i \gamma |A|^2 A$$

(2.2)

where $A$ is the pulse envelope and $\gamma$ is a material-dependent constant. This equation describes the propagation of a pulse in a $\chi^{(3)}$ medium and is sometimes referred to as nonlinear Schrödinger equation. Now we assume that $k' \epsilon_0 = 0$ (no dispersion effects) and focus on the effects of the right hand side on eq. 2.2. We want to find a solution for:

$$\frac{\partial A}{\partial z} = -i \gamma |A|^2 A$$

(2.3)

which can be solved by the following ansatz:

$$A(z, t) = A(0, t) e^{i \phi_{NL}(z, t)}$$

(2.4)

which assumes that the propagation modifies only the phase of the pulse envelope $A(z, t)$. Substituting the ansatz in eq. 2.3 and simplifying:

\(^{1}\)Which means the same train of pulses interacting with themselves
\[
\frac{i \partial \phi_{NL}(z, t)}{\partial z} = -i \gamma |A(0, t)|^2
\]  \tag{2.5}

It follows:

\[
\phi_{NL}(z, t) = -i \gamma |A(0, t)|^2 z
\]  \tag{2.6}

Substituting this in the previous ansatz definition we obtain:

\[
A(z, t) = A(0, t)e^{-i \gamma |A(0, t)|^2 z}
\]  \tag{2.7}

Eq. 2.7 shows that the phase of the field is modified by the field itself. The intensity profile remains unmodified. This effect is called self-phase modulation. To understand what happens to the instantaneous frequency we can write the expression of the electrical field at the beginning of the material \((z = 0)\):

\[
E(z, t) = A(0, t)e^{i(\omega_0 t - k_0 z + \Delta \phi_{NL}(z, t))}
\]  \tag{2.8}

Consequently the instantaneous frequency becomes:

\[
\omega_i = \frac{d}{dt}[\omega_0 t + \Delta \phi_{NL}(z, t))] = \omega_0 + \frac{d \Delta \phi_{NL}}{dt} = \omega_0 - \gamma z \frac{d}{dt}|A(0, t)|^2
\]  \tag{2.9}

Eq. 2.9 clearly shows that the instantaneous frequency changes with the time and depends on the field intensity. Because \(\omega_i\) is proportional to the propagation length \(z\), the more the pulse propagates the more the spectrum broadens. The generation of new frequencies is due to several nonlinear optical effects occurring at the same time [30]. If we consider a Gaussian shaped pulse, the spectral broadening appears at both higher and lower frequencies respect to the pulse. This can be proved noticing that the derivative of the pulse envelope is negative in the pulse leading edge (generation of red-shifted colors) and positive in the trailing edge (generation of blue-shifted colors). The self-phase modulation can be also explained as a intensity dependent refractive index modulation. A nonlinear change in \(\phi_{NL}\) implies a nonlinear change in the refractive index, called Kerr effect, which can be expressed as:

\[
n = n_0 + \Delta n(t) = n_0 + n_{NL}I(t) = n_0 + n_{NL}|E(t)|^2
\]  \tag{2.10}

This causes a spatial modulation of a refractive index which follows the pulse intensity shape. The quantity \(\gamma\) introduced in eq. 2.2 is actually defined as \(\gamma = 2\epsilon_0 n_0 \omega_0 n_2\). For a Gaussian pulse there will be a Gaussian phase and refractive index modulation.
A Gaussian modulation of the refractive index perpendicular to the propagation direction acts as a convergent lens. This specific phenomenon is called Kerr lensing and causes a self-focusing effect. It is fundamental to consider that all these effects (SPM and Kerr lensing) appear only with very high intensities, available from mode-locked high peak power lasers whose pulses are then further focused on the medium.

2.2.2 Experimental implementation of multiplex-CARS

The experimental multiplex-CARS setup is shown in fig. 2.4. The whole system is based on a Ti:Sapphire mode-locked laser (Coherent Mira) pumped by a diode-pumped solid-state (DPSS) laser at 532 nm (Sprout-G, Light-House Photonics Inc.). A Faraday isolator is placed right after the cavity output to avoid any back-reflected radiation to effect the mode-locking regime (not shown in the figure). The averaged output power is 1.20 W ± 0.3 W at 785 nm. The pulses have a repetition rate of 76 MHz and a duration of 160 fs (FWHM of 5.7 nm or \(90 \text{ cm}^{-1}\)). This corresponds to an energy per pulse of 14 nJ and a peak power of \(2.35 \times 10^7\) mW. If this pulse is directly used as a pump/probe pulse the resolution would be rather low compared to the vibrational linewidth. Therefore after a beam splitter (BS) a fraction of the emitted beam is filtered by a highly narrowband pass filter (F1). This is a notch optical filter centered at 783 nm with 1 nm spectral width. The output of this filter is used as pump/probe beam which now has a spectral width of around \(16 \text{ cm}^{-1}\) and a duration of 900 fs (see eq. 1.15). Clearly, as lot of pumping power is lost in the filtering process, the better the resolution (spectral narrowing) the lower the signal. In particular, while spectral resolution increases linearly with a narrower filter, the CARS signal drops quadratically (see eq. 1.44). A minor part of the beam power
Figure 2.4: MCARS experimental scheme: BS = beam splitter; F1, notch filter; PCF, photonic crystal fiber; F2, longpass filter; F3, short-pass filter; $\Delta \tau$ dashed box, delay line; Full black mirrors are silver coated reflective mirrors; dashed line mirrors represent flip mirrors.

(around 360 mW) is used to create the supercontinuum which is Stokes beam. The white light generation is achieved coupling the beam to a end-sealed polarization maintaining photonic crystal fiber (PCF), from NTK Photonics (FEMTO WHITE 800) with zero dispersion at 750 nm ± 15 nm. The whole fiber length is 12 cm ± 5 mm. The fiber output spectral range is 500-1100 nm. Both beams are aligned in a collinear way and recombine on a long-pass filter (F2), which blocks all wavelengths below 785 nm to cut off all background signals. The spectra of pump/probe and Stokes beam is shown in fig. 2.3

The role of the delay line ($\Delta \tau$) is to time delay pump and Stokes to optimize their temporal overlap. This can be done with a piezo controlled linear delay platform (Physik Instrumente GmbH). Adjustments on the order of 0.01 picoseconds are, in fact, impossible to achieve through manual alignment. The use of the delay line for time-resolved MCARS is faced in Chapter 3. The synchronized beams are used for two types of detection: epi-detection and forward-detection. In the first case the beams are coupled into a commercially available microscope and the backscattered radiation is measured. The epi-MCARS detection allows to raster scan a sample which is mounted on a 3D piezo nanopositioning (Physik Instrumente, P-545). In
Figure 2.5: (a) Epi-MCARS spectrum of PET sample. Strong bands around 1590 and 1700 are due to the carbonyl group \((C=O)\). (b) Epi-MCARS spectrum of PS sample. Bands at 3000 cm\(^{-1}\) are due to -C=H stretch; (c) Raman reference spectrum of PET, (d) Raman reference spectrum of PS. MCARS measurement parameters: total power injected in the microscope 67 mW, dwell time 200 ms, objective: Olympus LMPlan 50x IR (infrared compensated). Raman measurement parameters: dwell time 1 s, objective: EC Epiplan x50.

forward-MCARS (f-MCARS) the generated CARS signal is measured in transmission so two objectives are necessary. In this setup the forward detection is realized by a self-assembled microscope. Both detection system are part of the setup but cannot be used simultaneously. Two flip mirrors (mirrors with dashed lines) allows to switch between the two detection modalities very easily. After either crossing of backscattering from the sample the beams crosses a short-pass filter (F3) at 785 nm which ensures that only the spectral area of the blue-shifted CARS signal is measured. The detected beam is focused into the entrance slit of a grating spectrometer\(^2\) (Princeton Instruments Acton SP2300) which is coupled to a multichannel CCD camera (Andor Newton), Peltier-cooled at 70° to suppress thermal noise. The CCD detection system and the broad Stokes spectrum allow to test molecular vibrations up to 3500 cm\(^{-1}\) with a single shot acquisition.

