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**Bio-Functionalized Interfaces and
Graphene-Based Detection
for Single-Molecule Sensing:
Insights from Vibrational Spectroscopy**

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*To Ylenia,
To my family
To Paky and Sissi*

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Abstract

Large-area bio-functionalized interfaces are fundamental for determining the sensitivity and selectivity of high-performance biosensors. Numerous biomarkers have been detected at the physical limit of a single-molecule within 100 μL of the assayed solution (corresponding to a concentration of $\sim 10^{-20}$ M) using a sensing element with a high density of bioreceptors, $\sim 10^{12} \text{ cm}^{-2}$, immobilized onto a solid substrate. Remarkably, the affinity binding involving just one or a few analyte molecules induces an extended surface potential variation across the bio-functionalized sensing interface. This effect involves trillions of unbound molecules, in a domino-like cascade, ultimately generating a measurable signal that enables detection. The objective of this doctoral thesis is the study and the application of bio-functionalized interfaces, focusing on those encompassing a monolayer of antibodies physisorbed onto a gold substrate, typical of immunosensors. A novel biosensing approach is proposed, leveraging the capacitive coupling of a large-area interface bio-functionalized with human Immunoglobulin M antibodies (anti-IgM), and a single-layer graphene transducer inspected through Raman spectroscopy. Proof-of-concept experiments demonstrated the detection of IgM antigens at concentration as low as 10^{-19} M. The intrinsic sensitivity of phonons in the two-dimensional material enabled signal transduction, observed as a shift of the charge neutrality point of the graphene layer in response to the surface potential changes induced by the formation of a few antibody-antigen immunocomplexes. Furthermore, the properties of the antibody layer are investigated by polarization-modulation infrared reflection-absorption spectroscopy (PM-IRRAS). The prominent spectral features are recorded with a high signal to noise ratio (> 100), enabling the analysis of the amide bands. Specifically, the deconvolution of the amide I band, characteristic of proteins, provided valuable insights into the protein secondary structure. A method is developed to assess the structural order of physisorbed layer of antibodies, based on the comparative analysis of PM-IRRAS spectroscopy and attenuated-total-reflection (ATR) spectroscopy measurements. Our findings indicate partial order within the biolayer, primarily involving β -sheets units. Further analyses suggest that the densely packed layer of physisorbed proteins undergoes various metastable states, upon electrical and biological stimuli. These configurations could influence the biolayer's properties

on multiple scales, from macroscopic features such as the water contact angle, to the structure of the overall protein dipole moments, involving charge rearrangements and inter- or intra- molecular forces. Within this framework, this doctoral thesis contributes to the study and understanding of the complex network of interactions within a layer of physisorbed antibodies constrained in a two-dimensional (2D) non-physiological environment, offering new perspectives for enhancing the performance of biomaterials in biosensing application.

Abstract

Il ruolo delle macro-interfacce bio-funzionalizzate è fondamentale per ottenere sensibilità e selettività elevate in biosensori per applicazioni nella diagnostica medica. La rivelazione di un gran numero di biomarcatori è stata dimostrata, con performance al limite fisico di rivelazione di una singola molecola in 100 μL di soluzione ($\sim 10^{-20}$ M), utilizzando interfacce costituite da uno strato di bio-recettori immobilizzati su un substrato solido ad alta densità $\sim 10^{12}$ cm^{-2} . Il legame di una sola, o poche molecole di analita sullo strato di riconoscimento innesca una variazione estesa del potenziale superficiale dell'interfaccia di rivelazione. Questa modifica coinvolge trilioni di molecole distanti dal sito di legame, in un effetto di propagazione a cascata tipo domino, che produce un segnale misurabile. L'obiettivo di questa tesi di dottorato riguarda lo studio e l'applicazione di tali interfacce bio-funzionalizzate, focalizzandosi, in particolare, sull'analisi di strati biologici di anticorpi fisisorbiti su substrati d'oro, tipicamente impiegati in sensori immunometrici. Nella tesi è proposto un approccio innovativo per la rivelazione di proteine, basato sull'accoppiamento capacitivo tra una macro-interfaccia bio-funzionalizzata con anticorpi umani Immunoglobuline M (anti-IgM), e un trasduttore a grafene, interrogato tramite spettroscopia Raman. La trasduzione del segnale biologico è permessa dall'intrinseca sensibilità dei fononi nel grafene. Gli esperimenti riportati dimostrano preliminarmente il principio di funzionamento del biosensore, che è stato capace di rivelare antigeni IgM fino a concentrazioni di 10^{-19} M. Inoltre, le proprietà dello strato di anticorpi fisisorbiti sono investigate tramite spettroscopia di riflettività-assorbimento infrarosso con modulazione di polarizzazione (PM-IRRAS). La tecnica consente la misura dello spettro IR di film biologici di spessore nanometrico (~ 10 nm,) con un elevato rapporto segnale rumore (> 100). L'analisi delle bande originate dalla catena peptidica nelle proteine, con particolare attenzione alla deconvoluzione della banda amide I, fornisce informazioni rilevanti sulla struttura secondaria degli anticorpi adsorbiti. E' stato sviluppato un metodo per indagare l'ordine strutturale delle molecole nello strato biologico, basato sull'analisi comparativa delle misure di spettroscopia PM-IRRAS e di spettroscopia a riflessione-totale-interna (ATR), che ha permesso di verificare un ordine parziale dei β -sheets delle proteine adsorbite. Ulteriori analisi suggeriscono che lo strato di anticorpi fisisorbiti, ad elevata

densità, su oro incorre in transizioni tra stati metastabili, indotti da stimoli di natura elettrostatica e biologica. La spettroscopia PM-IRRAS contribuisce allo studio di tali stati con informazioni dirette sulle interazioni agenti a livello dei dipoli oscillanti. Questa tesi costituisce un supporto alla comprensione del complesso sistema costituito da molecole biologiche vincolate in un ambiente bidimensionale non fisiologico, offrendo prospettive per migliorare le prestazioni e l'integrazione di questi biomateriali in biosensori ultrasensibili.

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List of symbols

λ	wavelength
$\tilde{\nu}$	wavenumber
c	speed of light
k_b	Boltzmann's constant
ϵ	vacuum permittivity
$\hat{\epsilon}$	complex dielectric function
n	real refractive index
n_∞	real refractive index in the visible spectral range
k	absorption index
λ_D	Debye length
E_F	Fermi energy
v_F	Fermi velocity
d_p	penetration length

Introduction

Bio-receptors are essential molecules in living organisms, naturally selected to perform highly specific tasks and trigger prompt responses to harmful conditions. The immune system comprises a network of biomolecules, including antibodies, providing tailored reactions against antigens from pathogens, virus, cancers or diseases. Research and development in biomedical technologies for the early disease diagnosis takes advantage of the highly selective biomolecular interactions to design advanced biosensor platform for detecting clinically relevant biomarkers.

Single-Molecule Transistor (SiMoT) biosensors are bioelectronic devices capable of detecting biomarkers at concentrations as low as the zeptomolar (zM) range^{1,2}. Remarkably, a limit of detection of 10 zM corresponds to 1 ± 1 analyte molecule within 100 μL of the assayed solution. This ultra-sensitive performance is due to the incorporation of a bio-functionalized interface, consisting of a densely packed layer of bio-receptors, immobilized onto a sensing electrode. The single-molecule sensitivity of the SiMoT technology opens a double perspective. On one side, it inspires the possibility of developing novel biosensing platforms exploiting large-area sensing interface through various transduction mechanism. Furthermore, the molecular mechanism taking place across the biolayer of receptors, underlying the huge SiMoT signal upon a single binding unveils general behaviour of two-dimensional protein networks and demand an in-depth investigation and understanding.

This manuscript focuses on the study and proof-of-concept of bio-functionalized sensing interfaces which are the core of single-molecule biosensors. Specifically, it examines large-area, millimetre-sized interfaces, which are significantly larger than the molecular footprint of the detected analytes, $\sim (10 \text{ nm})^2$. The discussion is limited to immunosensors, featuring a biolayer of capturing antibodies (human Immunoglobulins G or M) physisorbed onto a gold substrate for the detection of the corresponding affinity antigens.

The detection performance of single-molecule biosensors relies on the sensitivity of the biological interface to affinity bindings. 1 - 10 antibody-antigen binding events, impacting an area of $\sim (10 - 100 \text{ nm})^2$, trigger extended electrostatic variations across the biolayer, involving a number $>10^7$ of

unbound antibodies, in a collective phenomenon which originates a measurable signal enabling detection. It is worth mentioning here that the expression “few affinity antigens” (or “few affinity bindings”) is used throughout the text referring to an amount of analyte molecules (or binding events) at least 10^8 times smaller than the number of capturing antibodies onto the sensing interface. This elucidation is essential to clarify our focus is about biosensors operating in a range of analyte concentrations where the measured response of the device cannot be simply ascribed to an increase of mass onto the sensing surface (due to bound analytes), but molecular collective amplification phenomena must be in place to account for macroscopic signals.

This manuscript starts with an introductory Chapter 1 on biosensors, focusing on electrolyte-gated field-effect-transistor (EG-FET) biosensors and graphene-based biosensors. A section is dedicated to the SiMoT biosensing technology as it first reached the single-molecule limit of detection for protein biomarkers. Also, insights about the sensing mechanism underlying the SiMoT response are provided.

Chapter 2 provides an in-depth description of proteins (Immunoglobulins and bovine serum albumin) and graphene, which are the relevant materials for this thesis, focusing on their structural, vibrational and electronic properties.

Chapter 3 describes the experimental methods, apparatus, for the fabrication of samples, performance of vibrational spectroscopy experiments and processing of data.

Then, results are described in Chapters 4 and 5. Chapter 4 reports the design, working principle and proof-of-concepts on the newly developed graphene-based biosensor. It employs a large-area sensing interfaces, bio-functionalized with physisorbed human Immunoglobulin M antibodies (anti-IgM), coupled to a graphene transducer. This biosensor has proven capable of IgM antigens detection with a limit of detection of 100 zM, which is a state-of-the-art performance for graphene-based biosensors, using Raman spectroscopy as an innovative means for optical transduction.

Chapter 5 focuses on the investigation of the bilayer of proteins physisorbed on gold through advanced infrared (IR) spectroscopy techniques, such as polarization-modulation IR reflection absorption spectroscopy (PM-IRRAS) and attenuated-total-reflection (ATR) spectroscopy. A method to perform the orientation analysis of monolayers of proteins adsorbed on gold is reported. The analysis on human anti-Immunoglobulin G (anti-IgG), and bovine serum albumin (BSA) reveals a partial orientation of β -sheets upon physisorption. Also, the influence of an electric field annealing and the effect of few affinity bindings on the layer of capturing antibodies is assessed through the analysis of the amide I band in the IR spectra, suggesting the key role of conformational variation of the protein structure.

Finally, Chapter 6 summarizes the main results of the thesis providing a general picture and outlooks. Our findings support the hypothesis that antibody biolayers undergo multiple metastable states upon electrical or biological stimuli, by means of a cascade of intramolecular interactions mainly driven by hydrophobic forces. The infrared spectroscopy investigation revealed that the secondary structure of antibodies is subject to a minor influence in those processes, whereas the tertiary structure is mainly involved.

Chapter 1

Large-area interfaces for single-molecule detection

1.1 Biosensors for single-molecule detection - generalities

A biosensor is an analytical device that converts the information of a biochemical interaction into a measurable signal. According to the IUPAC definition, a biosensor uses specific biochemical reactions to detect chemical compounds. The typical architecture of a biosensor includes a biological component, the *sensing element*, coupled with a *transducer*.

The sensing element represents the core of the device hosting the *bioreceptors* (or *recognition elements*), which take part to the biorecognition reactions with analyte molecules. Bioreceptors are chosen to selectively bind specific analytes. Examples are DNA or RNA strands, antibodies or enzymes, which selectively form biochemical complexes with their complementary strands, affinity antigens or substrate molecules respectively.

On the other hand, the transducer determines the physical mechanism to convert the biological reaction into a measurable output. Different classes of biosensors can be distinguished based on the nature of the bioreceptors or the kind of transduction mechanism.³ Common detection methods use electronic, electrochemical or optical transducers. Electronic biosensors detect analytes through a variation of currents or electrostatic potential across the devices, as for field-effect-transistor (FET) biosensors.⁴ Electrochemical biosensors, instead, reveal analytes leveraging electrochemical

reactions, such as redox reactions, occurring on the sensing electrode into an electrochemical cell.⁵ Differently, optical biosensors detect target molecules on the base of changes in the optical response of the device upon formation of the bioreceptor-analyte complexes, for example through refractive index variations or changes in the fluorescence intensity.⁶

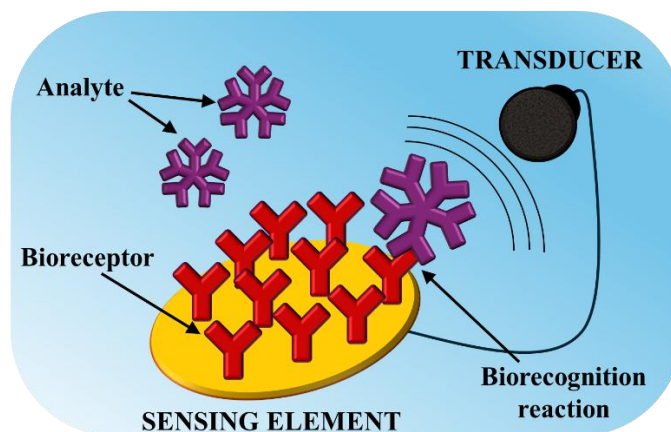


Figure 1.1 Biosensors. Schematic representation of the main component of a biosensor. The sensing element is typically constituted by bioreceptors immobilized on a substrate. Bioreceptors selectively bind analytes in a biorecognition reaction that originate a signal measured by the transducer.

Current research on biosensors is widespread for application in many fields, from healthcare, to environmental monitoring, to food quality control. New technologies and novel sensing approaches aim at boosting the performances in terms of sensitivity, selectivity and accuracy. In the field of medical diagnostic for point-of-care testing, a challenging goal is related with the development of technologies for the detection of clinically relevant molecules, namely *biomarkers*, at low concentrations, from the femto-molar level (10^{-15} M), down to the physical limit of a single analyte in the assayed sample volume. Indeed, the availability of biosensing platform capable of detecting biomarkers with enhanced sensitivity is crucial for the early diagnosis of progressive diseases, through minimally invasive analysis of peripheral biofluids.

The relevant figure of merit, to evaluate the diagnostic power of a biosensor is its limit of detection (LOD). It corresponds to the minimum concentration of analyte that can be detected with a 99.7% confidence level.² Accordingly, single-molecule detection performance correspond to a LOD of 17 zM ($17 \cdot 10^{-21}$ M), that is the concentration of solution containing a single analyte in a drop of 100 μ L. Here, 100 μ L is taken as a typical reference volume in biosensing assay, as it represents the minimum volume that can be dispensed via a pipette with a precision of 1%.⁷

Technologies leveraging nanometre-sized transducing interfaces, such as nanopores or nano-transistors structures have proven able to spot single molecules.^{8–10} Those devices are addressed as *near-field* biosensors, because of the volume probed by the transducer, on the order of (~ 10 nm)³, which is comparable to the size of an analyte molecule. Despite they reach the single-molecule resolution, near-field biosensors are limited to work at concentrations above the nano-molar picomolar level ($> 10^9 - 10^{-12}$ M), as at lower concentrations, the probability of an interaction between a target molecule, diffusing by random Brownian motion in the assayed solution, with the nanometre-sized probe becomes extremely low. This issue is known as the *diffusion barrier* and represents a major limit to the LOD of near-field biosensors.²

On the other hand, sensing approaches overcoming the *diffusion barrier* have been developed exploiting millimetre-wide bio-functionalized sensing interfaces. Indeed, the use of large active area, hosting trillions of biorecognition elements increases the interaction cross section between analytes and bioreceptors, enabling detection at concentrations below the picomolar range. Those sensing strategies are addressed as *wide-field*, opposite to the elicited ones.

On the other hand, since the sensing interface in *wide-field* biosensors has dimensions of (~ 1 mm)², considerably larger than the nanometre-sized footprint of a biomarkers, a further limiting factor to the LOD is the challenge to measure signals standing out from the noise produced by all the bioreceptors not involved in the bio-recognition reaction at low analyte concentrations.

Currently, the two best-performing sensing platforms for biomarker detection available on the market, are based on *wide-field* approach, namely next-generation sequencing (NGS)¹¹ and Single-molecule-array (SIMOA)¹². NGS technology enables the detection and sequencing of DNA strands based on polymerase chain reaction (PCR) amplification mechanism.¹³ It is the gold-standard technique for genomic biomarker detection reaching a limit of detection as low as 10^{-20} M. On the other hand, SIMOA represents the established analytical technique for the detection of protein markers. It uses bio-functionalized magnetic micro-beads (μ -beads), suspended in biofluids to capture. Then detection is performed following an ELISA-type strategy: the biomarker binds a secondary antibody conjugated with a fluorescent enzyme. The μ -beads are distributed across a SIMOA array and fluorescence images are acquired to count the number of captured antigens.¹⁴ This technology has reached a limit of detection of $220 \cdot 10^{-21}$ M, corresponding to few proteins in $100 \mu\text{L}$.¹⁵

1.2 Sensing element: bio-functionalization strategies

The effectiveness of a biosensor in detecting biomarkers is largely determined by its sensing component, as it encompasses the bioreceptors responsible for the capturing of target molecules. Immunosensors, which are the main topic within this thesis, use interfaces bio-functionalized with antibodies to selectively bind affinity antigens. Anchoring biomolecules on a substrate reduces their mobility and could induce molecular deformations or inconvenient orientation of the binding sites, thus compromising the bioactivity of receptors. Therefore, choosing the proper functionalization approach is of great importance to retain the conformation and functionality of bioreceptors. Three main immobilization strategies can be identified, illustrated in figure 1.2: chemical immobilization, physical immobilization, and bioaffinity immobilization.²

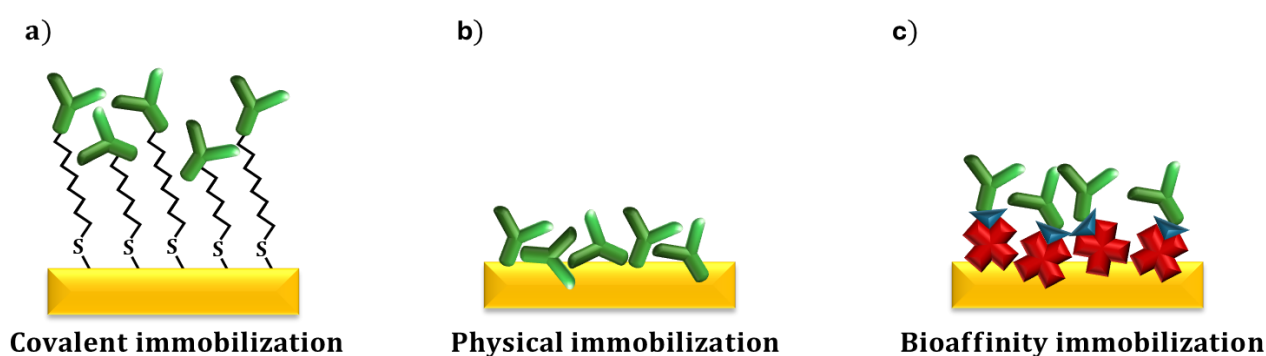


Figure 1.2 Bio-functionalization strategies. a) Chemical immobilization through covalent bounds with a SAM; b) physical immobilization through electrostatic, van der Waals and hydrophobic interactions; c) immobilization through bioaffinity biotin-streptavidin (or avidin) binding.

Covalent immobilization is achieved by covalent binding of bioreceptors to the surface via a self-assembled-monolayer (SAM) of polymeric molecules. In the case of gold surfaces (which is the usual case for bioelectronic devices) the SAM is anchored by thiol groups on gold, while exposing tail groups outward, available for the covalent binding with bioreceptors. Examples are SAM terminating with a carboxyl or amine functional group are used to immobilize antibodies.¹⁶ Covalent immobilization is the favourite approach for protein immobilization as it leads to stable and highly reproducible layer of a highly packed bioreceptors, though it does not ensure an oriented biofilm as the binding could involve several residues on the protein. On the other hand, physical immobilization exploits the spontaneous adsorption (*physisorption*) of biomolecules on the substrate, mainly driven by van der Waals, electrostatic and hydrophobic interactions. It is a convenient and simple functionalization strategy as it does not require any surface modification of the substrate. However, the molecule-substrate binding is considerably weaker compared to a covalent binding, hence physisorption is generally deemed as an uncontrolled process forming heterogeneous and unstable biolayers. The third immobilization strategy exploits the large affinity constant of specific

biomolecules, such as the biotin-avidin (or streptavidin) complexes. The bioreceptors are biotinylated (coupled with biotin molecules) while the substrate is prepared with an avidin layer; the immobilization occurs through the formation of highly stable biotin-avidin complexes holding bioreceptors on the surface.

Depending on the nature of the bioreceptors and the substrate material the optimal bio-functionalization strategy is selected. Within this thesis, we bio-functionalized solid surfaces through physisorption. This bio-functionalization strategy has proven effective for fabrication of sensing interfaces of biosensors, showing similar detection performances compared to those encompassing covalently immobilized capturing antibodies. The rationale beyond the choice of utilizing physisorbed biolayer lays in the evidence that it retains the fundamental properties enabling single-molecule detection, while being the simplest biolayer possible, both in terms of fabrication protocols and composition.

1.3 Electrolyte-gated field-effect transistor biosensors

Field-effect transistors (FETs) are electronic devices offering promising perspectives for biosensing applications. FETs are three-terminal devices including a source electrode and a drain electrode connected by a semiconductor channel, and a gate electrode separated from the channel by an insulating layer. A gate voltage (V_G), modulates the charge carrier density in the channel thus enabling the control of the source-drain current (I_{DS}) at constant drain potential (V_D). The electronic figure of merits of a FET are its output characteristic (I_{DS} vs V_D) and its transfer characteristic (I_{DS} vs V_G). The incorporation of a layer of bioreceptors within the FET architecture, renders it a bioelectronic device endowed with sensitivity and selectivity toward a target compound.¹⁷ The fabrication of FET biosensors using inorganic/organic semiconductors, or 2D and 1D materials, combined with the use of flexible substrates offers great versatility for the production of miniaturized, wearable and smart devices for healthcare.

Among them, electrolyte-gated field-effect-transistor (EG-FET) biosensors represent a promising bioelectronic technology for the label-free detection of biomarkers operating in aqueous environment.^{18,19} The structure of an EG-FET is in figure 1.3a, for the case of a top-gated device. EG-FETs incorporate a liquid electrolyte connecting the gate electrode with the transistor channel. A layer of bioreceptors is integrated within the EG-FET architecture, through the bio-functionalization of the channel, or the gate surface. The latter case is the interesting one within the scope of this thesis.

Mobile ions in the electrolyte accumulate at channel/electrolyte and gate/biolyer/electrolyte interfaces, forming two electrical double layers (EDLs) as depicted in figure 1.3b. The two EDLs act as two parallel plate capacitors, establishing a capacitive coupling between the bio-functionalized gate electrode and the transistor channel. Figure 1.3c features the equivalent circuit of a gate-functionalized EG-FET, including the two EDLs capacitance, and a voltage generator (V_{Bio}) accounting for the biolayer.¹⁹ The detection mechanism of a EG-FET biosensors is based on variations of the electrostatic configuration at the sensing electrode upon biorecognition reactions.

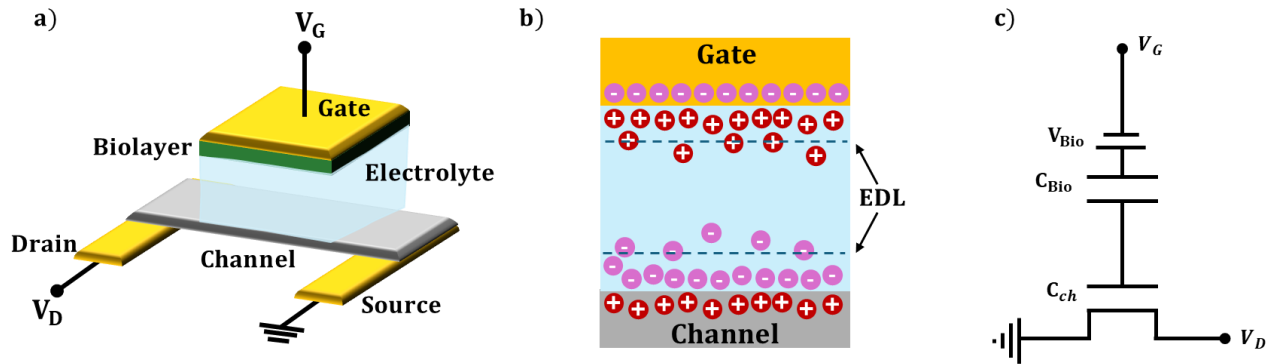


Figure 1.3 EG-FET structure. **a)** Schematic representation of a top-gated EG-FET with a bio-functionalized gate electrode; **b)** Schematic representation of the EDLs forming at the gate/electrolyte and electrolyte/channel interface in a EG-FET; **c)** Equivalent circuit of a gate-functionalized EG-FET, including the two EDL capacitances, C_{Bio} and C_{ch} , and a voltage generator V_{Bio} accounting for electrostatic potential drop across the biolayer.

Specifically, the presence of analyte may alter the morphological structure of the EDL capacitance at the gate/biolyer/electrolyte interface resulting in a different EG-FET transconductance, or induces a surface potential variation of the sensing interface resulting in a shift of the EG-FET threshold voltage.^{1,20}

EG-FET biosensors have proven stable operation in aqueous electrolyte which is important to ensure a physiological environment to the biomolecules. However, it is important that the applied voltages remain within a safe range, 0.1V - -0.5V, to avoid water electrolysis and any faradic reactions that may degrade the bio-functionalized interface.

1.4 Graphene-based biosensors

Novel materials have accompanied the development of new biosensing approaches and offered perspectives to improve the performance of existing technologies, pursuing the identification of relevant biomarkers with increased precision, efficiency and reliability. 2D materials have attracted

the attention of researchers for their interesting physico-chemical properties. Graphene and graphene-related materials, including reduced-graphene-oxides,²¹ carbon-nanotubes,²² graphene quantum-dots,²³ have found numerous successes in the field of label-free biosensing thanks to a high surface area-to-volume ratio, biocompatibility, mechanical strength and high conductivity. A variety of detection strategies have been demonstrated implementing graphene as an electrical or optical transducer, leveraging the highly sensitive modulation of its electrical conductivity and refractive index in response to biochemical recognition reactions on its surface.^{24,25} For example, graphene has been employed to enhance the sensitivity of surface-plasmon-resonance (SPR) platforms. Also, it is implemented in fluorescence-based biosensors leveraging its quenching ability. Graphene-based electrolyte-gated devices have stood out for their ultrasensitive detection performances. Within this framework, graphene field-effect transistors (gFET) are sensitive bioelectronic sensors capable of detecting small molecules,²⁶ nucleic acids,²⁷ proteins,²⁸ and virus particles.²⁹ Those biosensors incorporate a layer of biorecognition elements anchored onto the graphene channel. The binding with analyte molecules alters the charge carrier density, through field-effect or direct chemical doping of the graphene layer, thus modifying its conductivity and enabling detection by measuring the corresponding variations in the transistor figure of merit. Limit of detection as low as the atto-molar (10^{-18} M) level can be reached by gFET biosensors. Additional modification strategies of the graphene layer can lead to even higher sensitivity, as demonstrated using corrugated graphene for DNA detection reaching a limit of detection (LOD) of $600 \cdot 10^{-21}$ M.³⁰

However, improving the sensitivity could require complex functionalization protocols which can hinder the broader applications of those devices. Covalent immobilization of bioreceptors on graphene could undermine the electrical properties of the carbon lattice. In contrast, the physical immobilization of graphene is possible by means of the π - π or hydrophobic stacking of specific linker molecules, such as 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBASE), coupled to the bioreceptors.

Also, another promising approach consists in the coupling of a graphene-based transducer with a bio-functionalized gate electrode.^{31,32} The functionalization of the Au gate electrode, rather than the graphene channel, allows to decouple the sensing interface from the graphene transducer, thus taking advantage of the many validated and reliable functionalization protocols known for Au bio-functionalization while preserving the structural integrity of graphene layer. Indeed, ultrasensitive detection has been demonstrated using a gate-functionalized gFET, incorporating an aptamer biolayer, for the detection of prostate-specific antigen, reaching a LOD of $3.5 \cdot 10^{-19}$ M.³³

1.5 Single-molecule detection with large-area electrolyte-gated field-effect transistor: SiMoT biosensors

The first biosensing technology capable of single-molecule detection of protein biomarkers was the Single-Molecule-Transistor (SiMoT) biosensor, showcased by the group of Professor Torsi in 2018.¹

The SiMoT biosensor is an EG-FET, incorporating a gold gate electrode bio-functionalized with a monolayer of capturing antibodies for the detection of the corresponding affinity antigens. The gate electrode is coupled to a P3HT organic semiconductor channel, through water serving as the electrolyte, as shown in figure 1.4.

The key element in SiMoT biosensors is the sensing electrode, represented in figure 1.4b. It is a gold electrode whose active area, of about 20 mm², encompasses a monolayer of antibodies highly packed with a density of $\sim 10^{12}$ proteins cm⁻².

SiMoT biosensor design is optimized to maximize the sensitivity of the source-drain current to electrostatic variations occurring at the gate electrode. Upon exposure to analyte solution, the transistor threshold voltage (V_{Th}) shifts by several mV, as schematically depicted in figure 1.4.

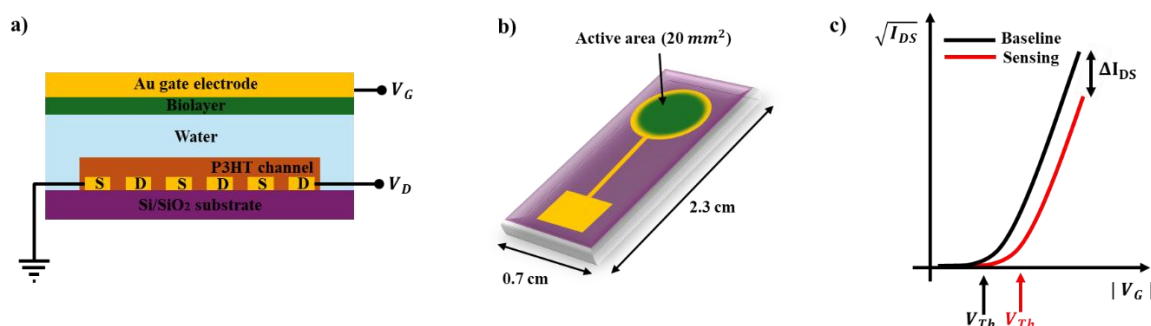


Figure 1.4 SiMoT biosensor. **a)** The SiMoT biosensor architecture. It is that of top-gated EG-FET with a Au gate electrode, interdigitated source and drain contacts, an organic semiconductor channel, and water serving as electrolyte. The gate is bio-functionalized with a layer of bioreceptors. **b)** Sensing gate electrode in a SiMoT biosensor. It consists of an Au electrode onto a Si/SiO₂ substrate having a bio-functionalized circular pad (the active area) and a square pad for electrical connection. **c)** Representative response of the SiMoT biosensor upon exposure to analyte solution, showing a threshold voltage (V_{Th}) increase. Detection is accomplished reading the current variation (ΔI_{DS}) in the transfer curves.

Remarkably, SiMoT biosensor demonstrated detection at extremely diluted analyte solutions, up to 10⁻²⁰ M, comprising 1±1 antigen in 100 µL (the assayed volume). A single antibody-antigen binding

event onto the millimetre-wide sensing electrode of SiMoT biosensor, generates a measurable signal out of the noise produced by the remaining $\sim 10^{11}$ unbound antibodies.

Up to now, the SiMoT biosensor succeeded in detecting several biomarkers, with sensitivity at the zepto-molar concentration range. Table 1.1 reports a list of the target molecule detected with a SiMoT biosensor. The type of recognition element utilized, the immobilization strategy employed to functionalize the gate electrode and the reached LOD are also reported.

IgG¹ and IgM³⁴ antigens, HIV-1 p24 capsid proteins,³⁵ and cytokine IL-6³⁶ detections was demonstrated at the single-molecule limit, whereas C-reactive protein³⁷ was revealed with a limit of detection of $210 \cdot 10^{-21}$ M, corresponding to ~ 13 proteins in 100 μ L. Also, the SiMoT technology was employed to detect the *Xylella fastidiosa* bacterium,³⁸ revealing up to a single cell. For all those examples, the SiMoT biosensor incorporated a sensing gate bio-functionalized with capturing antibodies chemically immobilized on a SAM of alkanethiols (figure 1.5a).

A remarkable result is the single-molecule detection performance, employing a sensing interface bio-functionalized with a physisorbed antibody bilayer (figure 1.5b). Specifically, detection of IgG and S1 proteins was demonstrated using a physisorbed bilayer of anti-IgG and of anti-S1 (complementary to the S1 spike protein of the SARS-CoV-2 virus) respectively, with single-molecule sensitivity.³⁹ Indeed, the relevance of this result is two-fold: i) functionalization via physisorption represents a time-saving and cost-effective alternative to the chemical immobilization; ii) the single-molecule detection performance is endowed by some properties of the sensing interface, which is retained within the layer of physisorbed antibodies. This shed light onto the mechanism underlaying the huge signal amplification occurring at the sensing interface, suggesting that it must depend on the protein layer itself.

Table 1.1 Biomarkers detected by SiMoT biosensor. The table features the target molecule detected by SiMoT biosensor along with the kind of bioreceptors, the functionalization strategy and the achieved limit of detection (LOD).

Target	Receptors	Functionalization	LOD (# molecules)
IgG ^{1,39}	Antibodies	Physisorption and chemisorption	1 ± 1
IgM ³⁴	Antibodies	Chemisorption	1 ± 1
HIV-1 P24 ³⁵	Antibodies	Chemisorption	2 ± 1
CRP ³⁷	Antibodies	Chemisorption	15 ± 4
miR-182 ⁴⁰	1-strand oligonucleotide	Bio-affinity	1 ± 1

Xylella ³⁸	Antibodies	Chemisorption	1 bact. $\pm$ 1
COVID-19 ³⁹	Antibodies	Physisorption	LOI = 166 $\pm$ 13

Furthermore, the label-free detection principle of the SiMoT biosensor has proven effective also for the detection of the miR-182, a relevant genomic marker for the diagnosis of multiple sclerosis.⁴⁰ This represents a remarkable achievement, rendering SiMoT the only technology using the same platform for both protein and genomic markers. In this case, a single strand oligonucleotide sequence, complementary to the miR-182 marker, is immobilized onto the gate surface by means of bioaffinity immobilization (figure 1.5c), reaching the LOD of a single biomarker.

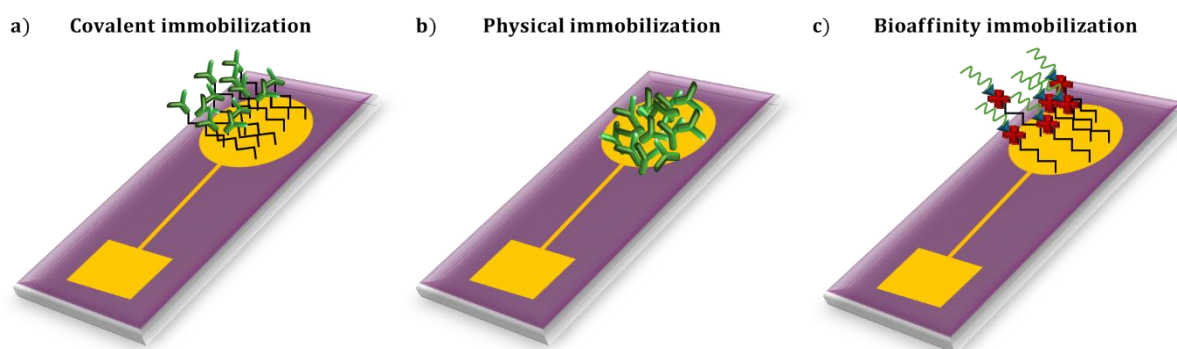


Figure 1.5 Functionalization strategies of the SiMoT sensing interface. **a)** Chemical immobilization: the gold surface is first prepared with a self-assembled-monolayer (SAM) of mixed alkanethiols (3-MPA and 11-MUA) terminating with carboxylic functionalities. After chemical activation, the carboxylic groups are conjugated with the amine groups of the proteins, leading to the formation of the bilayer. The functionalization is accomplished with the passivation of unreacted SAM molecules through a chemical and biological blocking. **b)** Physical immobilization: antibodies are directly adsorbed onto the electrode surface. **c)** Bioaffinity immobilization of DNA strands: the gold surface is prepared with a SAM of 3-MPA and 11-MUA alkanethiols, conjugated with a monolayer of streptavidin proteins. Then, the single strand oligonucleotide sequences are biotinylated and immobilized on the streptavidin layer by means of the affinity binding with biotin.

SiMoT holds great potential in view of the development of highly reliable, cost-effective, portable platform for point-of-care testing. The latest developed biosensing platform employing the SiMoT technology are a single-sensor device, and a 96-multiplexing array. The single-sensor is an easy-to-use platform, employing a disposable accessory cartridge conceived for operation by untrained individuals, whereas the multiplexing platform incorporate 96 sensing electrode biofunctionalized by different bioreceptors which enable the simultaneous detection of multiple biomarkers, suited for use

in clinical facilities by trained personnel. Both reached technology-readiness-level (TRL) 5-6 as they have been tested in relevant environment. The technology scalability and the low-cost manufacturing technique of those platform renders SiMoT one of the most promising technologies satisfying the requirement delineated by the World-Health-Organization for non-invasive point-of-care testing systems and pre-clinical studies are underway.⁷

1.6 Amplification mechanisms on large-area sensing interfaces

A common argument opposing the use of large-area sensing interface in high-performance biosensors concerned the too small portion of the sensing area engaged in a binding event compared to the total active area on the sensing element, which was believed to limit the signal to noise ratio. However, the single-molecule sensitivity of SiMoT technology implies the existence of an underlying domino-like amplification effect which propagates the information of a single biorecognition reaction, across the sensing interface. To have an idea of the amplification factor, consider the typical dimension of a target protein ($\sim 10\text{ nm}$)³, compared to the dimension of the sensing interface, about 20 mm^2 wide, encompassing $\sim 10^{11}$ capturing antibodies. For single-molecule detection, the signal generated upon single antibody-antigen binding must stand out from the noise of the remaining 10^{11} unbounded proteins.

Notably, nowadays SiMoT is not the only technology capable of single-molecule detection. Other bioelectronic approaches reached the same limit of detection, such as the single-molecule chiral sensor,⁴¹ electrochemical transistor for detection of COVID virus⁴² and FET biosensor for aptamers detection⁴³. All those biosensors integrate large-area sensing interface to reach ultrasensitive performance. Significantly, spontaneous amplification mechanisms should be in action in all the above devices. Nevertheless, the existence of collective effects has been identified and invoked to explain only SiMoT, so far.

Moreover, other systems exist in nature, able to sense single molecules encompassing a detecting surface order of magnitudes larger than the footprint of a binding molecule. Examples are rod cells in the retina detecting single photons⁴⁴, or sperm cell of sea urchins tracking the chemoattractants released by the oocyte in seawater⁴⁵. Those systems seem to rely on mechanism involving a cascade of enzymatic reactions and the propagation of electric signal through the cells membrane potential, being therefore considerably different compared to a biosensor architecture. However, they share a

common feature with the SiMoT platform, that is many bioreceptors, highly packed on their surface, with a density of $10^4 \mu\text{m}^{-2}$, which is comparable to the density of capturing antibodies in SiMoT immunosensors.

1.6.1 Extended surface potential shift triggered by a few affinity binding events

The experimental evidence that few antibody-antigen binding events generate an extended response across the bilayer onto a large sensing interface is provided by Kelvin-probe force microscopy (KPFM) experiments. KPFM is a variant of the atomic force microscopy (AFM) used to image the contact potential difference (CPD) between the metal coated AFM tip and the sample.⁴⁶ The CPD is related to the surface potential (SP) of the sample, hence this technique has been used to assess the SP of the monolayer of antibodies before and after the exposure to an antigen solution at 100 zM concentration.²⁰ Details on the technique are given in Appendix A.

The SP is a property of surfaces determined by the distribution and arrangement of surface charges and dipoles. Indeed, the bio-functionalization of the Au gate surface affects the SP. Consider a gold substrate half of which is bio-functionalized with antibodies, specifically Immunoglobulin M (anti-IgM) as it is depicted in figure 1.6a. The image obtained by KPFM, at the interface between the pristine and bio-functionalized surface, in figure 1.6b, clearly shows the effect of an additional molecular monolayer onto the surface. It represents the value of the SP relative to that of the bare Au (KPFM does not provide an absolute value of the SP but needs a reference value). The histogram in figure 1.6c shows that the SP of gold increase of about 176 ± 20 mV because of the macro dipoles introduced by the bio-functionalization.

The sample is then incubated with 100 μL of non-binding IgG antigen solution at concentration of ~ 3 fM, including $\sim 10^7$ IgG molecules, as a negative control measurement. The KPFM image (figure 1.6d-f) show that the SP of the sample is unchanged with respect to the pristine value.

Afterwards, the sample is incubated with 100 μL of IgM affinity antigen solution at a concentration of 100 zM, including ~ 6 IgM molecules. This results in a significant variation of the SP of the anti-IgM bilayer, (figure 1.6g-i): the bio-functionalized area features a SP 128 ± 20 mV higher than that of the bare Au, shifted of about 48 ± 28 mV with respect to the pristine value. The binding of ~ 6 IgM, out of $\sim 10^7$ unbounded capturing anti-IgM triggers a surface potential change extending across the whole inspected area. Increasing the concentration of IgM in solution leaves the SP almost unchanged (figure 1.6l-n).

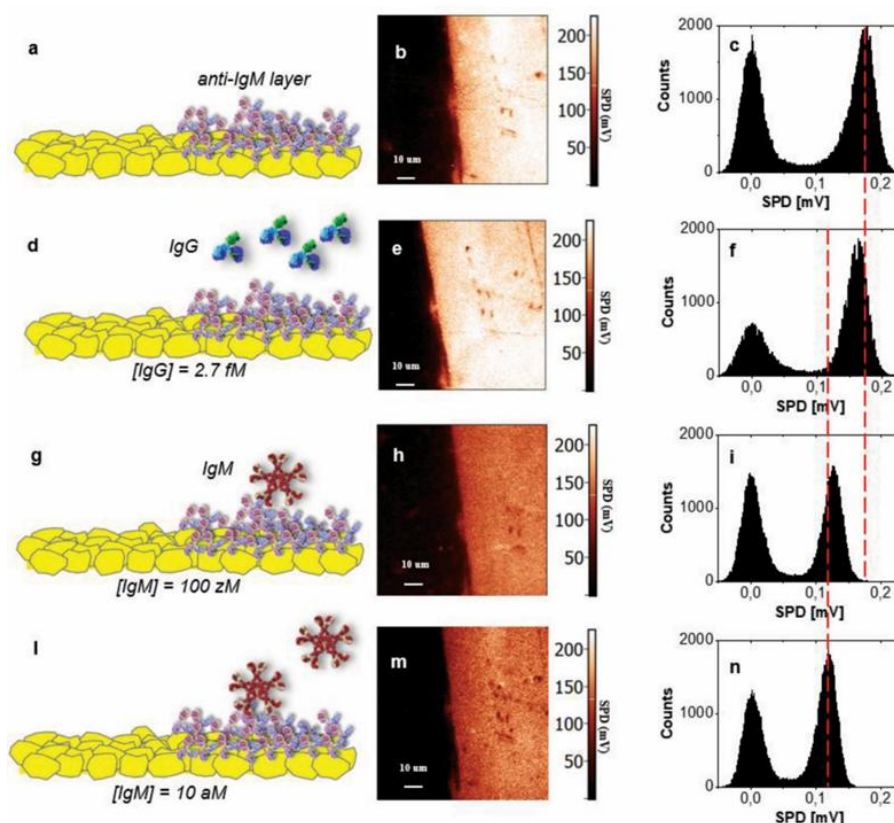


Figure 1.6 Surface potential changes of an anti-IgM functionalized gold electrode. **a)** Schematic representation **b)** KPFM image of the CPD of the patterned sample with the bare Au (left) and functionalized area (right) **c)** histogram of the SP with respect to the average value on bare Au, of the biofunctionalized electrode prior to exposure to any antigen solution. **d-f)** same as a- c) after exposure of the sample to non-binding IgG solution. **g-i)** same as a-c) after exposure of the sample to 10^{-19} M IgM solution. **l-n)** same as a-c) after exposure of the sample to 10^{-17} M IgM solution. Taken from Ref [18].

The described results have been correlated with the response of the SiMoT biosensor. Specifically, the magnitude of the SP shift is perfectly comparable with the threshold voltage shift measured upon detection of IgM at the same concentrations.

Moreover, further KPFM investigation of the SP shift induced by the binding of analyte have been conducted using different antibodies, such as anti-HIV-1-p24 physisorbed on gold used for the detection HIV-1-p24 antigens, showing a remarkable decrease of the biolayer SP of about 106 ± 16 mV after exposure with analyte solution at 10 zM, containing a single target molecule.⁴⁷

Those findings represent a direct proof that an amplification mechanism is in place across the biolayer of antibodies physisorbed onto a gold surface upon affinity binding. This mechanism is the base of the SiMoT single-molecule sensitivity. While the binding of a single analyte molecule affects the

local electrostatic properties of the antibodies on an area of about $(\sim 10 \text{ nm})^2$, a domino-like mechanism propagates the change of the dipole arrangements across the sensing interface $(\sim 100 \mu\text{m})^2$.

Hypothesis on the possible interactions underlying the amplification effect suggest, for example, the collaborative effects through a hydrogen-bonding network associated with the highly packed layer of capturing antibodies. However, newest results presented later in this thesis ascribe the propagation to a network of intermolecular interactions driven by hydrophobic forces.

Chapter 2

Proteins and graphene: material background

2.1 Proteins

Proteins are complex macromolecules essential for a variety of functions in living organisms. They constitute one of the most abundant class of biomolecules in living organisms. For example, a single human cell counts an average number of about 1-3 billion proteins.⁴⁸ They are constituted by long polypeptide chains folded and arranged into specific three-dimensional conformation. Proteins are involved in a plethora of biochemical reactions and have been naturally designed by evolution to fulfil their purpose through an intricate network of intra-molecular and inter-molecular interactions. The final shape of a protein is strictly related to its biochemical function therefore studying the structural basis of any process involving proteins is of crucial importance.

2.1.1 Protein structure

Amino acids are organic molecules comprising an α -carbon (C_α) atom bound to a carboxylic group ($C=O$), an amino group ($N-H$) and an additional functional group (*side chain*) which confers specific chemical properties. 20 different amino acids constitute the building-blocks of proteins.

Proteins have a modular organization⁴⁹ embodied in a hierarchical structure of four levels, depicted in figure 2.1. The *primary structure* is the linear sequence of amino acids (also called *residues*), bound through peptide bonds between the carboxyl group (C=O) on one amino acid and the amino group (N-H) of the following one to form long polypeptide chains. (figure 2.1a). The series of -(N-H)-C α -(C=O)- atoms forms the protein backbone. As the peptide bond does not have rotational freedom, C, O, N and H atoms are coplanar. On the other hand, the C α -C and C α -N bonds allow free rotations thus enabling the folding of the polypeptide chain into organized structure. Non-covalent interactions, such as H-bonds and hydrophobic forces between various segments of the chain, drive the folding of the protein.

The *secondary structure* refers to the folding of short segments of the chain into ordered units (figure 2.1b) constituted by regular structures stabilized by H-bonds between C=O and N-H groups in nearby amino acids. Secondary structure elements are α -helix, β -sheets, loops and turns. α -helix are segments of the polypeptide chain folded into helical structures. β -sheets are extended flat-like patterns, made of straight polypeptide strands adjacent to one another. Turns are short segments, typically involving four amino acids, engaged in a 180° inversion of the polypeptide chain; for instance, turns connect adjacent strands in a β -sheet. Loops are structures connecting two regions of α -helix or β -sheets. They contain more amino acids than turns, beyond the minimum necessary number to connect two regions, therefore assume irregular conformation though stabilized by H-bonds and hydrophobic interactions with other regions of the protein. The secondary structure of a protein also includes unordered units, characterized by a higher conformational flexibility. Those regions often participate in binding reaction with ligand molecules.

The secondary structure units arrange in space into the *tertiary structure* (figure 2.1c). It refers to the overall three-dimensional conformation of a single polypeptide chain and define the way different secondary structure regions relate to each other. Finally, the assembly of multiple polypeptides chains (sub-units) forms the *quaternary structure* of the protein (figure 2.1d).

The final conformation of a protein in aqueous physiological condition is named *native conformation*. It corresponds to the structure that minimizes the energetically unfavourable interactions among the protein structural units in water. Specifically, hydrophobic forces are often the dominant ones in aqueous environment. Those forces are due to chemical groups that interact unfavourably with water molecules and tend to exclude them by packing together.⁵⁰ In general, the native conformation of a protein exhibits a hydrophobic core made of non-polar residues surrounded by polar and hydrophilic residues, exposed to the aqueous environment. Note that, proteins do not have a static and rigid structure, rather their conformation is determined by a dynamic network of interactions between with

other molecules in the surrounding environment. Proteins may unfold and re-fold, while retaining their functionality or undergo large conformational changes and be denatured. For instance, conformational changes are expected upon formation of protein complexes with other proteins, such as upon binding with ligands.

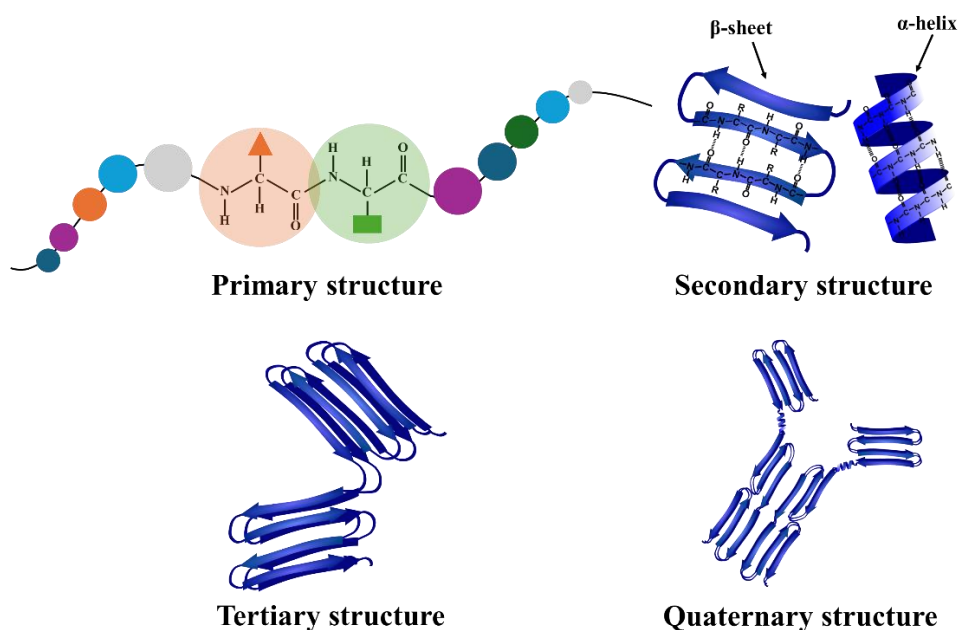


Figure 2.1. Protein structure. The sequence of amino acids constitutes the primary structure of a protein. The secondary structure refers to the folding of the amino acid chain into ordered structures (such as β -sheets and α -helix), stabilized by hydrogen bonds between the amino groups and the carboxyl groups in neighbouring regions. The spatial arrangement of different secondary structure domains constitutes the tertiary structure of the protein. The quaternary structure refers to the union of multiple polypeptide subunits.