The choice of the photonic crystal fiber (PCF) for the white light generation is non-trivial. The broadest output spectra is obtained when the input pulses are

\(^2\)See Appendix B
close to the zero-dispersion wavelength. A schematic representation of a transversal section of a PCF is shown in fig. 2.6.

The fiber core size, in pure silica, has a core-size of $1.8 \pm 0.3 \, \mu m$ which makes the alignment very challenging. The mounting and the coupling, realized with two coupling objectives $40x$, must be carefully implemented as any contamination of the end cleaved facets can easily destroy the fiber performances. Several layers protect the fiber core, in order quartz (in contact with the active material), stainless steel and aluminium. All components have a cylindrical shape. The chosen fiber is adapt for a Ti:Sa system as it can handle more than 1 W of average power. The careful choice of the PCF allows to pump it in the anomalous region: in this way the device itself can operate a dispersion compensation on the Stokes pulse (chirping it negatively) and make it roughly match the pump/probe time duration. The pulses also match in polarization. As the fiber is (mostly) polarization maintaining the input light is previously coupled to a $\lambda/2$ plate. All the fiber structure is mounted on a computer feedback controlled piezo positioning stage to compensate for any mechanical shock that can occur which is always relevant due to the fiber core dimension. Some PCF offer a dispersion compensation structure: half of the generated light is undergoing normal dispersion and the other half a negative one. In this way the global chirp is around zero. A similar PCF is used in this work, but the zero dispersion wavelength of the fiber ($750\,nm$) is different respect to the one used to generate the supercontinuum ($785\,nm$) so the Stokes pulse is chirped. Consequently its frequency components do not all arrive at the same time. The spectral coverage of vibrational modes is then varying respect to the time delay $\Delta \tau$ between the narrow pump and the broadband Stokes. Fig. 2.5 shows two comparisons between the MCARS spectra of

\footnote{Representative image only, not related to the real structure of the utilized fiber. Internal texture can be found at: https://en.wikipedia.org/wiki/Photonic-crystal_fiber.}
polyethylene terephthalate (PET and Polystyrene (PS) compared to their respective Raman reference spectra. Considering the analysis made in fig. 1.7 it is possible to analyse the MCARS signal behaviour. The bands at 1590 and 1700 cm$^{-1}$ in (a) and 3028 cm$^{-1}$ in (b) are Lorentzian. In these cases Re$\{\chi^{(3)}_R\} \gg \chi^{(3)}_{NR}$. When, instead Re$\{\chi^{(3)}_R\} \approx \chi^{(3)}_{NR}$, CARS bands are dispersive. An example is the area from 800 to 200 cm$^{-1}$ in (b). Because of the Stokes chirp, the temporal overlap pump/Stokes implies that not all bands are visible. Comparing fig. 2.5(a) with its Raman spectrum in (c) it is evident that bands beyond 2500 cm$^{-1}$ do not appear in the MCARS spectrum. On the other hand for the PS spectra the pump/Stokes overlap tends to hide the low wavenumbers bands. This is because the pump/Stokes delay has been adjusted to investigate the characteristic bands around 3000 cm$^{-1}$.

For 2D imaging of a sample the output data is stored in a hyperspectral matrix in which at every point (in X,Y coordinates) a whole MCARS spectrum is allocated. In fig. 2.7 an intensity map of a PET sample and an epithelial human tissue is shown. The result in (a) has been obtained by integrating the peak around 2900 cm$^{-1}$ corresponding to the CH-stretching band associated to lipids which is particularly strong in the intercellular area [19]. Cells borders and distribution are recognizable. In (b) the area around 1590 cm$^{-1}$ of the PET spectrum has been integrated.

### 2.2.3 Forward and Epi-detection

CARS and MCARS microscopy are distinguished from the corresponding spectroscopy technique because the laser beams are tightly focused through objective lens with high numerical aperture (NA). The phase matching condition relaxes and the setup implementation is easier. The whole setup used in the present work uses a collinear input beam geometry and two detection modalities: forward detection (F-MCARS)
Figure 2.8: Characterization spectra obtained in epi-detection from a PET sample with three different objectives: (a) OlympusMPlan 50x NA 0.55 (b) UPlanFLN 20x NA 0.5 (c) OlympusMPlan IR 50x NA 0.55. Each acquisition has been performed with a dwell time of 200 ms and is the average of 50 spectra.

and backward detection (epi-MCARS). There are advantages and drawbacks for each of them. Backward (epi) CARS signal detection was originally implemented to suppress the solvent background and increase the sensitivity of CARS microscopy \[34\]. This method makes it possible to measure signals from scatterers smaller than the wavelength of light and is particularly useful for intracellular imaging \[19\]. F-CARS radiation, instead, is efficiently produced by objects whose size is comparable or larger than the excitation wavelength. For its purposes epi-CARS does not allow to analyze liquid solutions. When a forward detection system (F-CARS) is used, signals are usually higher but so is the nonresonant contribution. An advantage is, of course, the possibility of studying liquid samples, as it will be done during whole Chapter 5. The existence of a backscattered signal is not immediately understood.

As previously said, very small objects can produce a high epi-signal as the solvent background is suppressed. There are other two mechanism responsible for this signal to arise: (i) the crossing of the interface between two media with different $\chi^{(3)}$ can avoid a complete destructive interference and (ii) some forward generated radiation is back reflected inside an heterogeneous sample \[35\].

For epi (backscattered) MCARS measurement the beams are coupled into a commercial microscope. Around 82% of the signal is lost after crossing the microscope. In particular the pump/probe beam loses 91% of its intensity and the Stokes beam 68%. This difference is due to the different transmission properties of the optical elements composing the microscope. After coupling into the objectives other losses
must be add (from 10 up to 40%) as the objectives transmittance drops dramatically after 700 nm. Three different objectives have been compared to chose the one from which the best signal response is obtained (fig. 2.8). Consequently all measurements have been performed using an Olympus MPlan 50x with infrared compensation, numerical aperture (NA) of 0.55.

In the MCARS self-assembled microscope for forward detection the input objective is an UPlanFL 20x with 0.5 NA (Olympus); the collection objective is Plan 20x with 0.4 NA (Olympus). As the implementation has been done in the vertical direction, but the detection is horizontal, investigating different samples requires a new alignment, but this is not a drawback of the detection method itself. When building the system a rough initial alignment has been done using a high fluorescent sample. This is necessary because the beam intensity is not only in the IR part of the spectrum but also very weak so infrared viewer or cards are not helpful. As comparison fig. 2.9 shows two spectra from the same PET sample. As already predicted the forward detected signal has a higher intensity but also a higher background.
Chapter 3

The maximum entropy phase retrieval method

3.1 Introduction to nonresonant background suppression techniques

The suppression of the nonresonant background (NRB) is of fundamental importance for improving the detection sensitivity of CARS microscopy, which results in faster acquisition time, especially compared to spontaneous Raman microscopy. Several suppression methods have been developed for this purpose. A proper choice of laser sources can already make the difference. As the non-resonant electronic contribution is enhanced by two-photon electronic resonance [33], the use of near-IR excitation light reduces this effect, improving image contrast [36]. Another important choice is the pulse duration. Raman vibrations have a spectral line width around 10 cm$^{-1}$, but a pulse of 160 fs is already 90 cm$^{-1}$ broad. This basically means that most of the pulse generates NRB. For a pulse to be comparable to the Raman linewidth, picosecond pulses have been successfully used [37] but the background is not completely eliminated and the process can be efficient only in a small spectral window.