The final conformation and the amino acid composition of a protein, also determine its electrostatic properties. Different regions of the protein could be differently charged, according to the protonation state of the functional groups therein. In aqueous solution, the charge of a functional group is modulated by the pH: low (high) pH favours the protonation (deprotonation). For a given molecule the isoelectric point (IP) is defined as the pH value at which its overall net charge is zero. At $\text{pH} > \text{IP}$ the molecule carries a negative net charge whereas at $\text{pH} < \text{IP}$ it carries a positive net charge. In a protein, different domains may have different IP, hence be differently charged. It is possible to associate an overall electric dipole to account for the distribution of charges across the structure of a protein. Since the pH determines the net charge and its distribution, it influences the entity and the direction of the associated dipole.

2.1.2 IR spectroscopy of proteins

The IR absorption spectrum of a protein contains a few characteristic bands. The main vibrational normal modes of a protein originate from the polypeptide backbone. The coupling of the stretching and bending vibrations of C, O, N and H atoms involved in the peptide bond gives rise to the main spectral features in the range between 1000 cm^{-1} and 4000 cm^{-1} . Figure 2.2. features a representative IR spectrum of Immunoglobulin G antibodies, showing the main absorption bands. In order of decreasing wavenumber we found: the amide A band ($\sim 3300\text{ cm}^{-1}$) originating from the localized stretching of N-H bond; the amide I band ($\sim 1650\text{ cm}^{-1}$) arising mainly from the C=O stretching; the amide II band ($\sim 1550\text{ cm}^{-1}$) ascribed to the out-of-phase combination of N-H in plane bending and C-N stretching; and the amide III band ($\sim 1400\text{--}1200\text{ cm}^{-1}$) mainly due to the in-phase combination of the N-H bending and the C-N stretching. Additional spectral features may arise from the IR absorption of chemical groups in the amino acid side chains. Examples are the S-H group of Cys ($\sim 2550\text{--}2600\text{ cm}^{-1}$), the C=O stretching in Asp and Glu ($\sim 1712\text{--}1716\text{ cm}^{-1}$), the NH_2 bending in Asn ($\sim 1612\text{--}1622\text{ cm}^{-1}$), or the symmetric stretching of the $(\text{CN}_3\text{H}_5^+)$ group in Arg ($\sim 1614\text{--}1663\text{ cm}^{-1}$).^{51,52} In general amide bands are informative on the protein structure, whereas spectral features from side-chains are of main concern in the study of protein reactions mechanism.

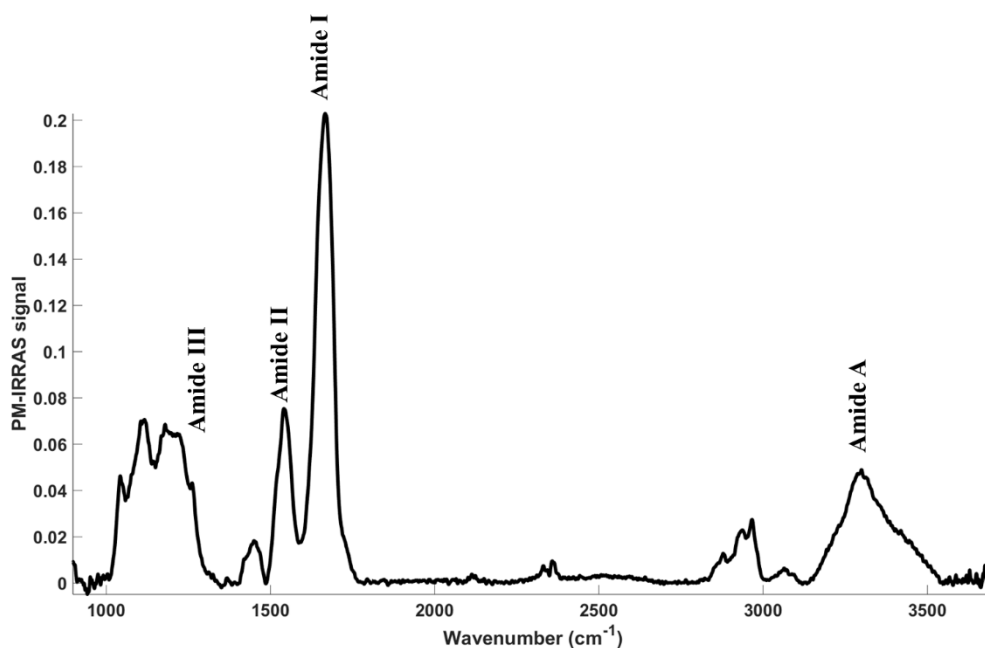


Figure 2.2. IR spectrum of anti-IgG. The spectrum shows the characteristic spectral feature in the IR spectrum of a protein: amide A ($\sim 3300\text{ cm}^{-1}$), amide I ($\sim 1650\text{ cm}^{-1}$), amide II ($\sim 1550\text{ cm}^{-1}$) and amide III ($\sim 1260\text{ cm}^{-1}$). The peaks in the region between 1000 cm^{-1} and 1300 cm^{-1} are due to

deposited HEPES salt from the solution used to prepare the sample. The spectrum is measured by PM-IRRAS spectroscopy on a monolayer of anti-IgG deposited onto a gold surface.

The transition dipole moment (TDM) of a given vibrational mode in a protein is sensitive to the surrounding environment of the vibrating bonds. H-bonds between nearby residues, or with water molecules, influence the natural frequency of the vibrations.⁵³ For instance, the resonant frequency of the amide I mode may downshift by 20-25 cm^{-1} upon formation of a single H-bond involving the oxygen of the C=O group. Moreover, vibrational frequencies are further affected by the coupling of multiple adjacent oscillating dipoles. Since amide I vibrations does not involve the C α atom of the polypeptide chain, the coupling among neighbouring peptide bonds mainly occurs through Coulomb interaction between adjacent oscillating dipoles. This coupling influences the oscillating frequency of amide vibrational modes depending on their IR absorbance, relative orientation and distance. It turns out that the resonant frequency of amide vibrational modes depends on the local surrounding and structure of the protein where it is located. This is the crucial aspect at the base of the amide I band sensitivity to the protein structure. Since amide I vibration has a large associated transition dipole moment (TDM), the effect of the coupling of nearby oscillators is larger than for other amide vibrational modes, rendering the amide I band the most sensitive to the secondary structure. The coupling delocalizes the energy of an excited vibrational mode, along a distance of few bond length, at the level of the secondary structure, whereas variations on the scale of the tertiary structure does not have an influence on the molecular vibrations. The amide I mode in α -helix, β -sheets, turns and other secondary structure units resonates at slightly different frequencies, thus the amide I band in the IR spectrum of a protein appears as a strong and broad band in the 1600-1700 cm^{-1} spectral range arising from the overlapping contributions of all the secondary structure elements. A few seminal papers, based on the analysis of the amide I band of proteins of known crystalline structure (determined by X-ray scattering), established the correlation between the fine structure of the amide I band and the protein secondary structure content.⁵⁴⁻⁵⁶ Proteins encompassing a major α -helix content show an amide I band picked around $\sim 1648\text{-}1657\text{ cm}^{-1}$ whereas those mainly composed of β -sheets show the amide I band picked around $\sim 1623\text{-}1641\text{ cm}^{-1}$, with a weak shoulder at higher frequency $\sim 1674\text{-}1695\text{ cm}^{-1}$. The increasing availability of IR spectroscopy data allowed the identification of spectral ranges to discriminate the amide I contribution from various secondary structure elements. Table 2.1 summarizes the spectral ranges associated to the most relevant ones. The listed ranges must not be intended as rigid boundaries.

Note that multiple bands could arise from the same structure, due to variations in the geometry, dimensions and influence of the surrounding environment. An example are intramolecular β -sheets

formed in aggregated structures which are associated to amide I components at frequencies below that of native β -sheets.⁵⁷

The sensitivity of the amide I band to the secondary structure renders the interpretation of its lineshape a complex task, though enables structural analysis and the monitoring of unfolding and denaturation processes.

Table 2.1. Amide I secondary structure components. Frequency range of the IR absorption from protein secondary structures in H₂O. [barth]

Secondary structure	Average (cm ⁻¹)	Extremes (cm ⁻¹)
β -sheet [B2]	1633	1623 - 1641
Unordered	1654	1642 – 1657
α -helix	1654	1648 – 1657
Turns	1672	1662 – 1686
β -sheet [B1]	1684	1674 – 1695

Transition dipole moments in β -sheets and α -helix

The TDMs associated to the amide I vibrational mode have been calculated for β -sheets and α -helix structures.^{52,58,59} This is possible for ordered structures which can be regarded as crystals. The description of the amide I modes β -sheets and α -helix is carried out within the framework of the transition dipole coupling (TDC) theory, developed by Abe and Krimm (1972)⁶⁰, which accounts for the coupling of neighbouring oscillating dipoles through intra-chain and inter-chain coulomb interactions. In β -sheets the amide I vibration generate two IR active vibrational modes, having orthogonal TDM in the β -sheets plane (figure 2.3a). The dominant one (B2 mode), is polarized perpendicular to the polypeptide chain resonating at frequency ~ 1630 - 1640 cm⁻¹ whereas a weaker TDM (B1 mode), is generated with polarization parallel to the polypeptide chain, vibrating at higher frequency ~ 1680 - 1690 cm⁻¹. Because of the TDC the amide I mode in β -sheets splits into two orthogonal modes ~ 50 cm⁻¹ far apart, explaining the evidence of an asymmetric amide I lineshape in fully β -sheets protein spectra. For α -helix the TDC model predicts the generation of two TDMs for the amide I mode, polarized parallel (A mode) and perpendicular (E1 mode) to the helix axis (figure 2.3b). Their TDMs are almost equally intense and little separated (~ 5 cm⁻¹), therefore they are not resolved by IR spectroscopy and contribute to the amide I intensity as a unique α -helix band at ~ 1654 cm⁻¹.

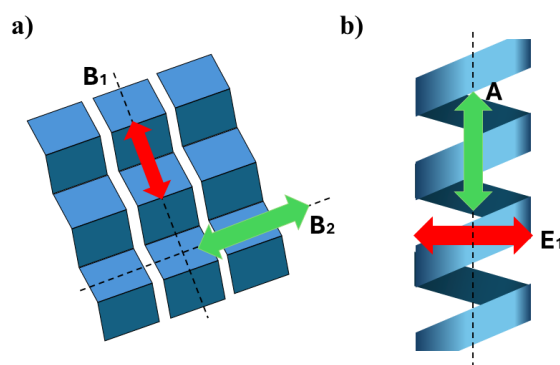


Figure 2.3. Amide I transition dipole moments in β -sheet and α -helix. **a)** Schematic representation of a β -sheet featuring the direction of the amide I TDMs: B2 mode is dominant (larger green arrow) perpendicular to the β -strands, B1 mode is weaker (smaller red arrow) parallel to the β -strands; **b)** Schematic representation of a α -helix featuring the direction of the amide I TDMs: A mode (green arrow) parallel to the helix axis and E1 mode (red arrow) perpendicular to the helix axis. The similar dimension of the arrows suggests that the A TDM and the E1 TDM have similar strength.

2.2 Immunoglobulins

Immunoglobulins (or antibodies) are proteins belonging to the immune system, whose main function is to recognize extraneous substances in the body, namely *antigens*, and trigger the immune system response to eliminate them. All immunoglobulins share a canonical Y-shape structure. The recognition function involves the antigen binding site of the protein. This region is highly variable among immunoglobulins and is designed to recognize *affinity* antigens with high selectivity. The variety of possible binding sites correspond to many recognizable antigens. Because of their highly specific activity, immunoglobulins are relevant biomolecules for therapeutic use and for implementation in biosensors, as excellent bio-recognition elements for the detection of their affinity antigens.^{61,62}

Structure

The basic Y-shaped structure of immunoglobulins is schematically represented in figure 2.4. It consists of four polypeptide chains: two identical heavy chains (H), ~50kDa, and two identical light chains (L), ~25 kDa, held together by disulfide bonds (S-S). The dimensions of the Y structure are

about 14 nm x 8 nm x 4 nm.⁶³ The two arms of the Y are also addressed as antigen binding fragments (Fab) because they are primarily involved in the binding with antigens. The stem of the Y is called crystallizable region (Fc) and is not specific for the target antigen. The two Fab regions are connected to the Fc region by a hinge region which confer mobility to the Fab to properly orient in space toward the binding antigen. Immunoglobulins are classified into five groups according to different type of hinge and Fc regions. The five class are IgG, IgD, IgE (Y-shaped monomer), IgA (dimer) and IgM (pentamer). The Fab fragments are responsible for antigen binding (one antibody can bind two antigens, one for each Fab fragment), whereas the Fc fragment is involved in the activation and regulation of the immune response. IgG is typically taken as a representative immunoglobulin, and all the others are addressed as IgG-like proteins as they share the same basic structure. Each chain is divided in domains. The terminal domains of the Fab fragments constitute the variable regions (V), which contains the antibody-antigen binding site, whereas the remaining domains of the Fab fragments along with those of the Fc fragments constitute the constant region (C). According to this nomenclature domains are identified as CH, VH, CL, VL. Each domain comprise ~110 amino acids folded to form two β -sheets sandwiched together into a β -barrel. Figure 2.4c shows a schematic representation of V and C domains. They are not identical as V domains contain additional β -strands and irregular and flexible loops forming the antigen-binding site.⁶²

The structure of variable domains can be further divided. The great heterogeneity of those domains is localized in few regions named hyper variable regions, located at the very top of the Fab arms. The spatial arrangement of those regions, from both the H and L chains, form the antigen-binding site, identical for the two Fab fragments. The hypervariable regions are also called complementary-determining regions (CDRs). The structure of the CDRs is sustained by less variable regions called framework regions (R).⁶⁴

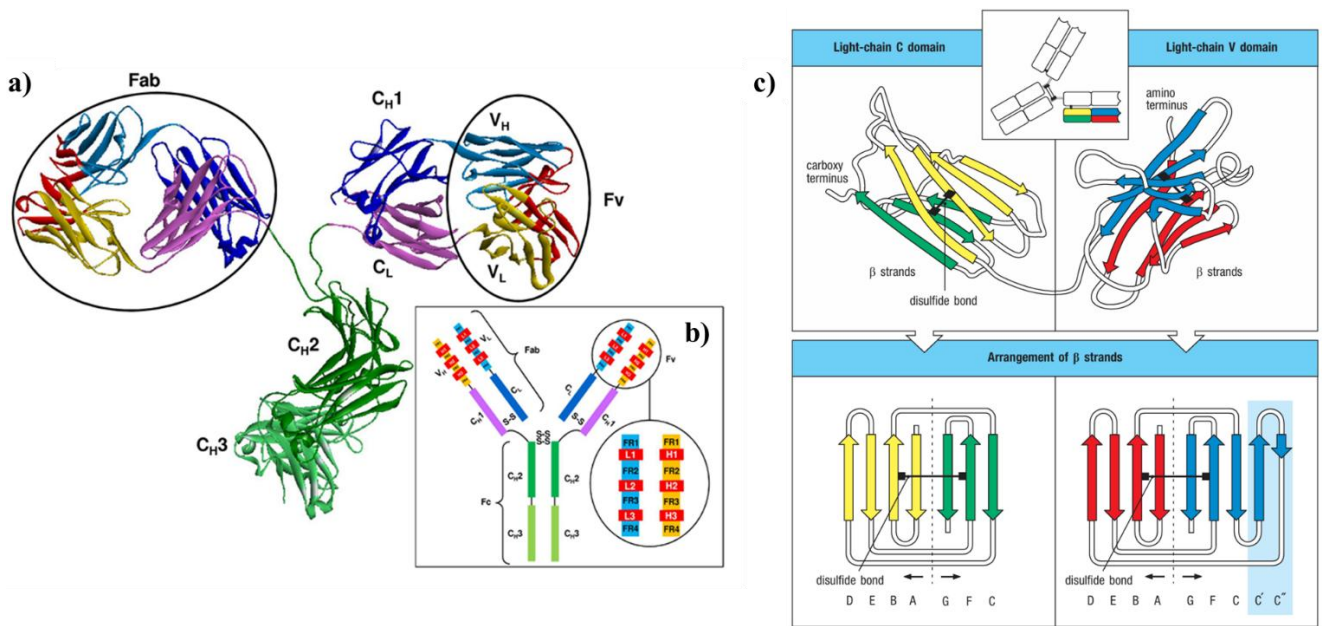


Figure 2.4 Immunoglobulin structure. a) Representative structure of an antibody b) schematic representation of the antibody structure; symbols stand for C=constant, V=variable, H=heavy, L=light, Fab=antigen-binding fragment, Fc=crystallizable fragment, FR=framework region; the same color code is used to identify the same regions in panel a) and b). Taken from [59] c) structure of the Immunoglobulin constant domains (4 + 3 β strands) and variable domains (4 + 5 β strands), made of two β-sheets joined into a barrel structure. Taken from [60]

Antibody-antigen binding

Antibodies exhibit an extraordinarily high affinity for binding affinity antigens. This specificity is endowed by the structure of the antigen-binding sites, also called *paratope* which is designed to bind a particular *epitope* (the region of the antigen which is recognized by the antibody) through greatly energetically favourable interactions while avoiding non-specific binding. The antigen-antibody interaction occurs through non-covalent bonds, such as H-bonds, van der Waals and hydrophobic forces, involving only a small fraction of the amino acid residues in the CDRs. Protein regions not included in the CDR play also a role in the binding to antigens, by providing structural support to the CDRs and orienting the CDRs correctly.⁶¹ Moreover, antigen binding induces conformational changes in the variable region that are transferred to the constant regions through allosteric effects. This phenomenon serves as a trigger for the activation of the immune system response against the antigen.^{65,66}

Electric dipole

Antibodies are typically described in terms of their associated overall electric dipole, schematically represented in figure 2.5. It is mainly defined by the different isoelectric points (IP) of the Fab and Fc fragments. Typically, the IP of the Fab fragment is larger than that of the Fc fragment.⁶⁷ For IgG they were measured to be $IP_{Fab} \sim 8.3$ and $IP_{Fc} \sim 6$.⁶⁸ Accordingly, at physiological pH ~ 7.4 the Fab fragments present a net positive charge whereas the Fc fragment is negatively charged. This corresponds to an overall dipoles oriented from stem (Fc) toward the two top arms (Fab) of the Y.

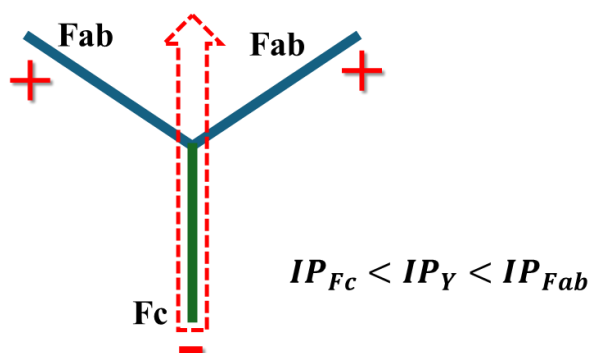


Figure 2.5 Immunoglobulin electric dipole. Schematic representation of the electric dipole of a Y-shaped Immunoglobulins, oriented from the Fc fragment toward the Fab fragments. The isoelectric point (IP) of the Fab fragment is typically larger than that of the Fc fragment, resulting in an larger positive charge on the two arms of the Y and more negative charged stem. IP_Y stands for the IP of the whole Y which is intermediate between IP_{Fab} and IP_{Fc}

2.3 Bovine serum albumin

Bovine serum albumin (BSA) is plasma protein typically used as model protein for human serum albumin, with whom share similar structure. Serum albumins are the most abundant plasma proteins in mammals, Its main biological function concerns the transport of fatty acids and the control of the osmotic pressure in blood. BSA is a globular protein consisting of a 583 amino acids long polypeptide chain (~ 66.3 kDa) folded into three α -helix domains. It has an ellipsoidal shape with dimensions of $15\text{nm} \times 3.8\text{nm} \times 3.8\text{nm}$.^{64,69} BSA is a relevant biomolecule with application in biosensors. It is often employed as a blocker in immunosensors. BSA binds to non-specific binding sites of bio-functionalized sensing interfaces, thus increasing the sensitivity and selectivity performance of biosensors.

2.4 Graphene

Graphene has been the first two-dimensional material to be produced, first isolated in 2004 by Andre Geim and Kostya Novoselov, using an adhesive tape to peel-off atomically thin layers from graphite.⁷⁰ Since then, graphene found several applications, thanks to its mechanical, electronic, optical and chemical properties. As it is thin, light, flexible and transparent, while exhibiting great mechanical strength, electrical and thermal conductivity it has been employed in the development of sensors, wearable devices, foldable electronics, for application in electronics, energy storage and healthcare.

Graphene consists in a single layer of sp^2 -hybridized carbon atoms arranged in a hexagonal lattice, as depicted in figure 2.6a. Each carbon atom is bound to the three nearest-neighbours by σ -covalent bonds between sp^2 electrons and by a π -bond with p_z electrons which form a metallic bond across the lattice.⁷¹ The graphene structure is that of a triangular Bravais lattice with a basis of two carbon atoms (A and B in figure 2.6b). The reciprocal lattice is also a triangular lattice, represented in figure 2.6c, featuring an hexagonal first Brillouin zone with three high-symmetry points Γ , K , M .

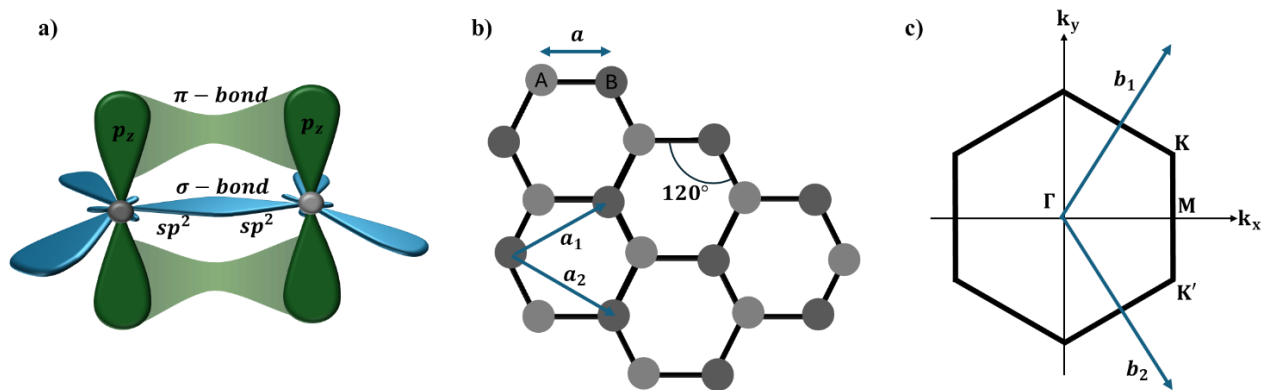


Figure 2.6. Graphene structure. **a)** covalent binding of sp^2 hybridized carbon atoms. Each atom has three sp^2 electronic orbitals (blue) and one p_z orbital (green). The sp^2 electrons of nearest-neighbour atoms form a σ -bond whereas the p_z forms a π -bond. **b)** Crystal structure of graphene. The interatomic distance of nearest-neighbour C atoms is $a=1.42\text{\AA}$. a_1 and a_2 represent the unit vectors of the corresponding triangular Bravais lattice. **c)** Reciprocal lattice of the graphene crystal lattice, featuring the high-symmetry point Γ , K , M and the lattice vectors b_1 and b_2 .

2.4.1 Band diagram and electronic properties

An approximation of the electronic energy band diagram of graphene can be derived from a tight-binding model considering π electron hopping between nearest-neighbour carbon atoms.⁷² The energy spectrum of the tight-binding Hamiltonian is

$$E_{\pm}(\mathbf{k}) = \pm t \sqrt{3 + f(\mathbf{k})} \quad (2.1)$$

where $t \approx 2.8 \text{ meV}$ is the nearest-neighbour hopping energy, $\mathbf{k}$ is the momentum vector and $f(\mathbf{k})$ is given by $f(\mathbf{k}) = 2 \cos(\sqrt{3}k_y a) + 4 \cos(\sqrt{3}k_y a/2) \cos(3k_x a/2)$. The plus and minus signs returns the upper (π^* -band) and lower (π -band) energy bands respectively. It turns out that the two bands cross at the $\mathbf{k}=\mathbf{K}$ and $\mathbf{k}=\mathbf{K}'$. Figure 2.7 reports the graphene band diagram in the first Brillouin zone. Expanding the energy spectrum close to $\mathbf{K}$ (or equivalently $\mathbf{K}'$) the energy dispersion is approximated by a linear relation describing two conical surfaces touching at the so called *Dirac point*.

$$E_{\pm}(\mathbf{q}) \approx v_F |\mathbf{q}| \quad (2.2)$$

being $\mathbf{q}$ the momentum relative to $\mathbf{K}$, and v_F the Fermi velocity of graphene, measured $\sim 10^6 \text{ m/s}$.

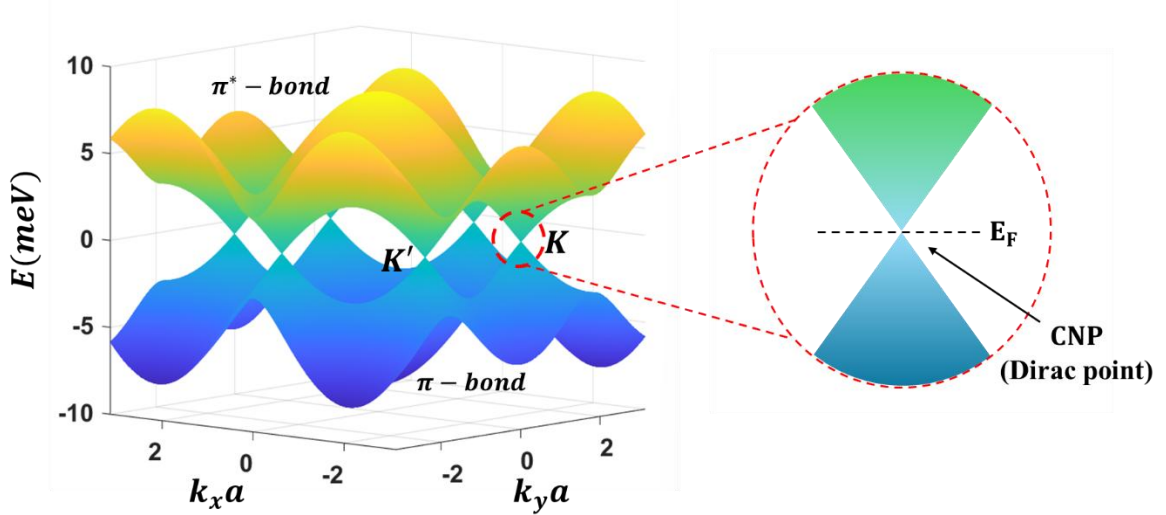


Figure 2.7. Band diagram of graphene. Electronic band diagram of graphene derived from the tight-binding method and considering only electron hopping between nearest-neighbour, according to equation 2.1. It features the valence and conduction bands, touching at the $\mathbf{K}$ and $\mathbf{K}'$ points. The zoom shows the energy dispersion in proximity of the CNP.

Electrons in a pristine graphene lattice fill all the available energy states in the π -band and the Fermi energy (E_F) set exactly at the Dirac point, also addressed as the charge neutrality point (CNP) of

graphene. Moreover, the linear energy dispersion close **K** (or **K'**) determines a linear density of states, vanishing at the Dirac point. E_F is related to the charge carrier density (n) in graphene according to the relationship

$$|E_F| = \hbar v_F (\pi |n|)^{\frac{1}{2}} \quad (2.3)$$

where $\hbar$ is the reduced Plank's constant, and E_F is measured with respect to the CNP. In real applications, graphene is hardly lacking a spurious doping introduced during the fabrication process or by contact with the substrate, therefore its Fermi energy is usually shifted from the CNP.⁷³

From equation 2.3 the quantum capacitance of graphene, C_q , can be derived. It is defined as the rate of change of charges in response to variation of the chemical potential (i.e. Fermi energy). For graphene it is

$$C_q = \frac{2e^2}{\hbar v_F} \sqrt{\frac{n}{\pi}} \quad (2.4)$$

being of the order of $\sim 10^{-7} - 10^{-6}$ F cm⁻². The quantum capacitance is a relevant quantity for the application of graphene in electronic devices.⁷⁴

2.4.2 Field-effect tuning of the Fermi energy

An additional peculiarity of graphene is the possibility of tuning its work function (i.e. its Fermi energy) through an electric field, as a direct consequence of the linear energy dispersion close to the Dirac point.⁷⁵ The effect has been extensively studied in back-gated graphene devices, consisting of a three-layer structure made of a conductive substrate, an insulating layer and graphene. An example is a doped-Si/SiO₂/graphene structure, schematically shown in figure 2.8a. By applying an electrostatic potential (V_G), the charge carrier density in graphene can be continuously tuned between electrons and holes reaching concentrations of $n \sim 10^{13}$ carriers cm⁻². In turn the Fermi energy is modulated according to equation 2.3. Modulation of $E_F \sim 500$ meV has been achieved by applying voltages up to 100 V.⁷⁶ The entity of the modulation depends on the gating capacitance of the device. Figure 2.8b features the equivalent circuit of a back-gate device, comprising a back-gate capacitance, C_{BG} , and the quantum capacitance of graphene, C_q . The circuit equation is

$$V_G = \frac{E_F}{e} + \phi \quad (2.5)$$

where ϕ is the voltage drop across the dielectric layer, whereas E_F/e is the Fermi energy shift (in Volts) induced by charge carriers upon application of V_G , determined by the quantum capacitance.

For back-gate devices, typical values of the gate capacitance are $C_{BG} \sim 10^{-8} \text{ F cm}^{-2}$, evaluated as the capacitance of a parallel plate capacitor filled with a dielectric layer ($C_{BG} = \frac{\epsilon\epsilon_0}{s} \sim 10^{-8} \text{ F cm}^{-2}$ considering $\epsilon = 4$ for SiO₂ and a $s = 300 \text{ nm}$ the SiO₂ layer thickness). In general, C_{BG} is small compared to C_q therefore prevails one in the series resulting in $\phi \gg E_F/e$. That is the reason why the field-effect modulation of the graphene Fermi energy requires large voltages in back-gate devices.

A much more efficient modulation has been achieved using electrolyte-gated (EG) devices, where the dielectric layer is substituted by an electrolyte. A representative structure of an electrolyte-gated device, with a top-gate architecture, is represented in figure 2.8c along with its equivalent circuit, figure 2.8d.

An electrolyte in contact with solid electrodes generates a characteristic structure known as electrical double layer (EDL). It is formed by the accumulation of mobile charges in the electrolyte at the electrode/electrolyte interfaces. A schematic representation of an EDL is shown in figure 2.8e. An EDL extends for a characteristic length, called *Debye length* (λ_D), which depend on the concentration of ions (z)

$$\lambda_D = \sqrt{\frac{\epsilon_0 \epsilon k_B T}{e^2 z}} \quad (2.6)$$

where k_B is the Boltzmann's constant, and T is the temperature. Typical values of λ_D ranges about 1-100 nm depending on the electrolyte. For example, for an aqueous electrolyte ($\epsilon \sim 80$) with $z \sim 150 \text{ mM}$ it is $\lambda_D \sim 25 \text{ nm}$. The role of the EDLs in electrolyte-gated device is that of screening all the electrostatic interaction in a nanometre-thin region. Accordingly, an applied DC bias, $V_G = 1 \text{ V}$, to the device, drops within $\sim 1\text{-}100 \text{ nm}$ generating electric field at the electrode's surface as intense as $10^7\text{-}10^9 \text{ V/m}$.⁷⁷ From the electronic perspective, an EDL behaves as a parallel plate capacitor, with a spacing s equal to λ_D . It turns out $C_{TG} \sim 10^{-6} \text{ F cm}^{-2}$ in the top-gate device, which is comparable or even higher than C_q . Consequently, V_G in an electrolyte-gated device distributes similarly across the electrolyte and as a Fermi energy shift enabling high modulation sub-volt operation.

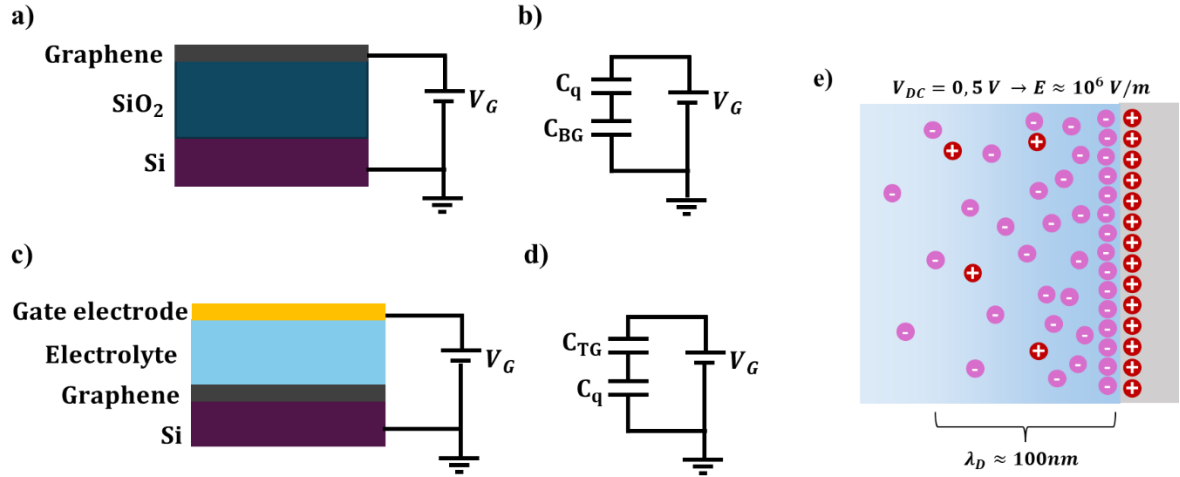


Figure 2.8. Back-gated and top-gated graphene. **a)** Back-gated graphene device comprising a SiO₂ insulating layer; **b)** equivalent circuit of the back-gated graphene device including the quantum capacitance of graphene C_q and the back-gate capacitance $C_{BG} = \frac{\epsilon\epsilon_0}{d_{BG}} \sim 10^{-8}\text{ F cm}^{-2}$ considering $\epsilon = 4$ for SiO₂ and a $d_{BG} = 300\text{ nm}$ the SiO₂ layer thickness; **c)** Top-gated graphene device comprising an electrolyte; **d)** representative equivalent circuit of the top-gated graphene device including the top-gate capacitance $C_{BG} = \frac{\epsilon\epsilon_0}{s} \sim 10^{-6}\text{ F cm}^{-2}$, considering for example water as electrolyte, having $\epsilon = 80$ and $s = \lambda_D \sim 100\text{ nm}$ equal to a typical EDL Debye length; **e)** schematic representation of the EDL forming at a solid electrode in contact with an electrolyte.

2.4.3 Vibrational properties: Raman spectroscopy of graphene

The analysis of the vibrational modes of graphene is informative about its structural and electronic properties, as they involve phonons while interacting with structural defects and charge carriers. Given the non-polar and aromatic chemical structure of graphene, Raman spectroscopy represents the optimal analytical technique to probe its lattice vibrations. Moreover, the Raman scattering process is enhanced in graphene as the absence of a band gap makes any incident wavelength resonant with electronic transitions.

Figure 2.9 shows phonon dispersion in graphene.⁷⁸ It comprises three acoustic and three optical modes. The vibrational modes at the Γ point are: $A_{2u} + B_{2g} + E_{1u} + E_{2g}$, being B_{2g} and E_{2g} the optical modes. Specifically, the E_{2g} mode correspond to Raman-active C-C stretching vibrations in the carbon lattice and appears in the Raman spectrum of graphene at $\sim 1580\text{ cm}^{-1}$. This peak is called the G-peak, as it is present in all the graphitic material. The B_{2g} is not active neither for Raman nor for IR spectroscopy.

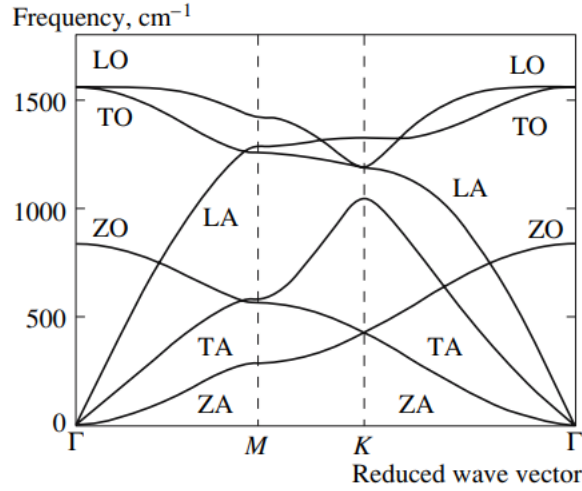


Figure 2.9. Phonon dispersion in graphene. Taken from ref [76].

A representative Raman spectrum of graphene is shown in figure 2.10. It features the G-peak ($\sim 1588 \text{ cm}^{-1}$) and the 2D-peak at $\sim 2700 \text{ cm}^{-1}$. The latter is an overtone of the D-peak ($\sim 1350 \text{ cm}^{-1}$), not observed. D-phonons correspond to the TO mode at the corner K of the Brillouin zone, which are not allowed to participate to Raman scattering with photons as momentum would not be conserved in the process. This vibration is associated to the breathing mode of carbon rings. In general D-phonons are revealed through the double-resonant scattering involving two D-phonons with opposite momentum to satisfy the momentum conservation law. Eventually, they produce a D-peak at $\sim 1350 \text{ cm}^{-1}$ in presence of lattice defects which lift the conservation of momentum (the name D-peak is because of Raman scattering with D-phonons is activated by defects). The absence of a D-peak in the Raman spectrum guarantees the high-quality of the lattice.⁷⁹

Furthermore, the position and the lineshape of those spectral features provides information about the doping of graphene and on the number of layers.^{75,80} The doping dependence of the Raman spectrum is the subject of the next paragraph. Concerning the number of layers, the profile of the 2D-peak differs from single-layer graphene, double-layer and multilayer. That is due to the sensitivity of the Raman scattering process to the electronic band structure, that changes with the number of layers. Single-layer graphene exhibits a sharp and symmetric 2D-peak, in general more intense than the G-peak, as for the spectrum in figure 2.10. Increasing the number of layers the G-peak become the more intense one, whereas the 2D-peak results from the overlap of sub-bands, appearing as an asymmetric large band.⁸⁰

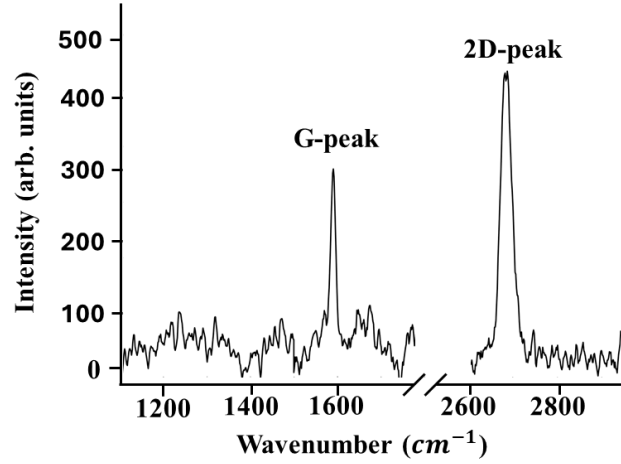


Figure 2.10 Raman spectrum of graphene. Raman spectrum of single-layer CVD graphene used in our device. The spectrum includes the G-peak ($\sim 1589 \text{ cm}^{-1}$) and the 2D-peak ($\sim 2685 \text{ cm}^{-1}$). The Raman spectrum of graphene is informative about the number of layers: the I_{2D}/I_G intensity ratio, >1 , and the symmetric lineshape of the 2D-peak confirms that it is a single-layer graphene. Moreover, the D-peak ($\sim 1350 \text{ cm}^{-1}$) is not observed suggesting a low density of defects in the carbon lattice.

2.4.4 Field-effect tuning of the Raman spectrum of graphene

G- phonons are markedly sensitive to the density of free charges in graphene. Indeed, phonons and charges are coupled. Lattice vibrations promote the creation of a virtual electron-hole pairs that recombine generating a phonon and the energy of G-phonons ($\hbar c \omega_G$) is renormalized in this process. Since the Fermi energy level determines the available states for hole and electrons, according to Pauli exclusion principle and to the conservation laws for energy and momentum, the final energy of G-phonons is influenced by the Fermi energy level. Moreover, G-phonons can also decay into a real electron-hole pairs. However, if $|E_F| > \frac{\hbar c \omega_G}{2}$ the transition is not energetically allowed anymore and consequently, the lifetime of G-phonons increases.

The electron-phonon interaction has been probed by measuring the Raman spectrum of graphene while modulating its doping level by field-effect. Peak position and bandwidth changes as a function of the Fermi energy level. Yan et.al measured the Raman spectrum of a back-gated graphene upon application of a gate voltage (V_G).⁷⁶ Their results are reported in figure 2.11. The G-peak position features a characteristic “V” trend. It is at $\sim 1583 \text{ cm}^{-1}$ for $V_G = 0 \text{ V}$ and blue-shifts for both positive and negative applied voltage, up to 1594 cm^{-1} when $V_G = -80 \text{ V}$. The field-effect tuning of the Fermi energy modulates the G-phonon frequency. The theoretical function describing the G-phonon energy

as a function of the Fermi energy can be worked out within the framework of a time-dependent perturbation theory under the approximation of a linear energy dispersion of electrons close to the Dirac point. It turns out

$$\omega_G - \omega_G^0 = \frac{1}{10^2 hc} \frac{\lambda_F}{2\pi} |E_F| \quad (2.7)$$

where $\omega_G^0 = 1580 \text{ cm}^{-1}$ is the theoretical energy of G-phonons at the CNP (when $E_F = 0 \text{ eV}$), whereas λ_F is a physical parameter describing the electron-phonon coupling strength found ~ 0.03 . Note that equation 2.7 holds for $100 \text{ meV} < |E_F| < 500 \text{ meV}$. Within this range the G-phonon's energy shift linearly with E_F and symmetrically with respect to the CNP. G-phonons stiffens as the doping increases, regardless of the doping sign, featuring a minimum energy, corresponding to the minimum of the “V”-shaped trend, when the Fermi energy is at the CNP. Furthermore, figure 2.11 shows that the G-peak bandwidth has a maximum value of $\sim 15 \text{ cm}^{-1}$ at the CNP and decreases to $\sim 6 \text{ cm}^{-1}$ as the doping increases. This narrowing is a direct consequence of the increased phonon lifetime for $|E_F| > \frac{hc\omega_G}{2}$.

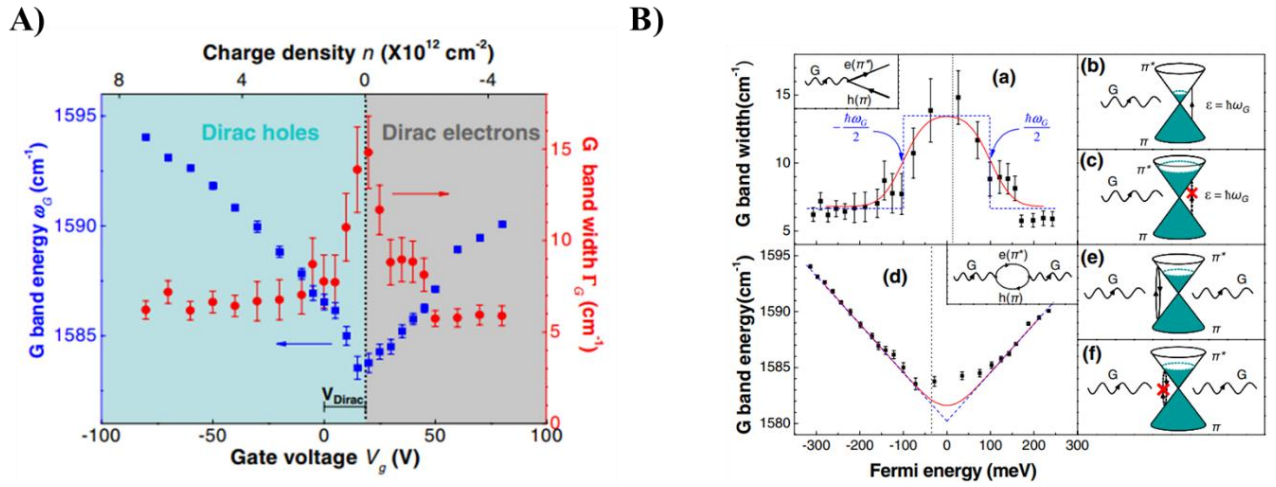


Figure 2.11. Field-effect tuning of G-peak. Taken from [Phys. Rev. Lett. 98, 166802 – Published 18 April, 2007] **a)** G-peak position and FWHM as function of the applied back-gate voltage. The minimum of the G-peak Raman shift, corresponding to a maximum bandwidth, identifies the CNP of graphene; **b)** a) G-peak bandwidth variation as a function of the Fermi energy.. re b) G-phonons decaying into real electron-hole pair; c) the decay process is forbidden if $|E_F| > hc\omega_G/2$; d) G-peak position variation as a function of the Fermi energy. The blue dotted line and the red line are the fit for ideal and non-uniform graphene e) G-phonons energy renormalization through virtual electron-hole pair transitions; f) virtual electron-hole pair transitions are forbidden for energy between 0 and $2|E_F|$.

The field effect tuning also influences the position of the 2D-peak in the Raman spectrum, though exhibiting a reduced sensitivity compared to the G-peak. One relevant difference is that D-phonons does not behave symmetrically for n- or p-doping. The 2D-peak blue-shifts in the case of p-doping, whereas red-shift for n-doping.

Remarkably, as great field-effect tuning of the Fermi energy is achievable by electrolyte gated device with low-voltage operation, as discussed in section 2.4.2, the tuning of spectral features in the Raman spectrum of graphene is also enhanced by the electrolyte. Using electrolyte-gated graphene, a shift $\Delta\omega_G > 15 \text{ cm}^{-1}$ has been measured upon application of gating voltage below 1 V.⁷⁷

2.5 Other materials

BSA lyophilized powder from Sigma Aldrich was used as received; Immunoglobulin G antibodies (anti-IgG), Immunoglobulin M antibodies (anti-IgM), IgG and IgM antigens buffered aqueous solutions from Sigma Aldrich was used as received. HEPES (MW $\approx 238.30 \text{ g mol}^{-1}$); sodium chloride NaCl, MW $\approx 58.44 \text{ g mol}^{-1}$, BioXtra, $\geq 99.5\%$, purchased from ATMerck; sodium hydroxide NaOH, MW = 40 g mol^{-1} , reagent grade, $\geq 98\%$, pellets, anhydrous purchased from Merk; HPLC-grade water, hydrogen peroxide 30%, VLSI grade, and sulfuric acid 96% VLSI Selectipur grade purchased from Avantor; Phosphate-buffered saline (PBS) tablets, ionic strength of 162 mM, pH 7.4, purchased from Sigma-Aldrich; 4-(2 hydroxyethyl)-1-piperazineethanesulphonic acid, HEPES $\geq 99.5\%$ purchased from Merk.

Chapter 3

Experimental methods, apparatus, and analysis of spectral data

3.1 Sample fabrication

3.1.1 Au electrodes and PM-IRRAS substrates fabrication

Au electrodes are fabricated by electron-beam deposition of 5 nm Ti adhesion-promoter layer and a 50 nm Au layer onto a Si/SiO₂ substrate, previously cleaned in sequential ultrasonic bath with deionized water, acetone and 2-propanol and dried under a nitrogen flux. A shadow mask is used to obtain the final Au pattern, with a 20 mm² circular pad, and a squared pad for electrical connection linked by a 0.25 mm wide channel.

PM-IRRAS reflective substrates are fabricated analogously, by e-beam deposition of 5/50 nm Ti/Au onto (2.5 x 5) cm glass microscope slides.

3.1.2 Buffer solutions preparation

Phosphate buffered saline (PBS, Sigma-Aldrich) solution is prepared by dissolving a tablet (phosphate buffer 0.01 M, KCl 0.0027 M, NaCl 0.137 M) in 200 mL HPLC water and used upon filtration on Corning 0.22 µm polyether sulfone membrane.

HEPES (4-(2-hydroxyethyl)-1 piperazineethanesulphonic acid; MW $\approx 238.30 \text{ g mol}^{-1}$) buffered solution is prepared starting from a 100 M solution obtained dissolving 2,383 g of HEPES dry powder into 100 mL of HPLC water. Then 250 mL are obtained by dilution at 5 M. The pH of the solution is adjusted by addition of NaOH 1M or HCl 1M solutions. About 400 μL of NaOH are added to take the pH at 7.4. The ionic strength is adjusted by addition of sodium chloride dry salt (NaCl; MW $\approx 58.44 \text{ g mol}^{-1}$).

3.1.3 Dilution of protein solutions

Anti-human Immunoglobulins (anti-IgG, anti-IgM) and Immunoglobulins (IgG, IgM) solution are prepared by sequential dilutions of the mother stock provided with a nominal concentration typically in the range 1-3 mg/mL by Sigma Aldrich. For Bovine serum albumin (BSA, MW $\approx 66 \cdot \text{kDa}$, Sigma Aldrich), first 50 mL of solution at 1 mg/mL is prepared dissolving 50 mg of BSA dry powder in 50 mL of buffer solution. Then sequential dilutions are performed.

A dilution of a factor k is prepared according to the equation $c_n = c_{n-1}k = c_{n-1} (V_{n-1}/V_n)$, being c_n and c_{n-1} the diluted and starting concentration respectively, and V_n and V_{n-1} the corresponding volumes. The uncertainty of the concentration is computed by error propagation, considering a 1% maximum uncertainty on the volumes, given by the supplier company of the micro-pipettes used. The uncertainty associated with the sampling of molecules is also taken into account, which is relevant at low concentrations. According to the Poisson's distribution, the number of sampled molecules has an associated standard deviation equals the square root of the expected value. The total uncertainty is given by

$$\sigma = \sqrt{\sigma_D^2 + \sigma_P^2} \quad (3.1)$$

where σ_D is the uncertainty from the dilution step and σ_P is the Poisson uncertainty. Note, σ_D depends on the number of dilution step and on the dilution factor used, increasing with the number of sequential dilutions. In general, the Poisson's uncertainty becomes comparable to σ_D for concentration below 1 aM.

The following table reports the concentrations, number of ligands and the associated errors for a typical dilution process up to the concentration of 10 zM.

Table 3.1. Sequential dilution. Ligand solution concentration with the corresponding number of ligands in 100 μL , dilution uncertainty σ_D , Poisson uncertainty σ_P and total uncertainty.