A whole class of techniques that either partially suppress or eliminate the NRB are connected to setup modifications. The advantages of epi-detection, which selectively suppresses background based on the objects size (and not on vibrational properties) have been analysed in the previous chapter. Polarization CARS (P-CARS) exploits the different spatial orientation of the resonant and nonresonant polarization components. The NRB can be totally eliminated but, because of the presence of an analyser, the signal is very weak [38].

The reason to suppress the NRB is not only to increase CARS sensitivity but to retrieve the peak position as they would appear in a Raman spectrum. In fact the
Raman resonant (R) signal is proportional to the imaginary part of $\chi^{(3)}$: \[ I_{\text{Raman}}(\omega) \propto \sum_n \text{Im}\{\chi_R^{(3)}\} \] (3.1)

where the sum is over all $n$ resonances. Finding a way of extracting $\chi^{(3)}$ imaginary part leads to full Raman spectrum reconstruction. This cannot be done using only pure CARS signal as the real part of $\chi_R^{(3)}$ is nonlinerly mixed with its nonresonant component (see eq. 1.54). In interferometric (or heterodyne) CARS (iCARS) the emitted coherent anti-Stokes signal $E_{\text{CARS}} \propto E_{\text{EX}} = E_p^2 E_S$ ($p = $ pump, $S = $ Stokes fields) is mixed with the electric field of a local oscillator ($E_{\text{LO}}$) (see e.g. [39]): \[ I_{\text{tot}} = |E_{\text{CARS}} + E_{\text{LO}} \exp(i\Delta \phi)|^2 = 2E_{\text{EX}}E_{\text{LO}}\{[\text{Re}\{\chi_R^{(3)} + \chi_{NR}^{(3)}\}]\cos(\Delta \phi) + \text{Im}\{\chi_R^{(3)}\}\sin(\Delta \phi)\} \] (3.2)

where $\Delta \phi$ is the phase difference between CARS signal and the local oscillator. By setting $\phi = 90^\circ$ the resonant contribution is maximized and the other suppressed. However the technique requires a minimum of two independently tunable laser which must also be locked in phase. In frequency modulation CARS (FM-CARS) the narrowband source is switched between two frequencies $\omega_p$ and $\omega_p'$. This frequency modulation results in a CARS signal amplitude modulation which depends on the frequency difference $\delta$: \[ I(\delta) = I(\omega_p) - I(\omega_p') \] (3.3)

The system then exploits the ability of a lock-in amplifier to eliminate CW components to get rid of the nonresonant background (which is a constant). Like iCARS this technique is difficult to implement as it requires fast frequency modulations (>500 kHz) and at least two tunable lasers sources. A solution which has been specifically developed for multiplex-CARS employs a pulse shaper for the Stokes beam (a spatial light modulator, SLM) to excite only specific vibrations [40]. Clear disadvantages are that spectral position of such vibrations must be known in advance and the NRB is not totally suppressed. In time-resolved CARS (tr-CARS) a time delay between the pump and the probe can be exploited for both NRB suppression and imaging through Raman free induction decay (RFID); this method is gaining popularity and its application are pretty successful ([41], [42]). The main disadvantage is that the probe delay leads to a decreased useful signal on the order of $\exp(-2\Delta t/T_{2v})$, where $T_{2v}$ is the molecule dephasing time, which can be limiting.

An alternative to setup modification is to use a post-acquisition data elaboration method. All of these methods are based on the retrieval of the $\chi^{(3)}(\omega)$ phase. Indeed
as eq. 3.1 shows, knowing the imaginary part of the electric susceptibility is the key to retrieve the Raman spectra. CARS measure is an intensity measure and is proportional to $|\chi^{(3)}(\omega)|^2$ from which only the absolute value can be calculated so we need to access in some way the spectral phase of the CARS signal. We can write the electric susceptibility with the exponential form:

$$\chi^{(3)}(\omega) = |\chi^{(3)}(\omega)| \exp\{i\theta(\omega)\}$$

(3.4)

where $\theta(\omega)$ is the phase of the electric susceptibility. Reminding that:

$$\chi^{(3)} = \chi_{NR}^{(3)} + \text{Re}\{\chi^{(3)}_R\} + i\text{Im}\{\chi^{(3)}_R\}$$

(3.5)

we can easily calculate the spectral phase contribution to $\chi^{(3)}$:

$$\theta(\omega) = \tan^{-1}\left\{ \frac{\text{Im}\{\chi^{(3)}_R\}}{\chi_{NR}^{(3)} + \text{Re}\{\chi^{(3)}_R\}} \right\}$$

(3.6)

whose left-hand side quantities are individually unknown. A possibility to calculate this $\theta(\omega)$ is to exploit time-domain Kramers–Kronig transform, in which real and imaginary part of a general complex function are connected with each other via integral calculations. It is possible to explicitly express $\theta(\omega)$ as a function of the absolute value of $\chi^{(3)}_R$ in the frequency domain [20]. The application of this formula can be tricky because it involves integrals over an infinite frequency range when the data are measured over a finite spectral range. A better solution, which avoids unlimited integrals, consists in an application of the maximum entropy method (MEM), called maximum entropy phase retrieval (MEPR). This algorithm will be discussed in the following section and has been employed as the background suppression method in the present work. MEPR is robust and efficient and we will prove that it is possible: (i) to recover Raman-like spectra without any previous knowledge about the substance under investigation, (ii) retrieve Raman spectra quantitatively and make concentration measures, (iii) to know the real Raman shift even when a time delay line has been used to adjust the pump/Stokes overlap, which can provoke big CARS peak intensity variations.

### 3.2 Maximum Entropy Phase Retrieval Method

The Maximum Entropy Phase Retrieval (MEPR) is an algorithm based on a specific application of the maximum entropy method (MEM) developed by Burg in 1978. It is not within the scope of this thesis fully develop the theory behind this method.
but nevertheless the main result on which the algorithm is based can be found in appendix B.

The power spectrum estimated with the MEM algorithm, in our case, corresponds to $|\chi^{(3)}|^2$. The signal is always measured between $\omega_1 \leq \omega \leq \omega_2$, so let’s define a normalized frequency as: $\nu = (\omega - \omega_1)/(\omega_2 - \omega_1)$, so that $0 \leq \nu \leq 1$. The power spectrum is then:

$$|\chi^{(3)}|^2 = S(\nu) = \frac{|\beta|^2}{1 + \sum_{k=1}^{M} a_k \exp(2\pi ik\nu)} = \frac{|\beta|^2}{|A_M(\nu)|^2}$$

(3.7)

where $\beta$ and $a_k$ are solutions of a Toeplitz system. The coefficient $C(m)$ in the Toeplitz system are defined by discrete Fourier transforms of CARS spectrum sliced into $M$ parts centred at the discrete set $\nu_m$ of the previously normalized frequencies $\nu$. We can then write:

$$\chi^{(3)} = \chi^{(3)}_R(\nu) + \chi^{(3)}_{NR} = \chi^{(3)}(\nu) \exp(i\theta(\nu)) = \sqrt{S(\nu)} \exp(i\theta(\nu)) = \frac{|\beta|}{|A_M(\nu)|} \exp(i\theta(\nu)) = \frac{|\beta| \exp(i\theta(\nu) - i\psi(\nu))}{|A_M(\nu)| \exp(-i\psi(\nu))} = \frac{|\beta| \exp(i\phi(\nu))}{A_M(\nu)}$$

(3.8)

where everything has been multiplied and divided by $\exp(-i\psi(\nu))$ to introduce the error phase $\phi(\nu)$:

$$\phi(\nu) = \theta(\nu) - \psi(\nu)$$

(3.9)

which is the difference between the true (unknown) phase from the measured CARS spectrum, $\theta(\nu)$, and the estimation of this phase $\psi(\nu)$ from the application of MEM. So $\psi(\nu) = \angle\{A_M(\nu)\}$. It is then necessary to quantify $\phi(\nu)$. The estimation of $\phi(\nu)$ is done by a polynomial fitting. In particular the assumption of the algorithm is that, as an MCARS spectrum consists of very narrow resonance band on a large background, it is possible to assume that in the part of the spectrum where there are no visible resonances $\theta = 0$ and so in this specific positions $\phi(\nu) = -\psi(\nu)$. The number (in our case 10) and positions of the fit points are free parameters of the algorithm and will be defined by the user (see fig. 3.1). For example, they can be set just before and after a very evident resonance band.