[ligand] (M)	# ligands	σ_D (M)	σ_P (# ligands)	σ (# ligands)
1,00E-05	6,02E+14	1,00E-06	24539764	6,02E+13
1,00E-06	6,02E+13	1,58E-07	7760155	9,52E+12
1,00E-07	6,02E+12	2,00E-08	2453976	1,20E+12
1,00E-08	6,02E+11	2,35E-09	776015	1,41E+11
1,00E-09	6,02E+10	2,65E-10	245398	1,59E+10
1,00E-10	6,02E+09	2,92E-11	77602	1,76E+09
1,00E-11	6,02E+08	3,16E-12	24540	1,90E+08
1,00E-12	6,02E+07	3,39E-13	7760	2,04E+07
1,00E-13	6,02E+06	3,61E-14	2454	2,17E+06
1,00E-14	6,02E+05	3,81E-15	776	2,29E+05
1,00E-15	6,02E+04	4,00E-16	245	2,41E+04
1,00E-16	6,02E+03	4,18E-17	78	2,52E+03
1,00E-17	6,02E+02	4,36E-18	25	2,64E+02
1,00E-18	6,02E+01	4,53E-19	8	2,83E+01
1,00E-19	6,02E+00	4,69E-20	2	3,74E+00
1,00E-20	6,02E-01	4,85E-21	1	8,29E-01
1,00E-21	6,02E-02	5,00E-22	0	2,47E-01

3.1.4 Bio-functionalization protocols

The bio-functionalization of gold substrates (sensing electrodes, and PM-IRRAS substrates) with a layer of physisorbed proteins is achieved according to the following protocol: i) sequential ultrasonic bath in HPLC water, acetone and 2-propanol and dried with nitrogen; if a more aggressive rinsing is needed the substrate are cleaned in a piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 = 3:1$), rinsed with HPLC water and dried under with nitrogen; ii) 10 minutes cleaning in UV-ozone chamber; iii) incubation with buffer solution ($\text{pH}=7.4$, $i_s=150$ mM) of proteins at concentrations of 50 $\mu\text{g/mL}$ or 100 $\mu\text{g/mL}$ for 2.5 hours at room temperature (according to the dimension of the substrate, the incubation is performed by dipping it into a vial with the protein solution, or by depositing a drop of solution onto its surface); iv) rinsing with plain buffer solution to remove unbound proteins. The bio-functionalization of KPFM samples follow the elicited protocol, with few differences: i) a polymeric mask is used to cover half of the surface, to obtain a sharp interface between the bio-functionalized area and the pristine Au area (used as internal reference); ii) the use of UV-ozone cleaner to keep the chemical inertness of gold. The ZnSe crystal used in ATR experiments, were gently rinsed with water, acetone, ethanol and 2-propanol, dried in air and further cleaned in an UV chamber for 30 minutes. Then the bio-functionalization follows the elicited protocol.

After the bio-functionalization, the sensing electrode are rinsed with HPLC water, whereas PM-IRRAS substrate and KPFM substrates and the ZnSe crystal are rinsed with plain buffer solution at

physiological pH=7.4 and low ionic strength $i_s=5\text{mM}$ to remove accumulated salt which interferes with the measurements. Also, samples for PM-IRRAS and KPFM experiments are dried using a spinner for 2 minutes at 1000 rpm. Whereas a flux of pressurised air is used to dry the bio-functionalized ZnSe crystal surface in the case of ATR spectroscopy measurements in air.

Surface plasmon resonance characterization

The elicited bio-functionalization protocol leads to the formation of a stable monolayer of highly-packed proteins, as demonstrated by surface plasmon resonance (SPR) measurements. SPR is a surface sensitive technique that enables the monitoring of surface processes through the assessment of refractive index changes within $\sim 300\text{ nm}$ from the inspected surface (appendix B). Specifically, layers of various proteins, such as anti-Immunoglobulin G, anti-HIV1-P24 and bovine serum albumin, physisorbed on gold according to the elicited protocol have been characterized through SPR analysis. The SPR sensograms show that proteins spontaneously adsorb on a bare Au surface, from standard buffer solution (pH=7.4, $i_s=150\text{ mM}$). As proteins tend to repel each other in normal condition, a single adlayer forms on the substrate, rather than multilayer aggregates. This is suggested by the adsorption kinetic (figure B2 appendix B) which is characterized by an exponential growth reaching a saturation value after about ~ 1 hour. Also, SPR data allows for the estimation of the protein film thickness of $\sim 10\text{ nm}$ (comparable to that measured by AFM), which further confirm the formation of a single monolayer. Furthermore, SPR analysis provides the density of adsorbed proteins. It results that our physisorption protocol leads to a density of $\sim 10^{11}\text{-}10^{12}\text{ proteins cm}^{-2}$ for all the investigated proteins.^{47,81} Note that $10^{12}\text{ proteins cm}^{-2}$ correspond to one protein in $(10\text{ nm})^2$ which is the molecular footprint of for a single protein, hence the our bio-functionalization strategy produces a monolayer of highly packed proteins.

3.1.5 Incubation with analytes

The incubation with ligands molecules is performed by dropping the ligand solution onto the bio-functionalized surface and leaving it unattended for 10 minutes at room temperature. A $100\text{ }\mu\text{L}$ drop is used for the sensing electrode a, whereas 1 mL is used for the PM-IRRAS substrate. After 10 minutes the surface is rinsed with plain buffer solution. sensing electrodes are further rinsed in HPLC water. The PM-IRRAS and KPPFM samples are further rinsed in buffer solution at physiological pH=7.4 and low ionic strength $i_s=5\text{mM}$ to remove accumulated salt which interfere with the measurements and dried using a spinner for 2 minutes at 1000 rpm.

3.2 Graphene electrodes fabrication

3.2.1 Chemical vapour deposition (CVD) growth of graphene

Single-layer graphene is grown by catalytic CVD. The methodology is as follow. A copper foil (10 x 10) cm² wide and 25μm thick is inserted into the quartz tube of the reactor thermal furnace. The pressure is lowered below 10⁻³ torr and the tube is heated at 990 °C under an H₂ gas flow of 10 sccm (0.05 torr). Then, the growth phase begins by injection of of CH₄ gas at 5 sccm for 20 minutes. The catalytic reaction of methane molecules onto the copper foil produces carbon atoms that arrange to form a very high-quality graphene layer. After the growth phase, the graphene/copper foil is rapidly cooled down by moving the quartz tube outside the growth zone.³²

3.2.2 Graphene transfer on glass

The graphene layer is transferred onto a Corning-glass substrate by a thermal release tape (TR-tape) procedure. A sheet of TR-tape is pressed on top of graphene using a laminator. The copper layer is removed in a ammonium persulfate solution (20 g L⁻¹) leaving a floating TR-tape/graphene sheet. The sheet is rinsed in deionized water and dried in air. Then, graphene is positioned onto the final glass substrate and hot-pressed to remove the TR-tape.

The electrical quality of the glass/graphene electrode is verified by measuring its sheet resistance, being in the range 850-1000 Ω cm⁻² whereas the lattice quality is assayed by Raman spectroscopy.

3.3 Experimental setups

3.3.1 Micro-Raman spectroscopy

Raman spectroscopy represents insightful technique for material identification, device characterization, analysis of structural and electronic properties and for fundamental studies on phonons. Novel carbon-based materials, above all graphene and its derivatives, have been extensively studied by Raman spectroscopy given the large cross-section for Raman scattering process. From the

analysis of the frequency, linewidth and intensity of Raman spectral features, information about the strain, defect density and doping level are provided. Raman spectroscopy is widespread in a range of applications and numerous diversified technological solutions are available to bring Raman spectrometers outside the lab for in-field applications.

Experimental setup

Raman spectra have been acquired by a NT-MDT NTEGRA Spectra Raman spectrometer coupled with a laser scanning confocal microscope. The optical scheme of the spectrometer is represented in figure 3.2. It has a modular design composed of: laser, optical-mechanical unit (OMU), microscope, detection system.

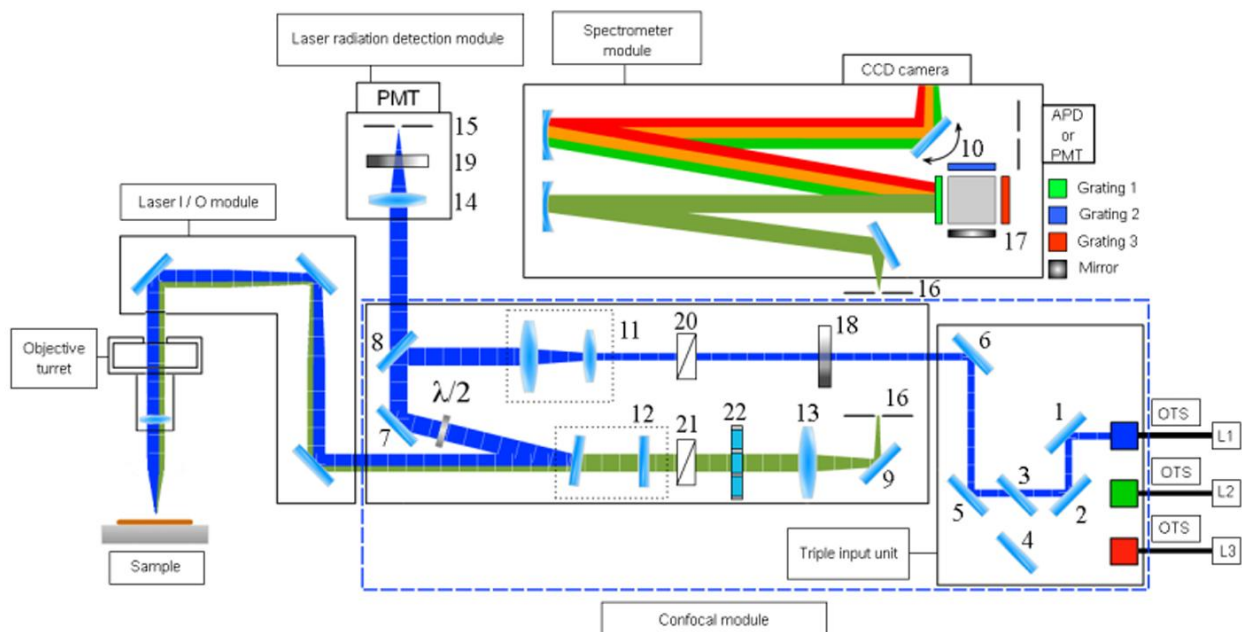


Figure 3.1. Micro-Raman spectroscopy setup. Optical scheme of the micro-Raman spectrometer NT-MDT NTEGRA Spectra. 1-7,9,10 mirrors; 8 97% mirror; 11 beam expander; 12 changeable edge filters (one per wavelength); 13,14 lenses; 15,16 adjustable pinhole; 17 diffraction gratings; 18,19 adjustable density neutral density filter; 20,21 polarizer; 22 filter cartridge; L1,L2,L3 laser APD avalanche photodiode; PMT photomultiplier tube; OTS optical fiber transport system.

4 input laser are available (786 nm, 633 nm, 532 nm, 488 nm), coupled with the spectrometer via optical fiber. The OMU includes all the optical and mechanical components designed to control the beam quality and define the parameters such as incident power and polarization. The OMU is couples to a confocal microscope by an input/output module. The microscope includes five objectives with magnitude 5x, 20x, 50x, 50x long-working distance, 100x. The sample is placed onto a motorized

scanning stage, with x-y scanning precision of 100 nm. Laser light is focused on the sample and backscattered radiation is collected again by the microscope objective and redirected toward the detector. On its way to the detector, radiation goes through an edge filter which transmits only radiation with wavelengths larger than that of the laser. Then a pinhole lets light into the detection module. It contains a grating turret including four gratings (75 lines/mm, 150 lines/mm, 600 lines/mm, 1800 lines/mm) and a Peltier-cooled CCD camera working at -61°C. The camera has a matrix of 1024x255 pixels 26 μm^2 wide, with a vertical scanning rate of 16 ms.

The microscope is also equipped with an optical viewing system, that includes a white LED and a camera for the acquisition of optical images of the sample. Changing the position of a mirror in the input/output module it is possible to select the detected radiation: backscattered radiation for the acquisition of Raman spectra and reflected visible light for recording the optical image.

Measurements of the Raman spectrum of graphene, were performed at room temperature using unpolarized green light was used ($\lambda=532$ nm), focused on the sample by a 20x microscope objective (NA = 0.25). Light was focused on an area 5 μm in diameter with an incident power is always below 3 mW, over an exposure time of 100 s. Collected backscattered radiation was dispersed by the 600 lines/mm grating having a resolution of 4 cm^{-1} (estimated as the full-width-at-half-maximum of the Rayleigh peak at 0 cm^{-1}).

Fundamentals of Raman spectroscopy

Raman spectroscopy is based on the inelastic scattering of photons by phonons. The incident radiation on the sample perturbs its electronic wavefunction inducing a temporary transition to an excited state. In general, the energy of the incident photons does not match the energy of an electronic stationary state hence the system is excited to a virtual state, whose lifetime is of the order of picoseconds, before decaying again to a stationary state with the emission of a photon. The process is schematically described in figure 3.2. Three scattering processes are possible depending on the final energy level. The Rayleigh scattering occurs if the system returns to the original state; this is an elastic process as the energy of the emitted photon ($\hbar\omega_s$) equals that of the incident one ($\hbar\omega_i$). Raman scattering takes place when the electronic wavefunction decays to a stationary state different from the original one, because of the interaction with phonons. The system could return to a stationary state in a higher energy vibrational level (corresponding to the excitement of a phonon in a solid material). In this case the emitted photon is less energetic than the incident one ($\hbar\omega_s = \hbar\omega_i - \hbar\omega_R$) and the difference equals that of the excited phonons ($\hbar\omega_R$). This is called *Stokes scattering*. Also, if the system starts

from an excited vibrational level and decays to a lower energy level, the emitted photon has higher energy than the incident one, equals to $\hbar\omega_i + \hbar\omega_R$. This is known as *anti-Stokes scattering*.

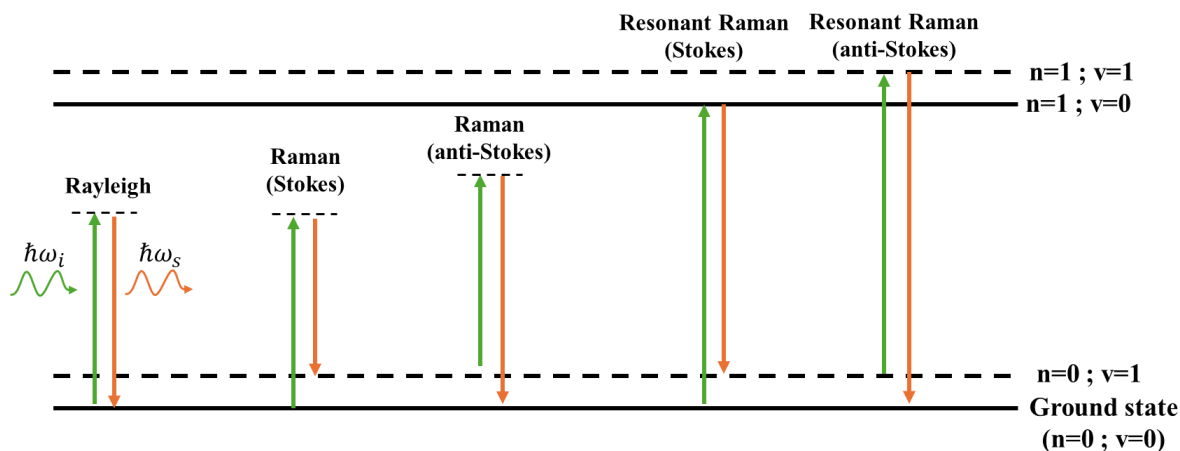


Figure 3.2. Raman scattering. Schematic representation of (from left to right): Rayleigh scattering, non-resonant Raman Stokes scattering, non-resonant Raman anti-Stokes scattering, resonant Raman Stokes scattering, resonant Raman anti-Stokes scattering. n and v are the electronic quantum number and the vibrational quantum number respectively.

The probability of Raman scattering is generally 10^6 - 10^8 times lower than that of a Rayleigh scattering, therefore, most of the incident photons on the sample are re-emitted at the same energy. The probability of Raman scattering is increased in the case of resonant processes, happening if the energy of incident photons matches that of an excited electronic state.

Raman spectroscopy measures the intensity of scattered radiation as a function of the *Raman shift* that is, the energy difference between the scattered photon and the incident ones. Peaks in the Raman spectrum correspond to Raman active vibrational modes, and its position, linewidth and intensity are informative on the structural properties of the sample. Note that the Raman scattering process involves only vibrational modes associated to a variation of the electronic polarizability of the system. Therefore, Raman spectroscopy is complementary to IR spectroscopy, that on the other hand is sensitive to vibrational modes involving a change in the molecular dipole moment. Since the Raman scattering involves electronic transition, the features appearing in a Raman spectrum provide information on the electronic properties as well.

3.3.2 Polarization-modulation infrared reflection-absorption spectroscopy (PM-IRRAS)

Infrared reflection-absorption spectroscopy (IRRAS) has been extensively utilized to measure the IR spectrum of thin samples deposited onto reflective substrate. Specifically, IRRAS on metallic substrates leverage the amplification of the electric field at the metal surface and the differential absorption of s- and p- polarized radiation at grazing angle of incidence to enhance the surface sensitivity and study the organization of adsorbed molecules. To further improve the performance of IRRAS spectroscopy, polarization modulation has been introduced, via a photoelastic modulator (PEM). Modern polarization-modulation infrared reflection-absorption spectroscopy (PM-IRRAS) take advantage of the modulation of polarization and of a synchronous sampling demodulator (SSD) system to attain enhanced surface sensitivity, up to sub-monolayer nm-thin films. However, such advantages came at the cost of a more complex setup and output signal.

Experimental setup

The PM-IRRAS experimental setup is illustrated in figure 3.3. It consists of a Nicolet iS50 Fourier-transform IR spectrometer coupled with an external research module containing a wire-grid polarizer, a photo-elastic modulator (PEM) a sample holder and a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector. The whole apparatus is constantly purged with dry air to keep relative humidity below 17% and low concentration of carbon dioxide in the system. The PEM consist of a Zinc Selenide (ZnSe) crystal coupled with a piezoelectric unit. A periodic cycle of stretching and compressions of the ZnSe crystal is produced through the application of a sinusoidal voltage $V(t) = V_0 \sin(\omega_{PEM}t)$, with frequency $\omega_{PEM} = 50 \text{ KHz}$. The mechanical deformations generated modify the crystal refractive index along the stress direction, rendering the crystal birefringent. Light propagating through the PEM experiences a periodic phase change resulting in the modulation of its polarization between a p-linearly polarized state (with the electric field oscillating perpendicular to the sample surface) and a s-linearly polarized state (with the electric field oscillating parallel to the sample surface), at a frequency $\omega_m = 2\omega_{PEM} = 100 \text{ kHz}$, as two polarization cycle occurs over each compression and stretching cycle. In fact, only for $\tilde{\nu} = \tilde{\nu}^*$ the polarization is effectively modulated between a p- and an s-polarization state, whereas for all the other wavenumber the modulation occurs between a p-polarization state and generic elliptical polarization. Then, radiation is reflected at grazing angle of 82° on sample surface and is directed to the MCT detector. The PM-IRRAS sample consist of an Au coated substrate, on which surface the layer of adsorbed molecules under investigation is deposited. The interferogram recorded by the detector is fed into the input channel of a synchronous sampling demodulator (SSD), along with a reference periodic signal at frequency ω_m , for the simultaneous demodulation and digitalization of the signal. This process returns a difference

spectra, proportional to $(R_p - R_s)$, and a sum spectrum, proportional to $(R_p + R_s)$, where R_p and R_s are the reflected intensity of the p-polarized and s-polarized radiation. Finally, the output PM-IRRAS signal is proportional to the ratio of the two spectra.

The PM-IRRAS spectra reported in this thesis were collected with a resolution of 4 cm^{-1} and result from the average of 1000 scans.

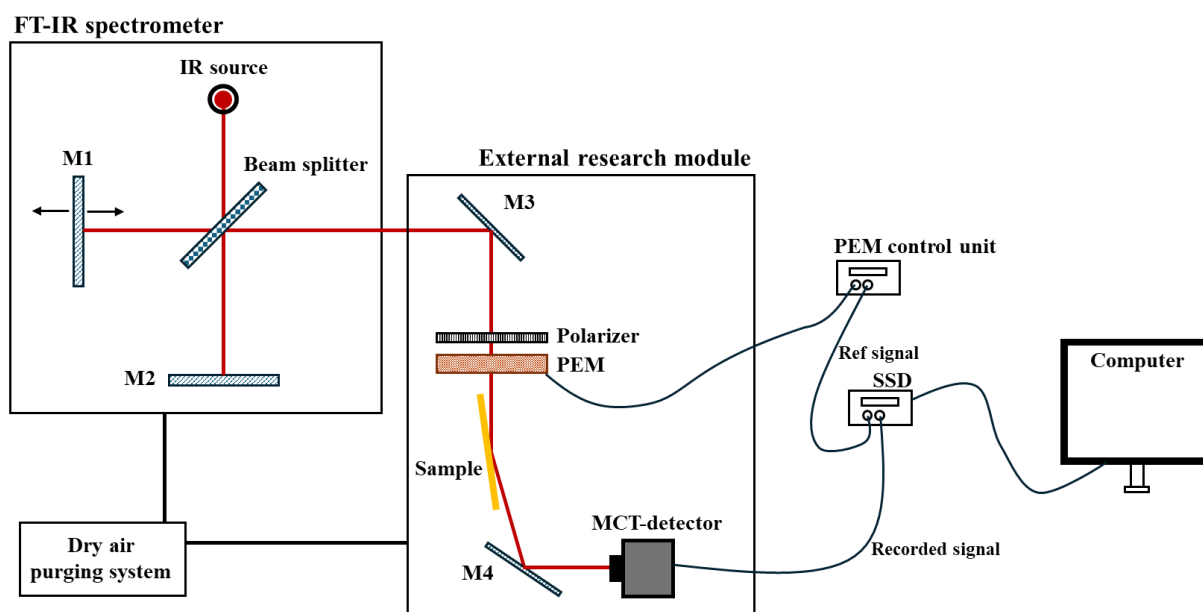


Figure 3.3. PM-IRRAS experimental setup. PM-IRRAS apparatus composed of a Nicolet iS50 FT-IR spectrometer coupled with an external research module. The figure features the main optical component of the FT-IR spectrometer including the IR source, one moving mirror (M1), a fixed mirror (M2), and a KBr beamsplitter. The external research module includes two parabolic mirrors (M3 and M4) the wire-grid polarizer, the PEM, the sample holder and the MCT detector. The whole apparatus is purged with dry air. The PEM is connected to a control unit. It provides a reference signal to the synchronous sampling demodulator (SSD), which also receive the recorded signal from the detector. Then the demodulated interferograms are processed and visualized by the OMNIC software on the computer.

Fundamentals of PM-IRRAS spectroscopy

PM-IRRAS spectroscopy on metallic substrate is based on the interference between the incident and reflected electromagnetic waves in proximity of a metal surface, illuminated by IR radiation at grazing angle of incidence (above 80°), that produce an amplified p-polarized electric field (oscillating normal to the metal surface) and an almost vanishing s-polarized field (oscillating parallel to the metal surface). Therefore, in presence of a thin film adsorbed on the reflective substrate, only the p-wave interacts and is absorbed by the layer of adsorbed molecules, whereas the s-wave is simply

reflected as for the case of bare substrate. This effect is known as the surface selection rule for the absorption of radiation on metal surfaces. The theoretical spectrum recorded in PM-IRRAS measurement comes from the differential reflectivity of s- and p-polarized radiation

$$\left(\frac{\Delta R}{R}\right)_{th} = \frac{R_p - R_s}{R_p + R_s} \quad (3.2)$$

containing features originated from the vibrational properties of the adsorbed ultrathin films. Note that this model holds for film thickness (d) smaller than 1/3 of the incident wavelength (λ). In fact, the experimental PM-IRRAS signal is complicated by the response of the experimental setup. Figure 3.4 features a schematic representation of a sample compartment of the PM-IRRAS spectroscopy setup.

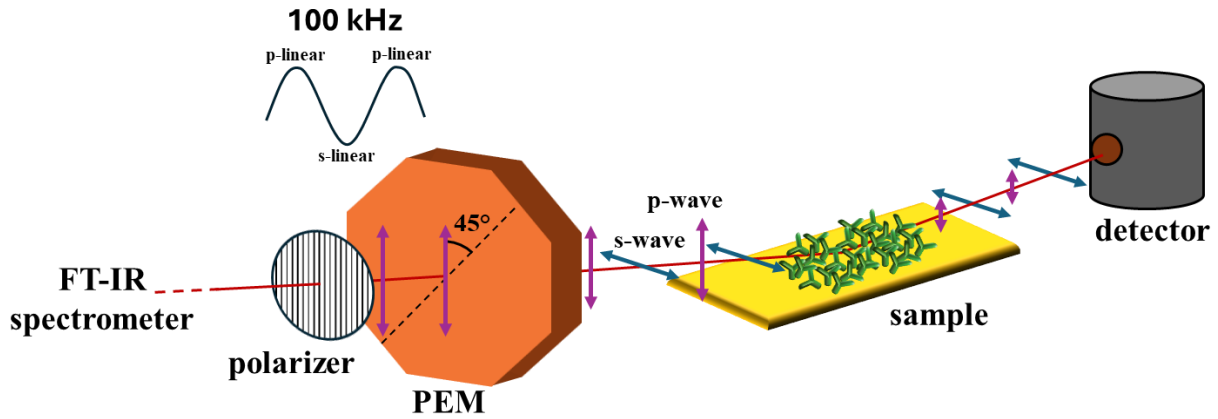


Figure 3.4. Polarization modulation. Schematic representation of a PM-IRRAS measurement, featuring the IR beam exiting the FT-IR spectrometer, passing through the polarizer and the PEM; the polarizer axis is mounted at 45° with respect to the PEM stress axis. The PEM modulates the polarization state of the incident beam at 100 kHz. The sample is depicted as a thin protein film adsorbed onto the metallic substrate. Blue and purple arrows correspond to the modulated s- and p-polarized electric field vectors. Radiation is reflected at grazing angle of incidence from the sample and recorded by a detector. The shorter purple arrows after reflection suggest the absorption of p-polarized radiation by the thin film.