The fit can be executed with different methods: least absolute method, least square method, bisquare method. The polynomial order of 4 or 5 is usually enough. After the fit we obtain the quantity:

$$\theta_{est}(\nu) = \phi_{est}(\nu) + \psi(\nu)$$

(3.10)
and, supposing that this is the original true phase of the third order susceptibility, we can retrieve:

$$\text{Im}\{\chi_R^{(3)}(\nu)\} \approx \sqrt{S(\nu)} \sin(\theta_{est}(\nu))$$

To summarize, the MEM algorithm generated as an output a phase spectrum $\theta_{est}(\nu)$ that consists of a sample-specific resonant phase and another contribution which is independent of vibrational resonances and is called 'phase-error'. This error is a non-specific background phase, estimated by a polynomial fit of the retrieved phase spectrum making approximation about the regions where there are no resonancies. After the subtraction of the estimated error phase, the imaginary part of the third-order nonlinear susceptibility is calculated. The weakest point of the algorithm is the interaction with the user through this manual phase fitting. However, as it will be shown in the next section, results are in good accordance with measured spontaneous Raman spectra.
3.3 Experimental results and analysis

Reconstruction of Raman spectral information

Fig. 3.2 shows the retrieved Raman spectra of polyethylene terephthalate (PET) and polystyrene (PS) (in (c) and (d)). The spectra are compared to their respective multiplex-CARS ((a) and (b)) and Raman spectra ((e) and (f)). The parameter $M$ defined in eq. 3.7 has been set to the value 605. After the calculation of its single frequency components, the $\psi(\nu)$ function is interpolated with a spline. It can be observed that the narrower peaks are in the MCARS spectrum the easier is the reconstruction through the algorithm. The Raman reconstructed spectrum can be eventually filtered to de-noise it, but this procedure has not been used in the retrieval.

Figure 3.2: (a) MCARS spectrum of PET, (b) MCARS spectrum of PS, (c) MEM retrieved spectrum of PET, (d) MEM retrieved spectrum of PS, (e) Raman spectrum of PET, (f) Raman spectrum of PS. Raman spectra measured with input wavelength of 532 nm.
of these specific spectra.

As evident from fig. 3.3 the retrieved peaks are not exactly corresponding to the Raman ones. The first peak shown in (c.1) is appearing at 1614 $cm^{-1}$ in the Raman spectrum and at 1600 $cm^{-1}$ in the retrieved one. Considering that this peak is found at 1588 $cm^{-1}$ in the MCARS measured spectrum, there is anyway a correction respect of the blue-shifted CARS emitted wavelength. The red-shifting peaks corrections for PET are around 12 $cm^{-1}$ and for PS they reach 23 $cm^{-1}$ . The peak differences between Raman and retrieved Raman spectra are due to several reasons. As already said in the previous section, the error phase fitting is itself a cause of error. In the MCARS systems the calibration of the setup can influence the peak position. Besides it is evident comparing (c.1) and (e.1) that the spectral resolution of the Raman microscope is higher and this can be another cause for the discordance.

Fig. 3.4 shows the application of the MEPR algorithm for imaging a biological sample. A 400 $\mu m$ x 400 $\mu m$ image of an epithelial human tissue has been acquired (fig.3.4 (a)) raster scanning the sample. As the maximum range of the piezo-controlled scanning stage is 200 $\mu m$, the whole image has been obtained assembling four different images. As the acquisition time per single spectra is 500 ms and the step size in both directions is 2 $\mu m$, the acquisition time for each sub-image is 1.5 hours and the total acquisition time is 6 hours. Part (c) shows the MCARS spectral area associated with the most important peak due to the $CH_2$ symmetrical
Figure 3.4: (a) 400 µm x 400 µm (400 x 400 pixels) image of an epithelial human tissue sample raster scanned with the MCARS setup, with 1 µm step. The picture results from integrating in the spectral area 2760-2920 cm⁻¹, with main peak around 2840 cm⁻¹. The integral value is used to create a normalized color map and scale. (b) same area of the sample whose hyper-spectral matrix has been elaborated with MEPR algorithm. (c) Average of 10 MCARS spectra from neighbouring points in the highlighted blue area. The peak at 2840 cm⁻¹ is associated with the CH₂ symmetrical stretch. Resolution 600 gr/mm. (d) Average of 10 retrieved Raman spectra from neighbouring points in the highlighted blue area (same points as in (c)). Data in (c) and (d) have been interpolated with an spline.

stretch, which appears at 2840 cm⁻¹. The whole hyperspectral matrix has been elaborated, point by point, with the MEM. The cursor adjustment must be done only once and is valid for every single point. In both images ((a) and (b)) the cell border can be identified, but the retrieved image has a worse signal/noise (S/N) ratio. This has two main reasons: (i) the error phase interpolation is not optimized for every single spectrum so the fit can be successful for some spectra but not good enough for others and (ii) the spectral resolution of the retrieved Raman signal is lower than the original MCARS signal by construction.

Finally, the validity of the algorithm, despite a temporal small delay between pump and Stokes beams has been demonstrated. Fig. 3.5 shows spectra retrieved from the PET sample. Between the spectrum acquired with a delay of Δτ = +2 ps and the one acquired at Δτ = -2 ps there is a shift of 5 cm⁻¹ which is small if compared to the global beneficial effect of the algorithm and if considering that the
Raman shift [cm$^{-1}$] Retrieved Raman intensity (Arb. Unit.)

$\Delta \tau = 0$

$\Delta \tau > 0$

$\Delta \tau < 0$

Figure 3.5: Retrieved PET spectra in the area 1000-2250 cm$^{-1}$ from MCARS spectra obtained with time delay $\Delta \tau$ between pump and Stokes ranging from +2 to -2 ps. Error phase fit has been, again, unmodified for each spectrum.

**Extraction of quantitative information from M-CARS spectra**

Spontaneous Raman spectroscopy is a linear technique and can be used for quantitative measurement. In particular, the Raman intensity is directly proportional to the scatterers concentration $N$. CARS emitted signal is a nonlinear spectroscopy technique and the measured signal is actually proportional to the second power of the scatterers concentration, as can be seen from eq. 1.51. It must be also considered that, as the Stokes spectrum has no flat shape, different peaks at different frequencies cannot be compared with each other.

Nevertheless it is possible to perform quantitative measures with an MCARS setup exploiting the phase retrieval algorithm. Fig.3.6 (c) shows the linear dependence of the spontaneous Raman peak intensity ($C\equiv N$ bond) and the molecule concentration. In the analysed case several solutions of n-(cyanomethyl)acetamide (fig.3.6,(a)) dissolved in a aqueous buffer have been prepared with different concentration ranging from 860 mM down to 20 mM which has been identified as the sensitivity limit for the Raman microscope. The linear fit converges as expected.
Figure 3.6: (a) Chemical structure of n-(cyanomethyl)acetamide, here often referred to as CN-I1; (b) MCARS spectra of CN-I1 860 mM (1) and 100 mM (2) dissolved in buffer solution. The C≡N bond, at 2273 cm⁻¹ becomes undistinguishable for lower concentration; (c) linear fit of the Raman peak from C≡N bond; (d) linear fit of the retrieved Raman C≡N peak concentration.

MCARS spectra from the same solutions have been acquired and elaborated with the MEM. Despite the non-linearity of the original signal, the linear fit applied to the retrieved Raman spectra converges. A disadvantage consists in the ultimate detection sensitivity of the MCARS system, which has been identified, for this specific bond, with a concentration of 100 mM. In fact, as shown in fig. 3.6 (b) 2, the peak becomes undistinguishable from the surrounding background due to the solvent.
Chapter 4

MCARS chemically selective imaging of brain tissue

4.1 Microspectroscopy for brain tissue medical imaging

Tumour typing and grading is of utmost importance for both choosing a proper therapy and removing the cancerous tissue as completely as possible. In neurosurgery the complete careful removal of tumour areas is as important as preserving the normal brain area to minimize any possible consequence on the patient after the surgery. The most commonly used standard method in neuropathology for cancer diagnosis is haematoxylin and eosine (H&E) staining. Eosine is a dye that colors proteins and cytoplasm (in bright pink) and haematoxylin stains DNA (in blu-purple). An example of stained sample is shown in fig. 4.1 (1). As the metabolism of a tumor cell is accelerated, it accumulates a higher quantity of dye so it appears with higher color density. The treatment can be only performed ex vivo on an excised tissue. Used in association with H&E staining, Ki67 immunohistochemistry exploits the protein Ki-67 as a cellular marker for proliferation (see fig. 4.1, A, B and C). This method can lead to misclassification [43]. The surgical removal of brain cancer exploits, at the moment, fluorescent particles that can be selectively accumulated in tumorous cells (this, in turn, because of their increased metabolism). The tissue is radiated with laser light and the fluorescent (tumor) part is removed. There are several disadvantages: (i) not all cancer tissue absorbs the fluorescent probe [44], (ii) the dyes are non-specific and do not allow for tumour classification (which is important for the choice of an appropriate therapy), (iii) fluorophores are affected by photo-bleaching. Recently, there have been interesting implementations of more powerful time-resolved multispectral fluorescence techniques which also use and identify more than one fluorophore, rising the amount of collected information (see e.g. [45]). However, even when adapted for studies in vivo, the presence of suitable markers is still required.
To overcome these limitations linear and nonlinear vibrational spectroscopy techniques, which are all label-free, have been widely applied to the study of brain tissue and, in particular, to brain cancer characterization. Linear techniques (Raman and IR) paved the way for data classification [46], but, in order to achieve better spatial resolution and faster acquisition speed, modern research is rapidly developing nonlinear methods. In particular, a new approach, called multimodal nonlinear imaging, consists in the use of several nonlinear technique, on the same sample, to gain as much information as possible exploiting different contrast mechanism. In particular CARS, second harmonic generation (SHG) and two photon excited fluorescence (TPEF) have been employed together for spectral identification and classification of brain matter [47]. This chapter is dedicated to the chemically selective imaging ex vivo of a mouse brain sample, containing solid tumorous area, with MCARS microspectroscopy. No other nonlinear technique has been used, but it is interesting to keep in mind the global direction in which medical imaging is moving nowadays with this multiple-technique approach.

\footnote{The studied samples as well as the representative images have been provided by: Neurochirurgie, Carl Gustav Carus Universitätsklinikum an der Technischen Universität Dresden, Germany.}
4.2 Raman and MCARS imaging of brain tissue

CARS signal covers the whole biologically relevant spectral window (500-3500 cm$^{-1}$). Chemoselective and quantitative information have been already recorded by researchers from mouse brain tissue, using MCARS setup, identifying component like myelin, grey matter, granule cells, Purkinje cells, white matter [48]. Here MCARS microscopy has been carried out to characterize solid tumor area and normal area in a mouse brain tissue specimen. Within the scope of this thesis we want to prove that it is possible to distinguish single spectra from cancer and normal area, after they have been properly elaborated with the maximum entropy method (MEM) explained in the previous chapter. Then, in the last section, a chemical mapping of brain tissue will be carried out in order to both identify tissue components and discriminate the border between cancer and normal tissue.

4.2.1 Sample preparation

A solid tumour is induced in mouse brain tissue by intracerebral stereotactic implantation of murine mK1735 melanoma cells. Couples of cryosections, 13 µm thick, are cut from different areas. The first sample from each couple is prepared as H&E stained sample and it is used as reference. The seconds is air-dried and it is used with the spontaneous Raman and the MCARS setups. The air-dried samples are delivered on a microscope slide. The sample structure is very fragile and sensitive. For this reason all received sample are stored at -70$^\circ$C. After defrosting, the sample loses water. If this loss is too high, some part of the sample can break, altering its integrity or its flatness. Flatness is an important property of a biological sample to be used for MCARS imaging. An excess of roughness can cause the loss of focus on

![Figure 4.2: H&E-stained reference of (a) sample T6-4 (area in the black box A contains tumorous cells) (b) sample T6-6 (presence of tumorous cells in the left darker area).](image)
the investigated plane. The H&E references of samples T6-2, T6-4 and T6-6, from which all the data have been acquired, are shown in fig. 4.1 and fig. 4.2.

### 4.2.2 CARS and MEM retrieved spectra

Several MCARS spectra have been measured in epi-detection with the objective OlympusMPlan IR 50x (NA 0.55). Fig. 4.3 shows two spectra measured respectively from a cancer area (a) and a normal area (b). Each spectrum is very noisy. The area under interest for pathological analysis is usually from 2700 up to 3500 $cm^{-1}$ (CH/OH region), where the most important spectral changes occur. This spectral area looks very congested and only an inhomogeneous peak can be identified. Data elaboration through MEM algorithm can overcome this problem. Fig. 4.4 shows a normal area of $200 \mu m \times 200 \mu m$ of sample T6-4 before (4.4, (a)) and after (4.4, (b)) MEM extraction. Spectra in fig. 4.4 (c) and (d) have been obtained averaging spectra from 10 neighbouring points (blue boxes in (a) and (b)). The algorithm is capable, once again, of suppressing the overwhelming background. As the signal is fluctuating very much during the acquisition, an excess of averaging can lead to characteristic peaks suppression. Anyway fig. 4.4 (d) shows a spectrum which has been acquired with a low spectral resolution (300 gr/mm), so it is difficult to make some biophysical analysis of the main peak. As shown in fig. 4.5 instead, if a proper reconstruction is performed, together with smoothing, filtering and the use of higher gratings number (600 gr/mm), the peak shows the typical shape of the white matter, in agreement with references in scientific literature [49]. The spectral vibrations between 2800 and 3000 $cm^{-1}$ can be recognised in both Raman and CARS spectra and are assigned to

![Figure 4.3](image)

**Figure 4.3.** Comparison between (a) MCARS single point spectrum of cancer cell in sample T6-4, (b) MCARS single point spectrum of normal area in sample T6-4. No extra averaging, smoothing or filtering has been applied to the raw data.
Figure 4.4: MCARS and MEM retrieved image of normal brain tissue, with respective spectra: (a) 200 µm x 200 µm (200 x 200 pixels) MCARS image of normal area in sample T6-4. Spectral integration for intensity color maps from 2640 to 3000 cm\(^{-1}\) (due to the peak correction), (b) MEM extraction from previous image with integration from 2770 to 3040 cm\(^{-1}\), (c) Average of 10 MCARS spectra from neighbouring points highlighted in the blue area (300 gr/mm) (d) Average of 10 MEM extracted Raman spectra from the same neighbouring point that in c.

Figure 4.5: Four retrieved spectra after normalization from four different points of a normal area in sample T6-4, resolution 600gr/mm, savitzky-golay filtering.