The intensity reaching the detector (I_d) can be expressed as^{82,83}

$$I_d = C \frac{I_0(\tilde{\nu})}{2} \left[(R_p + R_s) - 2(R_p - R_s) J_2(\varphi_0) \cos(\omega_m t) \right] \quad (3.3)$$

where $I_0(\tilde{\nu})$ is the intensity of radiation before the PEM, C is a proportionality coefficient, ω_m is the polarization modulation frequency, $J_2(\varphi_0)$ is the second order Bessel function and φ_0 is the maximum dephasing imposed by the PEM. The Bessel function $J_2(\varphi_0)$ is a sort of PEM response function accounting for its modulation efficiency at different wavenumber. The maximum dephasing φ_0 is

controlled by the PEM control unit and is set equal to π for a specific wavelength ($\tilde{\nu}^*$), at which the polarization is effectively modulated between a p- and an s-polarization state.

According to equation 3.3, the introduction of the PEM leads to a modulated detected intensity, given by the sum of two signals, one proportional to the average reflectivity $(R_p + R_s)/2$ and one proportional to the differential reflectivity $(R_p - R_s)$. Both terms are subjected to the Fourier modulation from the FT-IR interferometer, whereas the second contribution is further modulated at frequency ω_m . A synchronous sampling demodulator, tuned at frequency ω_m , extracts the average and differential reflectivity separately and delivers the two interferograms into separated channels. Then, they undergo the standard processing to obtain two single-beam spectra, in the wavenumber domain. Finally, the output PM-IRRAS signal is provided as the ratio of the two single beams.

Two major advantages of this method appear clearly at this point. In the first place, the two relevant quantities, the average reflectivity and the differential reflectivity are readily available from a single measurement. Contrary to other traditional IR spectroscopy approach, there is no need of a background measurement. Secondly, the isotropic absorption from molecules in the sample surrounding environment, such as atmospheric carbon dioxide and water vapour, affects equally both signals, thanks to the synchronous sampling, therefore their interference is almost perfectly corrected upon the ratio of the two channel spectra.

Hence, PM-IRRAS represents an extremely surface sensitive technique enabling measurements of nanometre-thin film with a excellent S/N (>200).

Conversion of PM-IRRAS signal into absorbance

Those advantages come to the price of an output signal which is not straightforwardly related to a physically meaningful absorbance ($A = -\log(R/R_0)$). Indeed, the experimental PM-IRRAS signal is affected by parameters such as the different gain experienced by the two signals in the two channels (C_+ and C_s), the different optoelectronic responses of the setup to p- and s- polarized radiation (C_p and C_s), and by the PEM response $J_2(\varphi_0)$, resulting in

$$PM - IRRAS \text{ signal} \equiv \left(\frac{\Delta R}{R} \right)_{exp} = \left| g J_2(\varphi_0) \frac{\gamma R_p - R_s}{\gamma R_p + R_s} \right| \quad (3.4)$$

being $g = C_+/C_-$ and $\gamma = C_p/C_s$.

Buffeteau et al.⁸² proposed a procedure for the conversion of the experimental spectral intensities into absorbance unit, consisting in dividing the PM-IRRAS spectrum of the sample by that measured with the bare substrate $((\Delta R/R)_{sub})$. The resulting normalized spectrum is then related with the absorbance according to the equation:

$$\left(\frac{\Delta R}{R}\right)_{norm} = \frac{\left(\frac{\Delta R}{R}\right)_{exp}}{\left(\frac{\Delta R}{R}\right)_{sub}} = 1 + \frac{2\gamma\rho}{1-\gamma^2\rho^2} \frac{1}{0.434} A \quad (3.5)$$

where $\rho = R_{p,sub}/R_{s,sub}$ is the ratio of the p- and s- reflectivity of the bare substrate.

Within this manuscript the conversion into absorbance has been performed according to the following assumption. First, the background signal has been calculated (see section 3.4.1) rather than measured experimentally. Also, for thin monolayer film deposited on gold substrate, which is the relevant case in this thesis, $\gamma \approx 1$ and $\rho \approx 0.95$ can be assumed.⁸⁴ Thus, absorbance spectrum is obtained according to the equation:

$$A = 0.0223 * \left[\left(\frac{\Delta R}{R}\right)_{norm} - 1\right] \quad (3.6)$$

3.3.3 Attenuated total reflection (ATR) spectroscopy

Attenuated total reflection (ATR) spectroscopy is a widespread technique for the measurement of the IR absorbance of a plethora of samples. It leverages the evanescent electromagnetic wave propagating at the interface between the ATR crystal and the external medium, upon reflection above the critical angle, to probe the sample in a micrometre thin volume. The main advantage of ATR spectroscopy is the almost complete absence of sample preparation and versatility. Indeed, it is particularly advantageous for the analysis of liquid samples since narrow cells are not needed facilitating the experiments and avoiding interference fringes as well. Also, the short path lengths of the evanescent wave within the sample renders it an ideal approach to acquire spectra in aqueous solutions as the absorption from H₂O molecules is minimized. Furthermore, ATR spectroscopy can be performed to study material surfaces and thin films. In this thesis, the ATR spectrum of protein monolayers, directly adsorbed on the ATR crystal surface was measured and analysed.

Experimental setup

ATR spectra reported in this thesis were collected via a Vertex 70, Bruker Optics, spectrometer. For measurements of bulk materials, a single-reflection diamond internal reflection element (IRE) was utilized. On the other hand, measurements of the ATR spectrum of thin protein film were performed using a ZnSe waveguide was utilized as IRE. It was a 7,2 cm long crystal allowing for 6 internal reflections, hosted within the measurement cell. The spectrometer was equipped with custom cell for liquid samples and with a liquid nitrogen-cooled mercury-cadmium-telluride detector. The spectrometer was constantly purged with dry air to keep low level of atmospheric water vapour and carbon dioxide. The custom cell was made of PEEK and stainless steel customized to fit the ZnSe IRE. Also, it includes an inlet and outlet connected to a peristaltic pump through microfluidic Teflon tubing. Figure 3.5 feature a schematic representation of the main component constituting the ATR experimental setup. The setup allowed to measure liquid samples, in static condition or in flow. Also, it allowed the bio-functionalization of the ZnSe surface with protein monolayers.

ATR spectra reported in this thesis have been acquired with a resolution of 2 cm^{-1} and results from the average of 1024 scans.

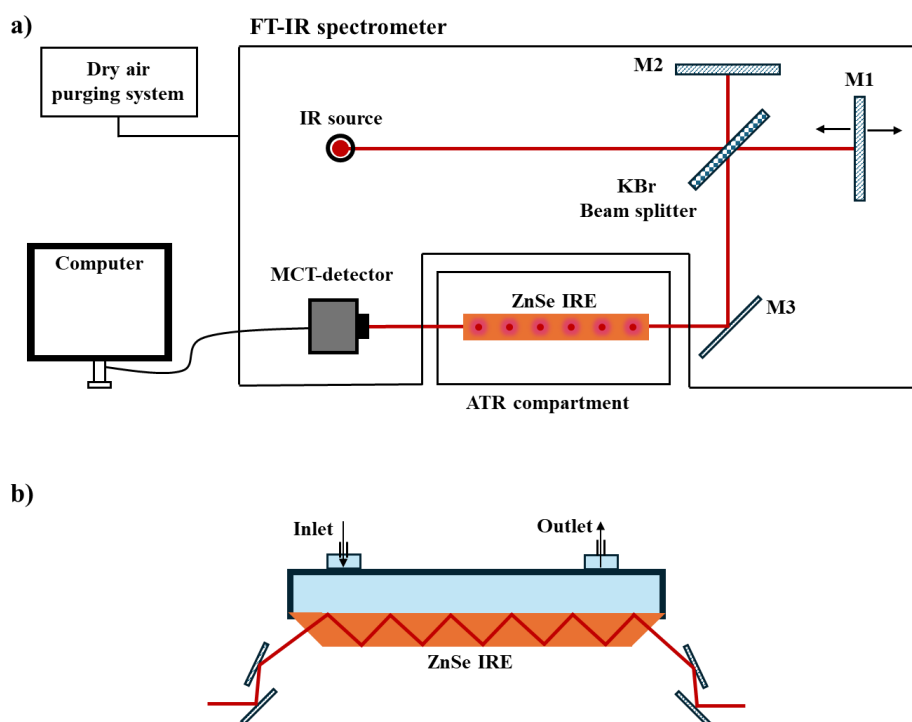


Figure 3.5. ATR spectroscopy experimental setup. a) ATR spectroscopy setup consisting of a Vertex 70 (Bruker Optics) FT-IR spectrometer coupled with the ATR compartment. The schematic representation of the FT-IR spectrometer features its main optical components including the IR source, one moving mirror (M1), two fixed mirrors (M2, M3), the KBr beamsplitter and the detector. The recorded signal is processed by OPUS 8.5 software on the computer. The orange rectangle inside the ATR compartment represents the ZnSe internal reflection element (IRE). The red dots represent

the six internal reflections of IR radiation at the ZnSe surface. **b)** Vertical section of the ATR compartment, featuring the ZnSe IRE placed inside the measurement cell. The ZnSe crystal has facet are cleaved at 45°. The inlet and outlet of the cell are also shown.

Fundamentals of ATR spectroscopy

ATR spectroscopy is based on the reflection of IR radiation at the interface between an optically dense material (with refractive index n_1), named the internal reflection element (IRE), and the external medium whose refractive index n_2 is smaller than n_1 .⁸⁵ For incidence above the critical angle ($\theta_c = \sin^{-1}(n_2/n_1)$) the transmitted electromagnetic field remains confined at the interface and an evanescent wave is generated in the rarer medium, as schematically showed in figure 3.6. This is clarified by the expression of the transmitted electric field upon reflection at supercritical angle

$$\mathbf{E}_t(\mathbf{r}, t) = \mathbf{E}_{0t} e^{-i\omega t} e^{ikn_1 \sin(\theta)x} e^{-kz\sqrt{n_1^2 \sin^2(\theta) - n_2^2}} \quad (3.7)$$

The field propagate parallel to the interface (along x) and its amplitude decays exponentially in the direction normal to it (z direction). The decay is characterized by a penetration length

$$d_p = \frac{\lambda}{2\pi\sqrt{n_1^2 \sin^2(\theta) - n_2^2}} \quad (3.8)$$

corresponding to the distance from the interface, where the electric field amplitude is reduced by a factor $1/e$, which is dependent on the incident wavelength, the refractive indices of both media and the incidence angle (θ). Typical penetration lengths for the geometries adopted in ATR experiments in the mid-IR spectral range, falls around few micrometres; for example, the total reflection of radiation with $\lambda = 1000 \text{ cm}^{-1}$ incident at 45° on a ZnSe/air interface ($n_{\text{ZnSe}} = 2.4, n_{\text{air}} = 1$) generates an evanescent field whose d_p equals $\sim 1.16 \text{ }\mu\text{m}$.

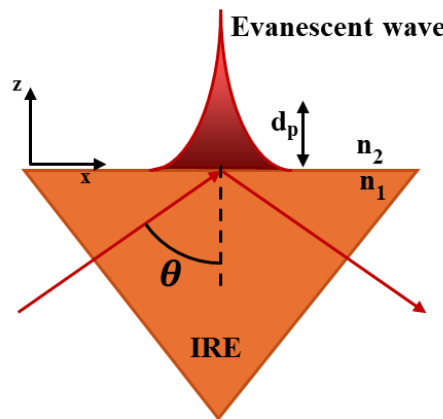


Figure 3.6. Evanescent wave. Schematic description of the total reflection of radiation incident at an angle θ at the interface between the internal reflection element (IRE), with refractive index n_1 and the external medium with refractive index $n_2 < n_1$.

If the external medium is transparent total reflection occurs, that is the electromagnetic energy carried by the incident beam is completely reflected. However, the case of interest for applications of ATR spectroscopy is that of absorbing external medium. It could be constituted by the sample itself, placed in intimate contact with the IRE surface or a by a medium containing absorbing molecules whose spectrum is going to be measured. That is described by a complex refractive index $\hat{n}_2 = n_2 + ik_2$ including the absorption index k_2 . Replacing it into equation 3.7, the transmitted field will still have a decaying amplitude in the z direction within the rarer medium, but also it will acquire a propagation term along z , corresponding to a wave propagating into the rarer medium at a slight angle while it is also absorbed at a rate defined by the absorption index k_2 . In this case, the reflection is “attenuated”. The reflected beam does not carry the total amount of incident energy, because part of it leaks through the interface. In an ATR experiment the signature of sample absorption follows from the energy loss of the reflected radiation, at the specific wavelengths resonant with the absorbing molecules, resulting in absorption bands in the reflectance spectrum. The ATR spectrum is generated by the ratio of the reflectance measured with the sample, R_{sample} , with respect to a background reflectance, R_0 (typically that of the bare IRE, or that of a plane solution). In absorbance units it is

$$A_{ATR} = -\log\left(\frac{R_{sample}}{R_0}\right) \quad (3.9)$$

Note that the sample is probed by the evanescent field in a few micrometre-thin volume defined by the penetration length. That brings the main advantage of minimizing the contribution from the bulk of the sample and from the sample surrounding environment. In particular, the latter is a crucial aspect for measurements in aqueous solutions (where radiation is completely absorbed within pathlength of $\sim 12 \mu\text{m}$), as the use of inconvenient narrow cell or deuterated water is not needed. ATR spectroscopy can be also adopted to measure the absorbance of ultrathin films, with thickness d smaller than the incident wavelength, such as protein monolayers adsorbed onto the IRE surface, which is the relevant case for this thesis. In presence of an ultrathin film, the critical angle is still defined by the refractive indices of the IRE and of the external medium, and the of s- and p- polarized radiation is described within the framework reported in Appendix C.⁸⁶ Considering the reflectance of s- and p- polarized radiation, $R_{0,s} = R_{0,p} = 1$ for the bare substrate at supercritical incidence angle, the ATR reflectance in presence of an ultrathin film adsorbed onto the IRE surface reads

$$R_s = 1 - \frac{8\pi d n_1 \cos(\theta_i)}{\lambda} \text{Im} \left[\frac{\hat{\epsilon}_{film} - \hat{\epsilon}_3}{\hat{\epsilon}_1 - \hat{\epsilon}_3} \right] \quad (3.10a)$$

$$R_p = 1 - \frac{8\pi d n_1 \cos(\theta_i)}{\lambda} \text{Im} \left\{ \left[\frac{\hat{\epsilon}_{film} - \hat{\epsilon}_2}{\hat{\epsilon}_1 - \hat{\epsilon}_2} \right] \left[\frac{1 - \hat{\epsilon}_1 / \hat{\epsilon}_{film} \hat{\epsilon}_2 (\hat{\epsilon}_{film} + \hat{\epsilon}_2) \sin^2(\theta_i)}{1 - (1/\hat{\epsilon}_2) (\hat{\epsilon}_{film} + \hat{\epsilon}_2) \sin^2(\theta_i)} \right] \right\} \quad (3.10b)$$

where $\hat{\epsilon}_1$, $\hat{\epsilon}_{film}$, $\hat{\epsilon}_2$ are the complex dielectric functions of the IRE, of the film, and of the external medium respectively.

3.4 Processing of vibrational spectra

3.4.1 Processing and analysis of Raman spectra of graphene

Background subtraction and Lorentzian fit

The analysis of the Raman spectra of graphene is focused on the G-peak and 2D-peak spectral region and is performed by custom Python scripts (see Appendix D). A second-order polynomial background is subtracted in the range 1550-1680 cm^{-1} and 2600-2830 cm^{-1} . A fit with a Lorentzian function is performed, to obtain the peak-wavenumber position, the full-width-at-half-maximum (FWHM) and intensity both the G-peak and 2D-peak. Figure 3.7 shows the background subtraction and Lorentzian fitting of a representative graphene spectrum in the G-peak spectral range.

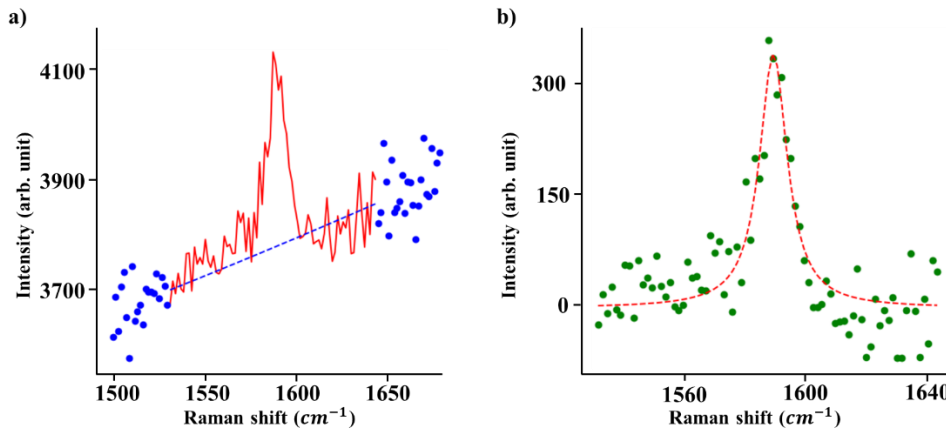


Figure 3.7. Analysis of the Raman spectrum. Background subtraction and Lorentzian fitting of Raman spectrum. **a)** Raman spectrum of graphene in the G-peak spectral region. The spectral background is computed by fitting the data far from the peak (blue dots) with a second order polynomial. **b)** Raman spectrum (green dots) after background subtraction. The dashed red line represents a Lorentzian fit of the peak.

3.4.2 Processing of PM-IRRAS spectra

Background subtraction

PM-IRRAS spectra are subject to a background correction in the relevant spectral range. A second order polynomial background is computed by a custom MATLAB script (see appendix D). The computed background accounts for the experimental response of the apparatus at different wavelength. Figure 3.8 shows a representative PM-IRRAS spectrum of a protein monolayer, with the computed background in the amide I amide II spectral range. The background is considered as the PM-IRRAS signal that would be measured with a pristine Au substrate, in absence of any adsorbed molecules on its surface. Further processing, such as smoothing and atmospheric correction is not needed () because of the high S/N ratio (>100) ensured by the synchronous sampling polarization-modulation methodology.

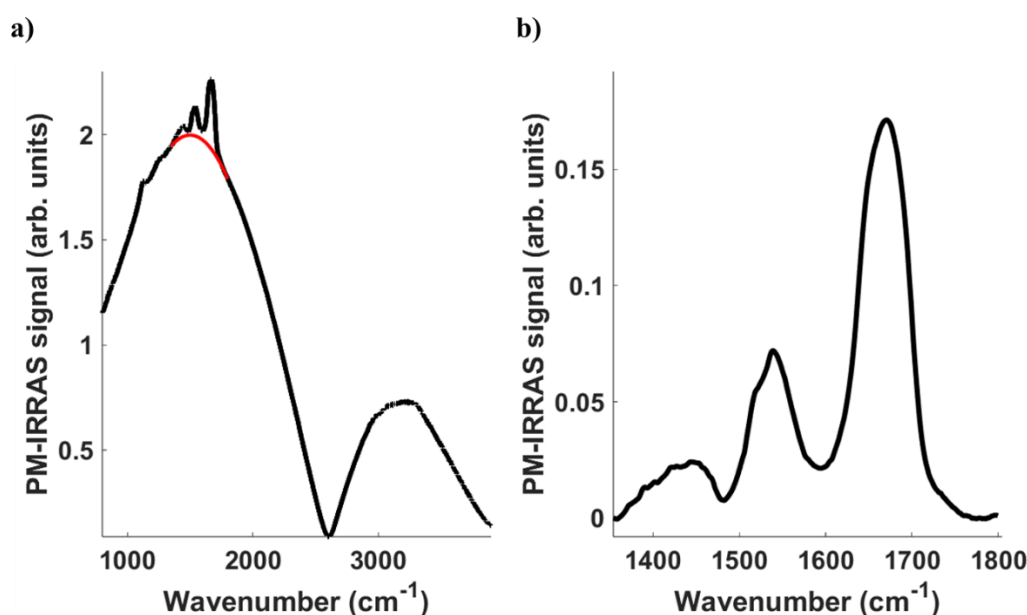


Figure 3.8. Background correction of PM-IRRAS spectra. a) Representative PM-IRRAS spectrum of a protein monolayer physisorbed on Au. The red line represents the second-order polynomial background computed in the relevant spectral range between 1350 cm^{-1} and 1800 cm^{-1} . b) PM-IRRAS spectrum after background correction. The original spectrum is divided by the computed background and vertically offset to zero.

3.4.3 Processing of ATR spectra

Background subtraction and smoothing

ATR spectra are processed by the OPUS 8.5 software. They are subject to a straight-line background subtraction in the amide I – amide II spectral range between 1495 cm^{-1} and 1710 cm^{-1} . Spectral smoothing is also performed to improve the S/N ratio (~ 30). The smoothing procedure is based on the Savitzki-Golay algorithm, using a moving window containing a number of points between 5-25.

Figure 3.9 Show a representative ATR spectrum before and after background subtraction and smoothing.

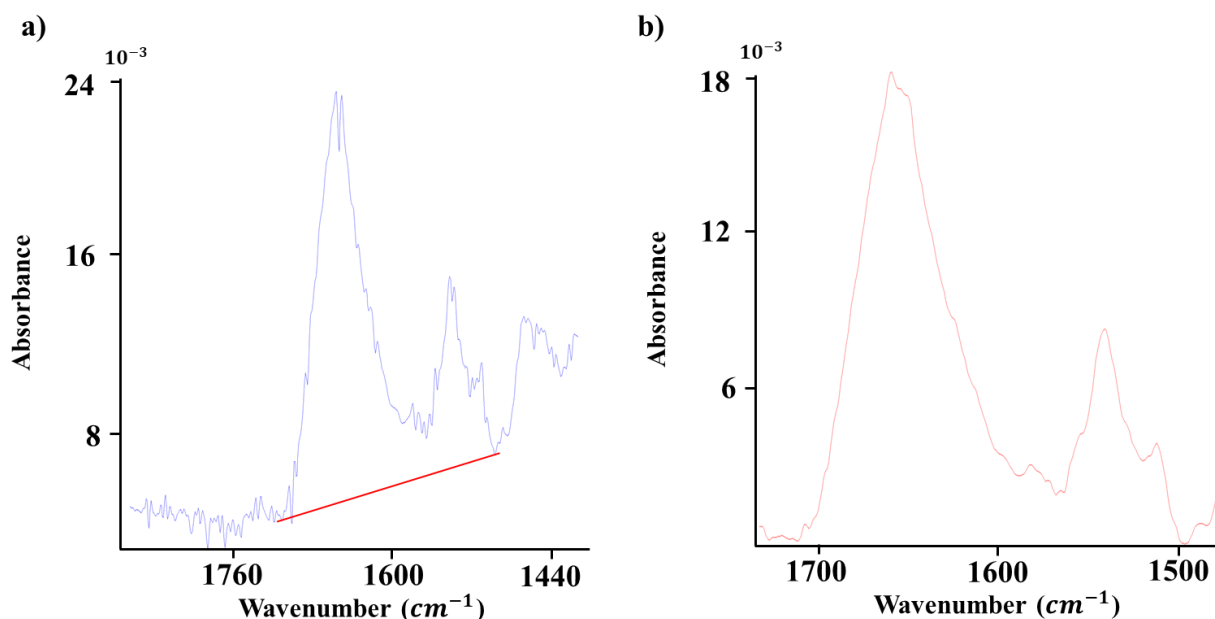


Figure 3.9. Background correction and smoothing of ATR spectra. **a)** Representative ATR spectrum of a protein monolayer physisorbed on the ZnSe IRE. The red line represents the straight-line background subtracted in the relevant spectral range between 1495 cm^{-1} and 1710 cm^{-1} . **b)** ATR spectrum after background subtraction and smoothing. The smoothing is performed with a 13 points moving window.

3.5 Analysis of IR spectra of protein films

3.5.1 Second derivative analysis

The analysis of the second-derivative spectrum is a mathematical approach utilized to resolve the overlapping contributions from various secondary structures to the amide I band intensity.⁸⁷ The method is based on the observation that the second derivative of a bell-shaped spectral feature (Lorentzian or Gaussian lineshape) shows a sharp negative peak, having a reduced FWHM and centred at the same frequency of the maximum on the original peak. Accordingly, two overlapping peaks, which are not resolved, may be distinguished looking at the second-derivative of the spectrum.

The second-derivative spectrum is computed using a Savitsky-Golay algorithm through a custom MATLAB code (appendix D). It operates within a moving window of fixed size, that scan the whole

frequency range: spectral data inside the window are approximated by a third-order polynomial and the second-derivative at the central frequency in the window is computed equal to that of the polynomial curve, according to the Savitsky-Golay algorithm.

Figure 3.10 represent an example of second-derivative deconvolution of a user-defined spectrum including two bands (panel a): a single Gaussian function having intensity $I=1$, FWHM=10 and positioned at $x=50$, and a broad band resulting from the sum of three Gaussian functions, with equal intensity $I=1$ and FWHM=10, positioned at $x=118$, 125 and 130. Panel b reports the second derivative, computed using two sizes of the moving window, 5 points (red curve) and 25 points (blue).