Lipids. In particular CH stretching vibrational mode at 2852 cm\(^{-1}\) (CH\(_2\) symmetric), 2885 cm\(^{-1}\) (CH\(_2\) asymmetric), 2938 and 2958 cm\(^{-1}\) are respectively CH\(_3\) symmetric and asymmetric |48|.
4.2.3 Comparison between Raman and MEM retrieved spectra

The MCARS spectra have been compared with spontaneous Raman ones acquired from the same sample area (but slightly different points). Fig. 4.6 shows a MEM retrieved spectrum of a normal area (in (a)) in comparison to a spontaneous Raman spectrum (in (b)) acquired from a neighbouring area. It can be seen that Raman spectra show a good specificity in a broad spectral area, but require long acquisition time. In fact to obtain a good quality is necessary to use a total acquisition time between 3 to 4 minutes. On the other hand MEM retrieved spectra from MCARS signal are obtained with high speed and high spatial resolution but with a contrast limited to a few Raman peaks. In fact the main sub-peaks of the area around 2800-3000 \( cm^{-1} \) are present in both spectra but the peaks in the fingerprint area are masked because of the excess of noise. The peak around 2400 \( cm^{-1} \) in the MEM spectrum is an artifact due to the phase error fitting. As already outlined, the peak shift between the Raman and the retrieved Raman signal are due to several reasons like setup calibration and phase error fitting. Fig. 4.7 shows MEM retrieved spectra from healthy (a) and tumour (b) tissue. Both of them show very good agreement with the peak shapes of spontaneous Raman spectra in (c) and (d).

![Figure 4.6: Comparison between spontaneous Raman and MEM retrieved spectra of normal brain area.](image)

Figure 4.6: Comparison between spontaneous Raman and MEM retrieved spectra of normal brain area: (a) MEM retrieved spectra from sample T6-4, no tumour area, resolution 600 gr/mm, dwell time: 600 ms. (b) Raman spectrum at 532 nm, dwell time 10 s, 20 accumulations.
4.2.4 Chemical mapping of brain tissue

It is interesting to map the different components of cerebral matter to study their distribution and eventually infer conclusions about associated pathologies. Fig. 4.8 shows the same area (100 x 100 µm) of sample T6-2 raster scanned with the MCARS microscope (a) and elaborated with an RGB mapping program implemented in LabVIEW. Following previous analysis in scientific literature [49] the RGB map is achieved in the following way: cells nuclei are shown in blue color (730 cm⁻¹, see 4.8, circle 1), lipid concentration is shown in red (2850 cm⁻¹, circle 2) and a specific fingerprint area assigned to red blood cells is shown in green (from 1540 to 1570 cm⁻¹, see circle 3).
Obtaining a contrast tumour/normal tissue is, instead, particularly difficult because the spectral areas of interest that undergo some changes are only slightly modified and are spectrally overlapping, as already shown in fig. 4.7. So, even if there are spectral modification, it is difficult to visualize them with a Raman or an MCARS spectrum and convert this chemical contrast into a visible optical contrast. Interesting works has been carried out to create better pseudocolor maps, but the spectra need further processing with PCA (principal component analysis) and/or an SVM (support vector machine) \cite{50}. Nevertheless it is possible to make some biophysical analysis integrating different spectral area of an MCARS raster scanned image and create an RGB map. In fact the ratio lipid/protein concentration in normal brain tissue is higher than in the tumour one. Fig. 4.9 has been created following this assumption. Lipids are shown in red (2850 cm$^{-1}$, see fig. 4.9 (c)) while the phenylalanine peak around 1000 cm$^{-1}$ is in green. An extra contrast is given again by the nuclei at 785 cm$^{-1}$. The right part of the picture appears with a higher red component and can be associated to the healthy area. The left part has, instead, a lower level of lipids and can be associated with tumour area. This result is in very good agreement respect to the H&E stained sample. It is interesting to notice that both mapping in fig. 4.8 and 4.9 have been realized without any MEM reconstruction. Consequently the MCARS technique iteself paves the way for rapid high-resolution label-free medical scanning of biological tissues.
Figure 4.9: 100 x 100 µm image at the border between normal and cancer area (a) MCARS scan. Sample: T6-4, irradiation power: 55mW, acquisition time 600 ms, 200 accumulations, 600 gr/mm (b) RBG map: red = lipids (2850 cm$^{-1}$), green = phenylalanine (1004 cm$^{-1}$), blue = nuclei (785 cm$^{-1}$).
Chapter 5

Studies on enzyme-inhibitor reactions

5.1 Introduction and motivation

Papain (papaya proteinase I) is a cystein protease enzyme present in Papaya which plays a crucial role in diverse biological processes and has medical uses (caries removal, minor sport injuries, applications to overcome gluten intolerance) and industrial applications (meat tenderizer) [51]. The activation of this enzyme is achieved by cleavage of three disulfide bonds, as three are the available active sites. Fig. 5.1 (a) shows the global structure of the Papain and the one of the disulfide bridge (b). The temperature for the enzyme activation is around $65^\circ$ in a pH environment between 6 and 7. The enzyme counts already ten verified inhibitors. This chapter presents a study on the inhibition reaction of Papain with a new experimental molecule. As the scope of this study is to clarify the inhibition mechanism, most of the solutions are realized only with the use of the molecule which is considered the active site of Papain, called L-Cystein, shown in fig. 5.1 (c). After this, the Raman microscope and the MCARS system are both used to follow the enzyme-inhibitor reaction in real time. Clearly, as already mentioned several times in previous chapters, MCARS is advantageous when a fast acquisition time is required.

The new inhibitor has been provided by Johannes-Gutenberg-Universität of Mainz. This molecule, called n-(cyanomethyl)acetamide (but referred to as CN-I1 from now on) is shown in fig. 5.2 (part (a), left side), in which the reaction mechanism with L-Cysteine (part (b), right side) is sketched. In this chemical reaction the C≡N

\[ \text{C} \equiv \text{N} \]

To get a list of Papain inhibitor and a insight into the activation mechanism, see data sheet at: [http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/papain.html](http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/papain.html).


\[ \text{C} \equiv \text{N} \]

Johannes-Gutenberg-Universität, Institut für Pharmazie, AK Schirmeister, Mainz (Germany).
Figure 5.1: (a) Papain schematic structure. Molecular weight: 23,406 Da. The yellow highlighted area is a possible position of the active site (only representative). The enzyme is hydrophobic. (b) Disulfide bridge (c) L-Cysteine, the active site of Papain in which the S-H bond is made available after disulfide bridge cleavage.

bond and the S-H bond respectively in the inhibitor and the active site, merge in a S-C=N structure. The evolution of this bonds will be studied into details.

5.2 Papain inhibition reaction

To prove that the global inhibition reaction can take place, a Papain sample has been mixed with the inhibitor CN-I1. Fig. 5.2 shows the Raman spectral interval of interest before (CN-I1 alone) and after the reaction takes place. In a1) the C≡N bond is well recognisable and is composed by two sub-peaks due to its symmetrical and asymmetrical stretch. The C=N spectral area in a2) is also shown as a reference. Then a solution of Papain is mixed with the inhibitor (final concentration respectively 100 mM and 400 mM) and the result is shown in b1) and b2). To obtain the best results the integration time has been set to 5 s, 200 accumulations. Figure C1) and C2) show the difference in the peak intensity before and after the reaction: the C≡N decreases as expected, due to the formation of C=N bond, which provokes an increase in the C=N area. For the reaction to take place, the final spectra have been measured after leaving the solution for one hour at room temperature. This result is very important as in scientific literature this reaction is not well characterized (only a few studies have been done, see e.g. [52] and [53]).

It is also possible, as shown in fig. 5.4 that the reaction products will assume
Figure 5.2: (a) n-(cyanomethyl)acetamide, referred from now as CN-I1 with the C≡N bond highlighted in red, (b) L-Cysteine, with S-H bond highlighted in yellow, (c) reaction products, with C=N bond highlighted in green.

a different configuration than the one previously described in fig.5.2 as pointed out in [54].

Figure 5.3: (a1), (a2) C≡N and C=N interval in CN-I1 spectrum 400 mM in aqueous buffer and DMSO. (b1),(b2) C≡N and C=N interval in CN-I1 and Papain mix spectrum (respective concentration 400 mM and 100 mM). (c1), (c2) differential spectra from before and after the reaction. Acquisition at 532 nm.
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Figure 5.4: Possible evolution of reaction products of CN-I1 and L-Cysteine according to reference [54].

5.3 MCARS sensitivity to C≡N bond

A preliminary study has been carried out to verify the capability of both the Raman microscope and the MCARS system to detect at least the C≡N bond and their relative sensitivity to it. Liquid solutions present a very weak signal in epifluorescence. Consequently a home-built forward detection system has been assembled for this study and has already been described in section 2.2.3 Fig. 5.5 (a) shows the MCARS spectrum of CN-I1 860 mM in aqueous buffer. The C≡N bond can be identified as well as the C-N-H bond. The latter bond is unaltered during the reaction. The high level of background is mainly due to water. The spontaneous Raman spectrum is recorded fig. 5.5 and, if compared with the MEM retrieval of the MCARS it is interesting to notice that the sensitivity of the MCARS system is, in principle, higher. In fact in fig. 5.5 (b) the peak of interest are very sharp and there is a lower background. Unfortunately the non resonant contribution of the MCARS system limits the sensitivity to a concentration of 100 mM, as already learnt in section 3.3. The Raman system can detect a concentration down to 20 mM.

5.4 Real-time detection of inhibition reaction

The reaction depicted in fig. 5.2 has been studied in real time with both experimental systems. It is possible to accelerate or to slow down the reaction speed modifying the pH level of the aqueous buffer. When the pH is adjusted to 7.4 the total reaction time can be more than one hour and all the main peaks variations can be detected with a normal Raman microscope. When the pH level reaches 7.8 the whole reaction takes place in about 3 minutes and the detection requires higher integration speeds. Both situations have been investigated. Before the reaction takes place all reference spectra from the two reagents have been recorded and are shown in fig. 5.6

Fig.5.7 shows the recording of the real time reaction between inhibitor CN-I1 and
Figure 5.5: Three different spectra of a solution of CN-II 860 mM in aqueous buffer (PBS): (a) MCARS spectrum (dwell time 400 ms, 100 averages), (b) MEM retrieved spectrum, (c) Raman spectrum (dwell time 5 s, 200 accumulations, at 532 nm)

The active cite L-Cysteine recorded with a Raman microscope. The spectra are taken every 10 minutes interval and the first spectrum has been recorded after 20 minutes from the beginning of the reaction. It is evident, once again, the consumption of both the triple bond and the -S-H bond together with the creation of a double bond. Fig. 5.8 shows the extrapolated peaks time behaviour from which it can be deduced that the reagents are probably undergoing a first or a second order chemical reaction.

The MCARS detection has been applied to the same reaction but accelerated, at pH level of 7.8. The estimated total reaction time is between 2 and 3 minutes. The two spectra shown in fig. 5.9 has been extracted respectively at the beginning (a) and a the end (b) of a matrix of 900 spectra measured in real-time every 200 ms for a total of 3 minutes. For practical reasons (manual mixing) the data acquisition starts around 30 seconds after the reagents mix. This very interesting result shows the capability of the MCARS experimental system to qualitatively follow the time evolution of all the peaks involved in the reaction even with a high amount of noise and background. This results is of fundamental importance and paves the way for future studies involving, in general, fast biochemical reactions.
Figure 5.6: Reference spectra at the time $t = 0$. (a) MCARS spectrum of CN-I1 430 mM in aqueous buffer (PBS), (b) MCARS spectrum of L-Cysteine 430 mM in aqueous buffer (PBS), (c) spontaneous Raman spectrum of CN-I1 430 mM in aqueous buffer (PBS), (d) spontaneous Raman spectrum of L-Cysteine 430 mM in aqueous buffer (PBS).

Figure 5.7: Quantitative spectral time evolution of the reaction depicted in fig. 5.2. The spectra are shown only in selected relevant spectral intervals, specifically C=N bond at 1640 cm$^{-1}$, C≡N bond at 2270 cm$^{-1}$ and S-H bond at 2580 cm$^{-1}$. First measure done after 20 min after the mix of the reagents. Solution consists of: CN-I1 and L-Cysteine 860 mM in aqueous buffer. Water has been substituted with deuterium $D_2O$ to follow the evolution of the -S-H vibration; pH 7.4. Acquisition parameters: dwell time: 500 ms, 100 accumulations.
Figure 5.8: Time evolution of: (a) C≡N bond in CN-I1, (b) S-H bond in L-Cysteine, (c) C=N bond in the products. First measure take after 20 min since the beginning of the reaction. Vertical scale: intensity in arbitrary units.

Figure 5.9: Time evolution of: (a) C≡N bond in CN-I1, (b) S-H bond in L-Cysteine, (c) C=N bond in the products. First measure take after 20 min since the beginning of the reaction. Vertical scale: intensity in arbitrary units.
Chapter 6

Conclusion and outlook

Multiplex-coherent anti-Stokes Raman scattering is a versatile and powerful technique. It can provide chemically selective maps of biological tissue with high resolution, on the order of $\mu$m, and high sensitivity. Being also non-invasive and label-free, its applications to biology and medicine are expected to increase in the future and possibly combined with other nonlinear spectroscopy techniques such as second harmonic generation (SHG), two-photon excitation fluorescence (TPEF) and third harmonic generation (THG). The forward (f-MCARS) and the backward (epi-MCARS) detection systems can be alternatively used to achieve the best measuring conditions. This work proves that chemical mapping, based on well-known and classified vibrational resonances, can distinguish between normal and tumorous tissue when the ratio between lipid and protein is taken into account. Through the utilization of the MEM algorithm it is also possible to distinguish specific spectral characteristics of healthy and cancer matter. The studies about a specific enzyme-inhibitor reaction prove (i) the good sensitivity of MCARS to biologically relevant bonds like the $\text{C}=\text{C}$ and (ii) the superiority of the MCARS system when applied to fast dynamics. The possibility of performing quantitative measurements is fundamental to follow in details fast dynamics in chemical reactions.

Despite of these advantages, MCARS systems are, at the moment, expensive and their use is mainly limited to research applications. On the other hand spontaneous Raman spectroscopy, together with its related techniques (SERS, RRS), is also rapidly improving and presents already a user friendly setup. Better optical components make Raman microscopes easily compatible with UV sources, improving signal levels. Tunable and pulsed laser systems allow to implement new techniques either more sensitive or able to test molecular dynamics. Consequently for an MCARS system to be competitive a lot of research must be done. The transition from non-collinear to collinear geometry, exploiting tight focusing conditions,
has already simplified CARS and MCARS implementation. The ultimate problem is still represented by the nonresonant background contribution, which has not been solved completely. The maximum entropy phase retrieval algorithm represents only a partial solution to the problem as some resonances are lost in the retrieval process. Besides resonances which are not visible in the MCARS spectrum will not be retrieved and a part of the algorithm results is user dependent. A possibility for non-resonant contribution suppression, not explored in the present work, is to modify the overlap between the Stokes and the probe beams. This approach, called time-resolved MCARS is of growing interests and it could be implemented in the experimental setup used in this work. Improving the system sensitivity leads to an increase of the acquisition rate, which is of fundamental importance if high resolution and fast scanning want to be achieved at the same time. Future studies can examine also the possibility of using fiber-based laser sources which could reduce the cost and the complexity of the system. Besides optics adaptation can in principle extend the penetration depth of the detection and eventually apply MCARS to intraoperative endoscopy.
Acknowledgement
Ringraziamenti

It is a must to thank Herr Prof. Dr. Marcus Motzkus who guested me in his group, giving me the possibility of developing this challenging and cutting-edge master thesis putting myself into test. A thanks also to Prof. Dr. Giulio Cerullo who suggested me this collaboration.