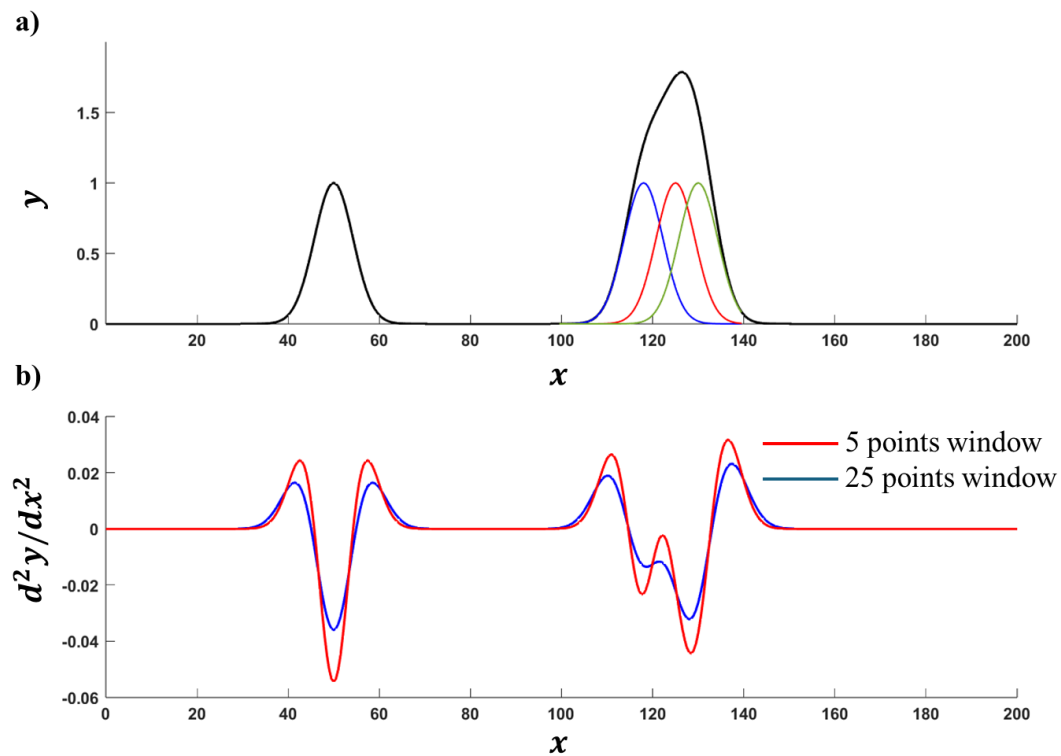


Figure 3.10. Second-derivative deconvolution. **a)** The black line represents a curve obtained by the sum of four Gaussian functions with intensity $I=1$, FWHM=10 centred at $x=10$, $x=118$, $x=125$, $x=130$. The three overlapping gaussians curves are represented as three blue, red and green curves. **b)** Second derivative of the black curve in panel a, computed by the Savitski-Golay algorithm with a moving window on 5 points (red curve) and 25 curve (blue curve).

The second derivative of the isolated gaussian peak features a sharp negative minimum centred at $x=50$, along with two less intense positive lobes. On the other hand, the second derivative band made of three overlapping components, clearly identify the presence of at least two of them. It shows a minimum at $x=118$ which correspond to the left-peak, and a second minimum at $x=128$, that does not correspond to a single underlying contribution. This example shows that the computation of the second-derivative spectrum helps in identifying overlapping spectral features, being extremely useful

in the analysis of the amide I band in protein spectra, however, it fails if the overlap is too large. A relevant aspect in performing a second derivative analysis is that the noise is amplified. The S/N ratio of the original spectrum must be high enough to allow for a reliable second derivative analysis. The interference of noise could be reduced working with a larger moving window, however, the larger the window, the more the second derivative appears smooth and distorted, as showed in figure 3.10b .

To analyse the amide I band in the IR spectrum of a protein, the second derivative is typically computed using a moving window about 10-15 cm^{-1} large, that is about half of the FWHM of all the amide I band components.

Figure 3.11 shows the ATR spectra of anti-IgG and BSA proteins, in the amide I and amide II spectral range. Those spectra were acquired by depositing protein through drop-casting directly onto the diamond IRE of the ATR compartment, forming a thick layer. The amide I band is peaked at shifted position for the two proteins, due to the different secondary structure composition. In the BSA spectrum, amide I is peaked at 1655 cm^{-1} , as expected for a protein with high α -helix content. Whereas amide I is peaked at 1640 cm^{-1} in the spectrum of anti-IgG whose structure is mainly constituted of β -sheets.

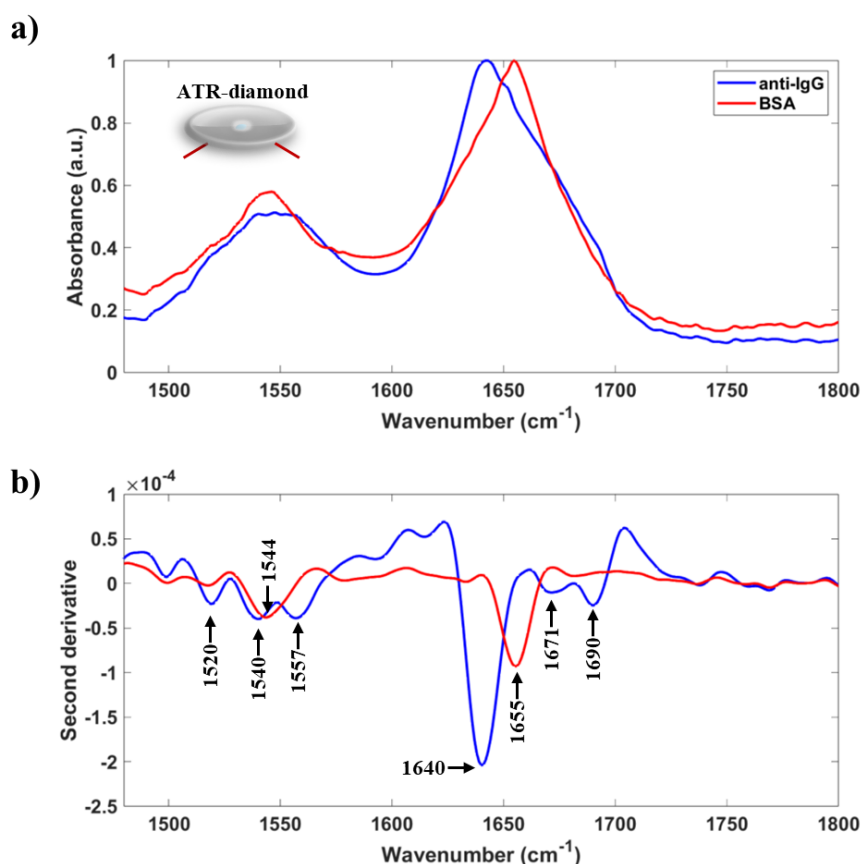


Figure 3.11. ATR and second-derivative spectrum of drop-casted anti-IgG and BSA thick film.

a) ATR spectra of drop-casted anti-IgG and BSA proteins in the amide I – amide II spectral range.

The spectra were acquired using a single internal reflection diamond IRE schematically depicted in the top left of the panel. **b)** Second derivative of the ATR spectra of drop-casted anti-IgG and BSA thick film computed through Savitsky-Golay algorithm using a 11 cm^{-1} wide moving window.

The second-derivative spectrum is also shown in the same frequency range. The second derivative of the BSA spectrum feature a single minimum in the amide I region centred at 1655 cm^{-1} corresponding to the main component from α -helix. Also, a minimum at 1544 cm^{-1} is identified, associated to the α -helix amide II vibrational mode. On the other hand, the second derivative of the anti-IgG spectrum show an absolute minimum at 1640 cm^{-1} associated to the main band from β -sheets (B2 mode), along with other two minima at 1671 cm^{-1} and 1690 cm^{-1} . Those are ascribed to turns and to the high-energy component arising from β -sheets (B1 mode), respectively. They appear as an asymmetric lineshape of the amide I band, though not clearly resolved, whereas they are identified from the second derivative analysis. The second derivative of the anti-IgG spectrum also feature three peaks in the amide II region, at 1520 cm^{-1} , 1540 cm^{-1} and 1557 cm^{-1} that could be ascribed to β -sheets and turns.

Note that, picking second derivative minima that are associated to secondary structure elements rather than to noise is not a systematic process and artefacts are possible especially with spectra having a $S/R < 500$.

3.5.2 Fit-deconvolution of amide I band

The amide I band is highly sensitive to the secondary structure composition of proteins hence it is the focus of conformational analysis. The deconvolution of the amide I band is accomplished by a curve-fitting procedure.^{54,87,88} A fit function, given by the sum of multiple Gaussians (or Lorentzian) functions, fit the spectral data in the range $1600\text{--}1700\text{ cm}^{-1}$. The number of Gaussian (or Lorentzian) components is generally determined looking at the second-derivative spectrum, assuming a secondary structure component for each minimum of the second derivative. However, the second-derivative fails discriminating amide I components if the overlap is too large, and component are added based on additional knowledge or different consideration, aiming at improving the fit. Given that each component has three parameters (intensity, FWHM and wavenumber position), and generally more than 4 are identified (β -sheets and turns could generate multiple components whereas α -helix and random structures are generally associated to a single band), it results that the fit-function includes many parameters. The fit algorithm iteratively adjusts the parameters and return the solution which minimize the root mean square error. However, several solutions could be found, depending on the set of parameter bounds and starting point utilized and selecting a unique solution is not

straightforward. The second derivative can guide the fit procedure by identifying the position of the various components. Also, the number of parameters could be reduced by assuming the same FWHM for all secondary structure elements.

The deconvolution of amide I band is essential for the quantitative analysis of the secondary structure composition of proteins. Based on the assumption that the IR absorbance of C=O stretching vibrations is equivalent for each secondary structure element, the protein composition is obtained as the percentage contribution of secondary structure components to the total amide I band. This spectrum-structure correlation has been proven as the composition of proteins of known structure, obtained from amide I analysis is in good agreement with that obtained through X-ray analysis.

Figure 3.12 reports the fit-deconvolution of the amide I band of the ATR spectra of drop-casted anti-IgG (figure 3.12a) and BSA (figure 3.12b) thick films. Five components have been included ascribed to β -sheets (two bands), α -helix and random structures and turns. Table 3.2 reports the position found for each component, along with the FWHM (equals for all bands). The deconvolution has been guided by the featured of the second-derivative spectra in figure 3.11, for the setting of the initial position of the main amide I components.

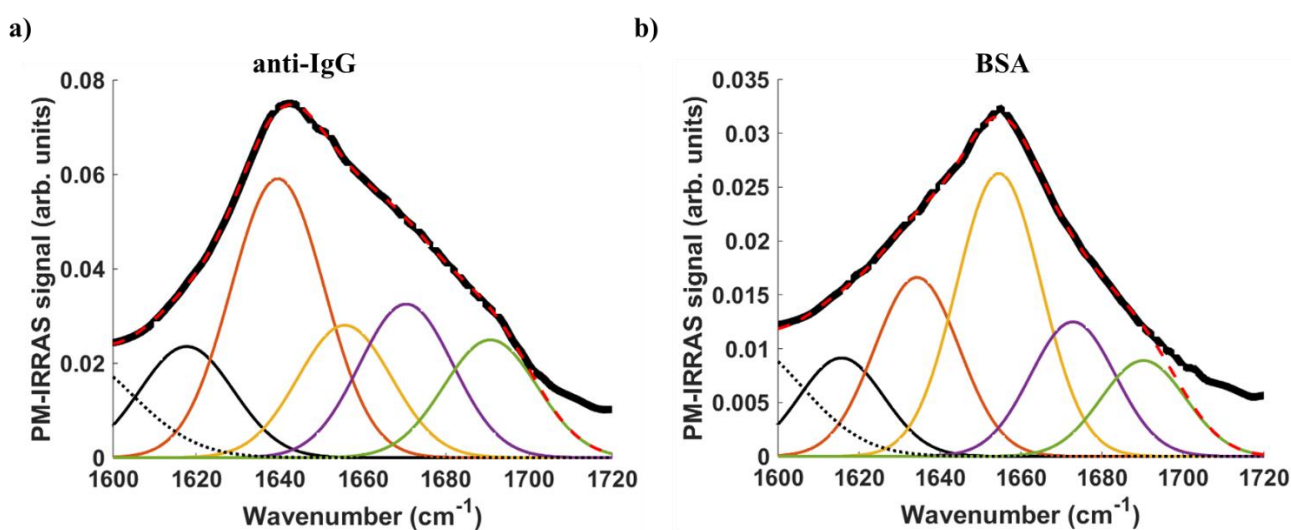


Figure 3.12. amide I fit deconvolution of ATR spectra of drop-casted anti-IgG and BSA. a) fit-deconvolution of the amide I band of anti-IgG drop-casted thick film. b) fit-deconvolution of the amide I band of BSA drop-casted thick film. The fit includes 5 gaussian contribution ascribed to side-chains (black), β -sheet (orange and green), α -helix/random (yellow) and turns (violet).

For the amide I of the anti-IgG spectrum, a band to account for unordered structure, and a low-energy band to account for the spectral intensity below 1620 cm^{-1} have been added. On the other hand, the second derivative of the BSA spectrum clearly identified the main α -helix band. However, BSA also contains β -sheets and turns structure, therefore a 5-gaussian model has been used in this case as well.

The fit model also includes a gaussian band to account for the high energy tail of the amide II band protruding beyond 1600 cm^{-1} .

The quantitative secondary structure analysis of anti-IgG shows that β -sheets are the prevalent structure, 50-64% whereas a lower quantity of turns (19%) and random (17%) is present. The amide I in BSA spectrum features main band ascribed to α -helix (36%) along with less intense bands associated to β -sheets (35%), turns (17%) and side-chain (12%).

Table 3.2. Amide I analysis of drop-casted anti-IgG and BSA. Amide I deconvolution of ATR spectra of drop-casted anti-IgG and BSA: position and percentage area of secondary structure components.

	anti-IgG		BSA	
	ν (cm^{-1})	Area (%)	ν (cm^{-1})	Area (%)
Side-chains/ β -sheet	1618	14	1616	12
β -sheet	1640	35	1634	23
α -helix/random	1656	17	1655	36
Turns	1670	19	1673	17
β -sheet	1691	15	1690	12
FWHM	27 cm^{-1}		25 cm^{-1}	

This fit model, with five secondary structure components, is used throughout the thesis for the amide I fit deconvolution of PM-IRRAS and ATR spectra. It is performed through a custom MATLAB code (Appendix D). If possible, the second derivative has been utilized to set the bounds and the starting points of the fit parameters, otherwise they have been set referring to the table 2.1⁵¹.

Comparison with literature: Amide I analysis of IgG-like proteins

X-ray crystallography of IgG crystals revealed that its structure contains β -sheets (67%), turns (18%), unordered regions (12%) and a little amount of α -helix (3%).⁵⁵ Accordingly, the IR spectrum of Immunoglobulins (IgG-like proteins) features an asymmetric amide I band, peaked around ~ 1635 - 1640 cm^{-1} with a high energy shoulder around 1690 cm^{-1} , as expected for a protein mainly constituted by β -sheets. Table 3.3 reports literature findings concerning the amide I band composition of IgG samples.

Table 3.3 Amide I deconvolution of IgG IR spectra.

Ref	Condition	Side-chains or Intra. β -sheets	β -sheets	Unorder ed	α -helix	Turns
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Azin Sadat et al.⁸⁹	In solution	-	1613-1636	1647	1654	1662-1684
V. Sathya Devi et al.⁹⁰	In solution	1611	1636- 1641,1685, 1692	1651	1651	1670
Susanne Matheus et al.⁹¹	In solution	1616	1639,1689	-	-	1663,1676
J. Bujis et al.⁹²	Adsorbed	1612	1623,1636,	1646	1654	1664,1688
Carla E. Giacomelli et al.⁹³	Adsorbed (on hydrophilic surface)	1613	1620- 1624,1637- 1640, 1670-1675	1647-1652	1647- 1652	1661,1688
Carla E. Giacomelli et al.⁹³	Adsorbed (on hydrophobic surface)	1613	1622-1624, 1635-1638, 1670-1674	-	-	1660,1688

Comparison with literature: Amide I analysis of BSA

The secondary structure content of BSA in native conformation, is dominated by α -helix units (~67%) with minor contribution from β -sheets (~21%) and turns/random elements.^{94,95} Table 3.4 reports literature findings concerning the amide I band composition of BSA samples.

Table 3.4 Amide I deconvolution of BSA IR spectra.

Ref	Condition	Side-chains or Intra. β -sheets	β -sheets	Unorder ed	α -helix	Turns
M. J. Desroches et al.⁶⁹	Adsorbed on stainless steel	-	1615-1639	-	1648- 1649	1663-1695
A. Bouhekka, et al.⁹⁶	Adsorbed on TiO ₂	1615	1624-1642, 1690-1696	1640-1648	1654- 1658	1675-1685
M. P. Schmidt et al.⁹⁷	Adsorbed onto Montmorillonite	1600-1620, 1660-1690	1620-1640	1644-1648	1650- 1660	1660-1690
M. S. C. Barreto et al.⁹⁸	Adsorbed on hematite	1612	1628,1637, 1662,1676	1644	1652	1662,1676

BSA is considered a protein with low conformational stability. Many studies confirms that it undergoes significant conformational change upon adsorption of solid surfaces, with the unfolding of α -helix and the formation of β -sheets.⁹⁹

3.5.3 Molecular orientation analysis

Surface sensitive IR spectroscopy enable the orientational analysis of adsorbed monolayer.¹⁰⁰ Information on the molecular orientation in thin films is accessible through measurement of the dichroic ratio by means of polarized IR spectroscopy. However, for molecules adsorbed on metal substrates this approach is not applicable because of the surface selection rule for IR absorption: molecular vibrations only couple to the electric field vector component perpendicular to the metal surface (z-direction), whereas the parallel component is screened by electrons in the metal.

IR radiation is absorbed by molecules on a metallic substrate according to the equation

$$A \propto |\mathbf{E} \cdot \mathbf{TDM}|^2 = E^2 TDM^2 \cos^2(\theta) \quad (3.11)$$

where A is the absorbance, E is the electric field on the surface, $\mathbf{TDM}$ is the transition dipole moment vector associated to the given vibrational mode and θ is the tilt angle between the $\mathbf{TDM}$ and the normal direction to the surface. Note that the absorbance A of thin film is provided by IRRAS spectroscopy measurements. Hence, if the absolute magnitude of the TDM is known, the orientation of oscillating dipoles can be obtained as

$$\cos^2(\theta) = \left(\frac{TDM_z}{|TDM|} \right) \quad (3.12)$$

being TDM_z the z-component of the $\mathbf{TDM}$ and $|TDM|$ its magnitude.

However, in general $|TDM|$ is not known a priori. An approach for the orientational analysis of adsorbates on metals has been developed by Allara, based on the estimation of $|TDM|$ in a bulk sample.¹⁰¹ The method consists in the following steps: i) measurement of the IRRAS spectrum of the thin film sample of molecules adsorbed on metal substrate; ii) transmission spectroscopy measurement of a bulk sample prepared as a mixture of the same molecule and KBr pellet; iii) calculation of the absorption index and real refractive index of the sample from the transmission spectrum; iv) simulation of a IRRAS spectrum of the thin film made of randomly oriented TDMs; v) orientation analysis according to the formula

$$\cos^2(\theta) = \frac{A_{film}}{3 A_{simulated}} \quad (3.13)$$

where A_{film} is the absorbance in the experimental IRRAS spectrum and $A_{simulated}$ is the absorbance in the simulated IRRAS one.^{102,103}

Here the method is described in major details. From the transmission spectrum ($T(\tilde{\nu})$) of the bulk sample, it is possible to compute a good estimate of the sample absorption index $k(\tilde{\nu})$, according to the Lambert-Beer law

$$T(\tilde{\nu}) = \frac{I}{I_0} = e^{-4\pi\tilde{\nu} k(\tilde{\nu}) s} \quad (3.14)$$

where s is the thickness of the KBr pellet and I/I_0 is the fraction of transmitted radiation. From $k(\tilde{\nu})$ in the relevant spectral range, the real refractive index can be computed through the Kramers-Kronig relation

$$n(\tilde{\nu}) = n_{\infty} + \frac{2}{\pi} P \int_0^{\infty} \frac{k(\tilde{\nu}') \tilde{\nu}'}{\tilde{\nu}'^2 - \tilde{\nu}^2} d\tilde{\nu}' \quad (3.15)$$

where P stand for the principal value integral, and n_{∞} is the refractive index in the visible range, below electronic absorption (the value at ~ 589 nm is usually considered, corresponding to the sodium D-line). The knowledge of the sample optical constants enables the simulation of an IRRAS spectrum. It is achieved by computing the reflectance of a three-layer system made of air, the adsorbates film which thickness is d and the metal, upon incidence at grazing angle, exactly matching that used in the experimental IRRAS measurement. Since the absorption index is that of an isotropic sample (molecule in the bulk sample are randomly oriented) the simulation returns the IRRAS spectrum of a thin layer of adsorbed molecules randomly oriented on the metal substrate. Indeed, the simulated spectrum must be corrected to account for the different density and thickness of the bulk sample compared to the thin film.

The IRRAS absorbance in an isotropic film is 1/3 with respect to the absorbance that would be measured if all the oscillating dipoles were aligned normal to the metal surface. In other words, $|TDM| = 3 A_{simulated}$. This is at the base of equation 3.13.

The ratio of the experimental IRRAS absorbance and the simulated one, for an isotropic film, allow for the determination of an average tilt angle of the TDM with respect to the normal direction. Furthermore, if the TDM vector is known of molecular coordinate system, orientation of molecules on the metal substrate can be assessed.

Figure 3.13 represent the tilt angle as a function of the absorbance ratio. Note that according to this model θ is an average angle but nothing is known about the shape of the angle distribution. This represent a limit of this approach. Indeed, a fully random orientation would return an absorbance ratio equal to 1, which also correspond to a fully oriented film at $\theta = 54,7^\circ$.

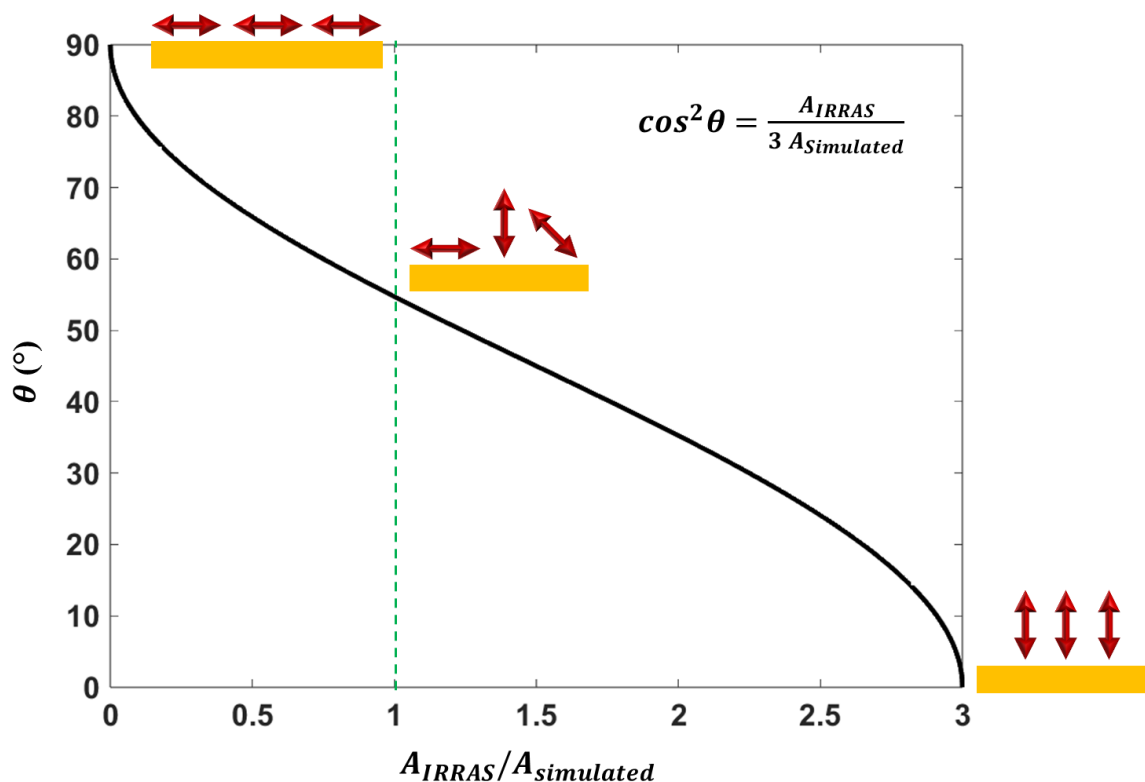


Figure 3.13. Tilt angle as a function of the IRRAS absorbance ratio. Tilt angle as a function of the ratio between the experimental IRRAS absorbance and the simulated one, according to equation 3.13. $A_{IRRAS}/A_{simulated} = 0$ correspond to a film whose dipole lays parallel to the substrate. $A_{IRRAS}/A_{simulated} = 3$ correspond to a film whose dipole are oriented perpendicular to the substrate. The dashed green line at $A_{IRRAS}/A_{simulated} = 1$ represents the case of an isotropic film, which correspond to a tilt angle $\theta = 54,7^\circ$ as well.

This method has proven effective for the study of SAM on metal substrates. However, it is unsuitable for the study of molecules whose structure could undergoes significant changes in the bulk form with respect to that in the thin film, which is the case for protein monolayers on gold. In this case the bulk spectrum cannot represent a reference isotropic spectrum. Later in this thesis an approach based on ATR spectroscopy of protein monolayers will be described to perform the orientation analysis of protein films.