A special thank to Prof. Dr. Hans Volpp who can be contagiously enthusiastic about crazy scientific subject and taught me lot of chemistry, about which he made me realize I knew absolutely nothing.

Dr. Tiago Buckup, you helped me and advised me in crucial moments even when you were overwhelmed of things to do. Thank you.

Nick Paul, thank you for having your door always open for discussion and advices.

Andreas Ludwig, Dr. Elisabeth Brühl, Daniel Herz thank you for all the funny office talks and for keeping me company. Dr. Lukas Brückner you have been a good teacher, thanks. Dr. Man Jiang, you have been and you are such a supportive friend and an incredibly motivated scientist. Mariana Ishikawa it was a pleasure to meet you and to work with you, that was team work.

Nicolas Wenzel, for your tireless and unconditional support and your IT and scientific advice, vielen dank.

Un grazie di cuore alle mie compagne di universitá Elisabetta Coppola, Ludovica Guarnieri ed Anastasia Leone per lasciarmi ricordi divertenti che offuscheranno, con il tempo, i miei incubi di studente pendolare.

Ringrazio Michele Miná, senza il quale il mio approccio alle questioni scientifiche non sarebbe lo stesso di oggi.

Un ringraziamento speciale va, infine, alla mia famiglia per il costante supporto morale e in particolar modo ai miei genitori per il loro costante e incondizionato sostegno economico. Per come é il mondo oggi, bisogna avere una gran fortuna alla nascita.
Appendix A

The Grating Spectrometer

In a grating spectrometer the light under analysis is focused on the entrance slit (fig.A.1, ES). The light then diffracts on a collimating (or focusing) mirror M1 and impact on a diffraction grating. A diffraction grating is a collection of reflecting elements whose distance is comparable to the detected light wavelength. A typical diffraction grating, shown into more details in the dashed box, consists of a substrate with a large number of parallel grooves ruled or replicated in its surface and overcoated with a reflecting material. To understand the working principle of a grating system let us consider the two represented rays 1 and 2, of wavelength \( \lambda \), impinging on two consecutive grooves. After reflection on the grooves, the path difference between rays 1 and 2 is:

\[ dsin\alpha + dsin\beta \]  \hspace{1cm} (A.1)

which clearly results in constructive interference only if:

\[ d(sin\alpha + sin\beta) = m\lambda \]  \hspace{1cm} (A.2)

where \( m \) is an integer (positive or negative) and is called diffraction order. This basically results in the fact that different \( \lambda \) have diffraction maxima at different angles:

\[ \beta_{max,\lambda} = sin^{-1}(sin\alpha + m\frac{\lambda}{d}) \]  \hspace{1cm} (A.3)

Taking all the other grooves into account does not change the basic equation but sharpens the peak. It is clear that the spacing of the grooves is crucial to the performance of the system and it is measured in gr/mm (number of grooves per millimeter). Typical diffraction gratings nowadays have 600gr/mm up to 2400 gr/mm. After being spectrally separated the different colors are focused on a detector. In particular each small color (frequency) interval is focused on a different pixel, which will be a CCD cell. Globally the point of the entrance slit of focused on each pixel of the
detector. The higher is the grating number the better is the spectral resolution. In some systems the light is dispersed so much from the gratings that most of it is lost (is not getting to the detector), but, on the other hand, the spectral resolution can be very high. The usual achievable resolution is a fraction of nm.

Figure A.1: Scheme of a grating spectrometer: ES = Entrance Slit; M1, M2 = mirrors; G = gratings; D = detector; in the dashed box: 1 and 2 = incoming rays; $\alpha$ and $\beta$ = angles respectively at the left (positive) side and at the right (negative) side of the normal to the grating surface; $d$ = distance between two consecutive grooves.

See [55] as a reference for a good explanation on how gratings work, are realized and characterized.
Appendix B

Maximum Entropy Method

The purpose of the Maximum Entropy Method (MEM) is to solve not well-posed (ill-posed) problems, that means problems lacking of at least one among the properties of existence, uniqueness or stability of the solution. MEM states that of all the feasible solutions, we should choose the one that has the maximum entropy. This choice is not obvious but there are several mathematical justification to this approach which can be found in [56]. The MEM used in this thesis work exploits the MEM1 school of thought and in particular its modeling and implementation made by J.P. Burg in 1978, which is commonly used in spectral analysis. This short appendix simply wants to recall the main mathematical results on which the whole MEPR is based.

Let us suppose we are fully sampling a time-varying signal, and we do it a number M of times. A single sample will then be \( y(t_m) \), with \( 0 < m < M \). This must be done under the hypothesis of weakly stationary process ¹. It is well known that the power spectrum \( S(f) \), with f frequency, of a time varying signal and its autocorrelation function \( R(m) \) are a Fourier-transform pair (Wiener-Khinchin theorem). The reconstruction of \( S(f) \) could be done by windowing \( R(m) \) (multiplying it by a rectangle function) and taking the Fourier transform (FT). This windowing process introduces truncation errors due to the fact that it is impossible to predict which windowing function gives the best result. From this problems arises the needs for better reconstruction algorithm.

First of all we can explicitly write \( S(f) \) and \( R(m) \) as:

\[
S(f) = \mathcal{F}[R(m)] = \sum_{m=-\infty}^{\infty} R(m) e^{-2\pi imf} \tag{B.1}
\]

\[
R(m) = \mathcal{F}^{-1}[S(f)] = \int_{-1/2}^{1/2} S(f) e^{2\pi m f} df \tag{B.2}
\]

¹It means that the mean and the variance of the quantity under study are independent of m.
the MEM problem is to find $S(f)$ through the maximization of the entropy $H$ which, according to the information theory, can be written as:

$$H = \int_{-1/2}^{1/2} \ln S(f) df$$

(B.3)

with the constraints given by equation (B.2) for $|m|\leq M$ as the data are time limited. The $1/2$ factor derives from choosing the signal bandwidth $B=1$ to simplify the calculations. The solution to this problem is unique [56]. It is possible to convert the constrained maximization to an unconstrained one, using Lagrange multiplier method. This reduces the problem to the maximization of the following functional:

$$Q = \int_{-1/2}^{1/2} \ln S(f) df - \sum_{m=-M}^{M} \lambda_m \left[ \int_{-1/2}^{1/2} S(f) \exp(2\pi ifm) df - R(m) \right]$$

(B.4)

where $\lambda_m$ are Lagrange multipliers. The problem is solved through variational method. Considering that we are interested in the variation of $Q$ respect to $S(f)$ (the unknown), we must solve:

$$0 = \delta G = \int \delta S(f) \left[ \frac{1}{S(f)} - \sum_{m=-M}^{M} \lambda_m \exp(2\pi ifm) df \right]$$

(B.5)

As $\delta S(f)$ is arbitrary, the quantity in square brackets must be zero:

$$S(f) = \frac{1}{\sum_{m=-M}^{M} \lambda_m \exp(2\pi ifm) df}$$

(B.6)

To obtain the expression used in Chapter 3 we must substitute $C_k = \lambda_m^*$, which is a sequence of $2M+1$ Hermitian coefficients. The Hermitian properties allow to decompose $C_k = \alpha_k * \beta_k$, where with $*$ we mean the convolution operator. Considering also that $S(f)$ is a real quantity it’s possible to rearrange the equation in the final expression (see [56]):

$$S(f) = \frac{P_M}{\left| \sum_{m=0}^{M} a_m \exp(i2\pi fm) df \right|^2}$$

(B.7)

The $P_M$ and $a_m$ coefficients are the solution of the Toeplitz system that can written in compact form as:

$$\sum_{m=0}^{M} a_m R(n-m) = P_M \delta_{nm}$$

(B.8)

with $n = 0,1,...,M$ and $\delta_{nm}$ is a Kronecker delta.
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