3.5.4 Principal component analysis (PCA)

Principal component analysis (PCA) is a multivariate data analysis technique used to reduce the dimensionality (the number of features) in a dataset. It is based on the identification of few *principal components* (PCs), that contain most of the information included in the original dataset. It is an

approach for an exploratory analysis of data by facilitating their visualization in a 2D or 3D plot, while preserving the integrity of data.⁴⁷

PCA has found applications in the analysis of spectroscopy dataset. Examples of PCA analysis of IR or Raman spectra concern the discrimination of chemical or biochemical compounds based on the analysis of their vibrational properties, also through the application of cluster identification algorithms that are more efficient for dataset of reduced dimensionality.¹⁰⁴

From the mathematical perspective PCA transform the original data into a new basis through an orthogonal linear transformation. The new basis is a set of mutually orthogonal vectors called *principal components*.

Here the fundamental concept beyond PCA is explained with concern to a dataset of IR spectra, including q spectra, acquired in a frequency range n (for example $\sim 1000 \text{ cm}^{-1}$) wide. The dataset can be represented as a matrix of q columns and n row

$$X_0 = \begin{pmatrix} a_{11} & \cdots & a_{q1} \\ \vdots & \ddots & \vdots \\ a_{n1} & \cdots & a_{nq} \end{pmatrix} \quad (3.16)$$

An IR spectrum is a n -dimensional column vector made of “absorbance” values. It corresponds to a point in a n -dimensional space whose coordinates are wavenumbers. Correspondingly, the whole dataset forms a cloud of point in this space. To perform the PCA analysis the dataset is first centred around the origin

$$X_1(i, j) = X_0(i, j) - \frac{1}{q} \sum_{h=1}^q X_0(i, h) \quad (3.17)$$

The PCA is based on the calculation of a set of base vectors ($V_1, V_2, \dots, V_n$), ordered so that the dataset exhibits decreasing variance along their directions, from V_1 to V_n . That is done by computing the eigenvectors and the corresponding eigenvalues ($\lambda_1, \lambda_2, \dots, \lambda_n$) of the covariance matrix

$$\sigma^2(i, j) = \frac{1}{n-1} \sum_{h=1}^q (X_1(i, h) - \langle X_1(i, \cdot) \rangle) (X_1(j, h) - \langle X_1(j, \cdot) \rangle) \quad (3.17)$$

where $\langle X_1(i, \cdot) \rangle$ is the average of the entrances of the i -th row in X_1 .

$V_1, V_2, \dots, V_n$ (normalized to unity) represent the PCs of the dataset. The amount of explained variance is given by the percentage cumulative sum of the eigenvalues. In general, few PCs ($p < n$) explain more than the 90% of the variance of the original dataset, therefore, a reduced set made of the first p PCs can be considered. Accordingly, the spectral dataset is projected onto the new base in a p -dimensional space following the equation

$$Y_{[pxq]} = V_{[pxn]}X_{1[nxq]} \quad (3.18)$$

where the matrix $Y_{[pxq]}$ represent the new dataset of q spectra described by p variables, called *scores*, whereas $V_{[pxn]}$ is the transformation matrix made of the first p PCs. Each spectrum is described with scores in the PCs space as a linear combination of the original spectral intensity multiplied by a weight. The weight for a PC constitutes the *loading vector* of the given PC. The convenience of PCA analysis lays in the fact that 2 (or 3) PCs are often enough to describe the dataset. Hence, spectra can be represented as a point in 2D (or 3D) space, having coordinates equals to the score of PC1 and PC2, and the whole dataset can be visualized in a 2D plot (PC1 vs PC2). Once difference among spectra have been identified in the PCs space, they can be correlated to real spectral features through the analysis of the loadings. Thus, hidden features could emerge.

Within this thesis PCA has been performed on a set of PM-IRRAS protein spectra as an exploratory approach seeking for relevant spectral features in the amide I region.

Chapter 4

Graphene-based optical detection of proteins at zeptomolar concentration

A novel biomarker detection strategy is presented in this chapter, based on an electrolyte-gated graphene electrode coupled to a large-area sensing interface. A monolayer of anti-Immunoglobulin M (anti-IgM) capturing antibodies serves as biolayer for the detection of IgM affinity antigens. The biosensing architecture shares similarities with the SiMoT immunoassay, as both rely on the capacitive coupling of a bio-functionalized electrode with a transducer through an electrolyte. Moreover, the detection mechanism relies on the surface potential changes at the sensing interface provoked by the antigen binding onto the antibody biolayer. In the proposed biosensor, graphene probes those electrostatic changes through the modulating of its Fermi energy level. The biochemical information is converted into a measurable signal, through Raman spectroscopy of graphene. Specifically the method is based on the extraction of the CNP position from the analysis of the field-effect tuning of the G-peak in the Raman spectrum of graphene, and we correlate the CNP shift with the presence/absence of analyte molecules. Our findings are supported by Kelvin-probe force microscopy (KPFM) measurements of the surface potential changes at the sensing interface, showing an effective ON/OFF-type response for the ultrasensitive detection of IgM at concentration of 10^{-19} M, being state-of-the-art performance for a graphene-based biosensor for protein detection. This proof-of-concept could serve as the foundation for the development of a benchtop system, integrating our graphene-based device with a portable Raman spectrometer, offering an accessible and reliable point-of-care diagnostic tool.³²

4.1 Biosensor design

The biosensor is based on the capacitive coupling of a sensing interface with a graphene transducer. The schematic structure of the device is shown in figure 4.1. It is a two-terminal device featuring an Au electrode (sensing element) and a single-layer graphene electrode (transducer), connected by a water channel in a lateral architecture. The sensing electrode is made of a 5/50 nm layer of Ti/Au deposited on a Si/SiO₂ substrate via e-beam evaporation, bio-functionalized with a monolayer of physisorbed anti-IgM capturing antibodies. The graphene electrode consists of a single-layer graphene fabricated by chemical vapour deposition (CVD) and transferred onto a glass slab. A stable connection between the two electrodes is sustained through a water-soaked paper strip, having one end dipped in a water reservoir. Water (HPLC grade) serves as the electrolyte, ensuring the capacitive coupling between the Au/anti-IgM and graphene interfaces. A DC voltage (V_G) is applied to the sensing electrode while keeping graphene grounded. The electrostatic potential distribution across the device is determined by the electrical double layers (EDLs), forming at the electrode/electrolyte interfaces. At steady-state, after relaxation of any transient currents across the electrolyte, the equivalent circuit of our device includes the double-layer capacitances, $C_{\text{bio/w}}$ and $C_{\text{g/w}}$ of the Au/anti-IgM/water and water/graphene interfaces respectively and the quantum capacitance of graphene, C_q , to account for the field-effect tuning of the graphene Fermi energy. Also, a voltage generator, V_{bio} , represents the biolayer, accounting for the electrostatic potential drop due to charge and dipoles within the anti-IgM layer, being therefore related to the surface potential of the sensing electrode.

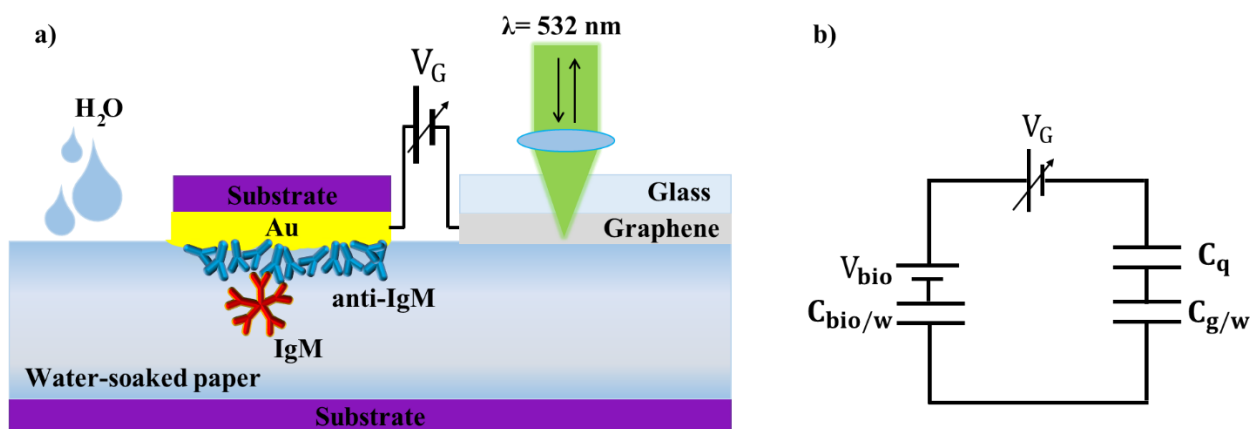


Figure 4.1: Graphene-based biosensor. a) Schematic cross-sectional view of the device. It includes an Au electrode biofunctionalized with a monolayer of physisorbed anti-IgM encompassing 10^{12} molecules cm^{-2} , and a single-layer graphene electrode capacitively coupled via a water-soaked paper strip. An external DC voltage (V_G) is applied between the Au and the graphene electrodes. The green

laser, $\lambda = 532 \text{ nm}$, used for micro-Raman spectroscopy measurement, is focused on graphene through the transparent glass substrate, in a backscattering configuration. **b)** Equivalent circuit of the device, at steady state. The EDLs at the Au/biolyer/water and graphene/water interfaces act as two capacitors, respectively $C_{\text{bio/w}}$ and $C_{\text{g/w}}$. The quantum capacitance of graphene, C_q , accounts for the field-effect tuning of its Fermi energy. The resistance of the electrolyte is negligible in steady state, as no current is flowing across the water channel. A voltage generator V_{bio} accounts for electrostatic potential drop determined by dipoles and charges in the biolyer; it could be considered the contribution of the surface potential of the biolyer to the device.

To account for the initial p-doping of graphene the quantum capacitance is considered initially charged with a carrier concentration n_{dop} . Accordingly, the DC voltage V_G distributes across the device following the equation

$$V_G = V_{\text{bio}} + \frac{A_g e (n_{\text{dop}} - n)}{C_{\text{gate}}} + \frac{E_F}{e} \quad (4.1)$$

where $A_g = 20 \text{ mm}^2$ is the graphene/water contact area, n is the charge carrier surface density in graphene, and C_{gate} is the capacitance equivalent to the series of $C_{\text{bio/w}}$ and $C_{\text{g/w}}$. A schematic representation of the electrostatic potential drop is featured in Figure 4.2.a. V_G partially distributes in the EDLs, and partially as a shift of the Fermi energy of the graphene electrode.

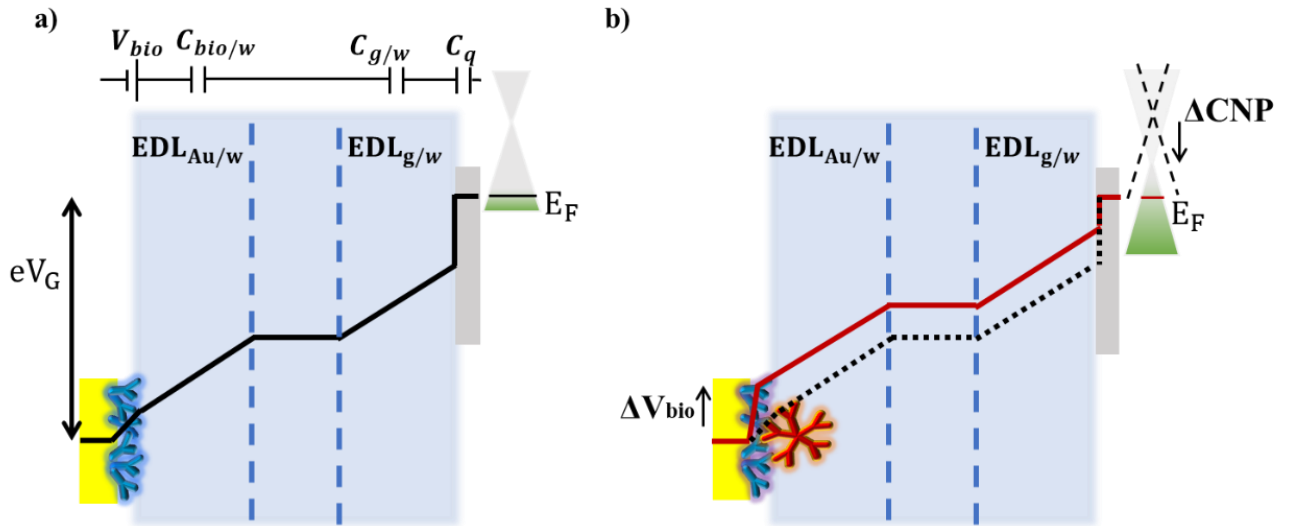


Figure 4.2 Electrostatic potential distribution across the graphene-based biosensor. a) Schematic potential energy diagram across the sensing device. The electrostatic potential energy (eV_G) provided by the external voltage generator, is distributed across the EDLs at the Au/bio/water and water/graphene interfaces, and as a shift of the Fermi energy in graphene. **b)** After the affinity binding with IgM ligands the voltage drop is redistributed due to a change in the surface potential of the biolyer, represented by a change in the voltage drop V_{bio} . This results in shifted Fermi level.

The band structure of graphene is represented on the top-right corner of panels a) and b), featuring the characteristic Dirac cone with the green area showing occupied states.

Eliminating n in equation 4.1, using equation 2.3 the relationship describing the field-effect modulation of the Fermi energy within our device is worked out

$$E_F = \text{sign}(V_G - V_{CNP}) \frac{\pi \hbar^2 v_F^2}{2e^2} \left\{ -\left(\frac{C_{gate}}{A_g}\right) + \left[\left(\frac{C_{gate}}{A_g}\right)^2 + \frac{4e^3}{\pi \hbar^2 v_F^2} \frac{C_{gate}}{A_g} |V_G - V_{CNP}| \right]^{\frac{1}{2}} \right\} \quad (4.2)$$

It depends on two parameters: C_{gate} and V_{CNP} . The former is related to the geometrical structure of the two electrodes and to the nature of the electrolyte. The latter depends on the initial doping of graphene and on the term V_{bio} according to the equation

$$V_{CNP} = \left[\left(n_{dop} e \frac{A_g}{C_{gate}} \right) + V_{bio} \right] \quad (4.3)$$

When $V_G = V_{CNP}$ the Fermi energy set at the CNP, ($E_F = 0$ eV). Notably, it results from equation 4.3 that the CNP sensitivity to variations of the interfacial capacitance of the Au/anti-IgM/water interface is reduced making the graphene/water contact area small with respect to the active area of the sensing electrode. Also, equation 4.3 explicates the sensitivity of the CNP of graphene to variations of the surface potential of the sensing electrode. That relationship is at the base of the sensing mechanism of our graphene-based biosensor. Figure 4.2.b. represents the expected effect of IgM affinity bindings onto the capturing anti-IgM layer: a modification of the surface potential of the biolayer and the transfer of the information of the biochemical reaction to the graphene electrode through the electrolyte, resulting in a shift of the CNP of graphene.

4.2 Transduction via Raman spectroscopy of graphene

To monitor the V_{CNP} shift upon biorecognition of ligand molecules, we use Raman spectroscopy of graphene. Indeed, phonon's energy in graphene is sensitive to the Fermi energy level, as discussed in section 2.4.3. Specifically, from equation 2.7 and equation 4.2 a relationship describing the modulation of the G-peak frequency (ω_G) as a function of the applied potential V_G within our device is obtained

$$\omega_G = \omega_G^0 + \frac{1}{10^2 \hbar c} \frac{\lambda_\Gamma \hbar v_F^2}{4e^2} \left| -\frac{C_{gate}}{A_g} + \left[\left(\frac{C_{gate}}{A_g}\right)^2 + \frac{4e^3}{\pi \hbar v_F^2} \frac{C_{gate}}{A_g} |V_G - V_{CNP}| \right]^{\frac{1}{2}} \right| \quad (4.4)$$

To verify the effective field-effect tuning of the Fermi energy and demonstrate the goodness of equation 4.4, the Raman spectrum of graphene has been acquired while varying V_G in the range -0.5V - 0V, at steps of 0.05V. Note that the voltage range is chosen within the electrochemically inert region for water-gated devices, to prevent any faradaic current which could alter the capacitive coupling between the graphene and the Au electrodes. Figure 4.3a-b feature the G-peak and 2D-peak in the Raman spectrum as a function of the applied voltage. At $V_G = 0V$ the G-peak starts at $\omega_G = 1589 \text{ cm}^{-1}$. It is blue-shifted shifted of 9 cm^{-1} with respect to the theoretical value at the CNP, as a consequence of the unintentional doping introduced during the transfer process and through the contact with gold and water within the device. As the voltage increases, the peak blue-shifts up to a maximum value of 1599 cm^{-1} when $V_G = -0.5V$. Those results are comparable to those reported in literature for electrolyte-gated graphene, confirming an effective field-effect tuning of the Fermi energy of graphene within our device with a G-peak modulation of about $20 \text{ cm}^{-1}/V$. On the other hand, the 2D-peak, in figure 4.4b, exhibits minor sensitivity to the applied voltage, as it blue-shifts of about 7 cm^{-1} , in agreement with previous reports. Notably, the 2D-peak shift points out the sign of the doping induced by the applied voltage. The 2D-peak blue-shift upon negative V_G between 0V and -0.5V correspond to a lowering of the Fermi energy level (p-doping) of the graphene electrode.

To extract the position of the CNP, equation 4.4 has been used to fit the ω_G vs V_G data. Figure 4.4c. reports the experimental data, along with the resulting best-fit curve (grey solid line). The fit shows a good agreement with the experimental trend and returns two fit-parameters: $V_{CNP} = (340 \pm 20)mV$ and $C_{gate} = (1.68 \pm 0.3)\mu F$. The V_{CNP} is found at a positive voltage, corresponding to an initial unintentional p-doping of the graphene electrode. Concerning the gate capacitance, the resulting value has the typical order of magnitude measured for water-gated devices $\sim 10^6 \text{ F cm}^{-2}$.

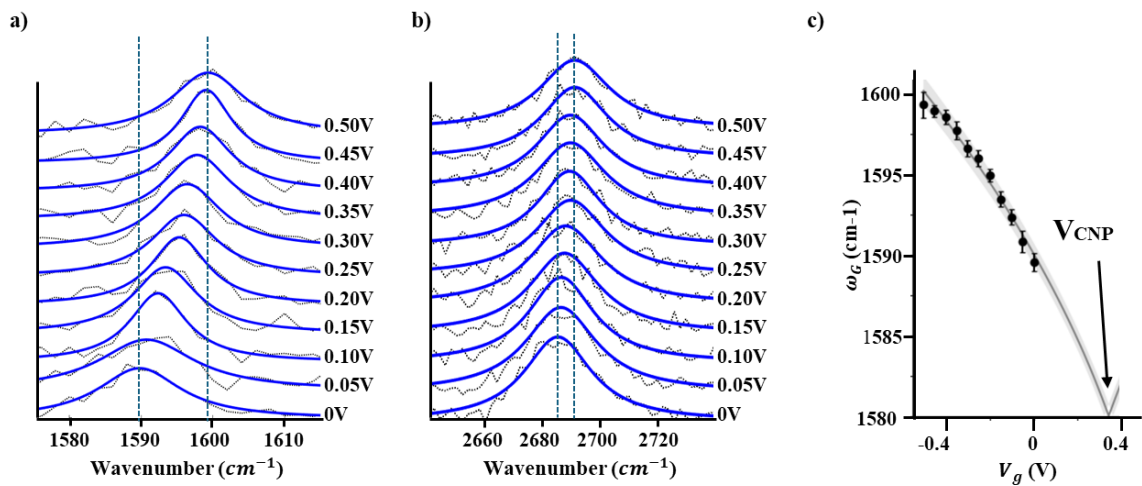


Figure 4.3 Field-effect tuning of the G-peak and 2D-peak. a) Field effect tuning of the G-peak of graphene. b) Field effect tuning of the 2D-peak of graphene. The dotted lines represent the

experimental spectra. Each peak has been subject to a Lorentzian fit to extract the peak-wavenumber positions, ω_G and ω_{2D} . The blue-solid lines represent the best-fit curves. Spectra in panels a) and b) are vertically offset for clarity. c) Voltage dependence of the G-phonons frequency (ω_G). The grey-solid line represents the best-fit curve obtained using equation 2.6. The arrow points to V_{CNP} which corresponds to the minimum of the fit-curve.

The recording of ω_G as a function of the applied voltage V_G enables the evaluation of the CNP position, V_{CNP} through the fit with equation 4.4. This method has been utilized to perform a sensing experiment, using a bio-functionalized Au sensing electrode, coupled to graphene.

4.2 IgM detection

A sensing experiment has been performed to detect Immunoglobulin M antigen (IgM) using a sensing electrode bio-functionalized with a physisorbed anti-IgM antibodies. The bio-functionalization protocol is described in section 3.1.4. The experiment consists in the monitoring of the CNP position of the graphene electrode upon exposure of the sensing electrode to analyte solution. Figure 4.4a-f shows the representative response of the device at each step of the sensing experiment. First, the starting position of the CNP is evaluated, while graphene is coupled to the anti-IgM functionalized electrode. Figure 4.4a represents the fit of ω_G vs V_G data acquired at this step, for the extraction of V_{CNP} as described in the previous section. Then, the sensing electrode is removed from the measuring cell and incubated with a 10^{-13} M PBS solution of IgG antigens for 10 minutes. This step constitutes a *negative control*, as IgG does not bind to the anti-IgM bioreceptors. The ω_G dependency on V_G is expected to be unchanged with respect to the baseline, as for the corresponding V_{CNP} . The incubations are performed ex-situ, to prevent the contamination of the graphene surface with non-specific adsorption of molecules, which is a relevant issue especially at high analyte concentration. Afterwards, the sensing electrode is rinsed with plain PBS solution and water and is reintroduced into the measuring cell for the acquisition of a new set of Raman spectroscopy measurements. Figure 4.4b represents the negative control step, with the ω_G vs V_G data and their fit. Then, the sensing electrode is again removed from the measuring cell and incubated with PBS solution of IgM affinity antigens at 10^{-19} M concentration for 10 minutes. Afterwards the electrode is rinsed, positioned into the device and the protocol is repeated for the extraction of V_{CNP} through Raman spectroscopy. The experiment goes on following this protocol for sequential incubation of the sensing electrode with increasing concentration of IgM, from 10^{-19} M to 10^{-10} M. Figure 4.4c-f shows the ω_G vs V_G data, acquired after each incubation step, along with the fit for the extraction of V_{CNP} . The results are summarised in

figure 4.4g. It reports the V_{CNP} values extracted from the fit of data in panel a-f. The CNP of graphene starts at $(340 \pm 20) \text{ mV}$ and remains almost unchanged upon incubation with unbinding IgG solution. Then, it shifts from $340 \pm 20 \text{ mV}$ to $305 \pm 19 \text{ mV}$ after incubation with IgM solution at 10^{-19} M concentration and remains quite stable around an average value of $297 \pm 9 \text{ mV}$ after the exposure to higher concentrations of IgM. It turns out that the device works as expected: the IgM binding occurring upon incubation at 10^{-19} M provoke a CNP shift enabling detection. Remarkably, just ~ 6 IgM molecules are present in $100 \text{ }\mu\text{L}$ of solution (incubation volume) at concentration of 10^{-19} M . This is a relevant result in the field of graphene based biosensor as there are no further technologies capable of detecting proteins with 100 zM sensitivity.

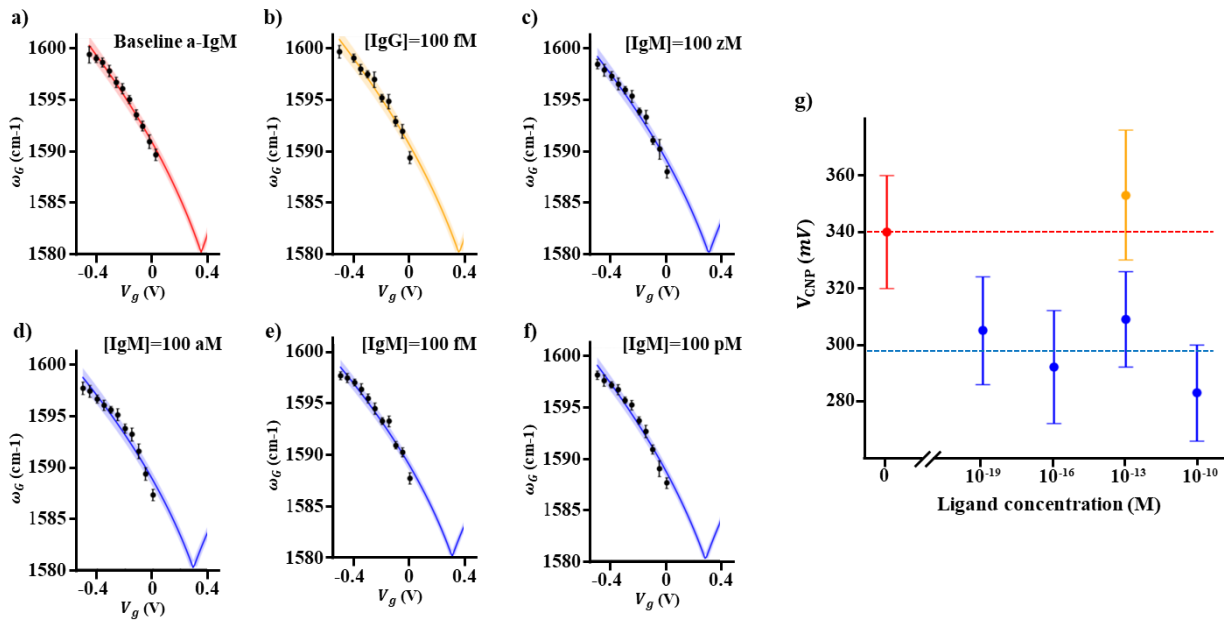


Figure 4.4 Sensing experiment. ω_G versus V_G measured: **a)** after anti-IgM functionalization of the gold electrode (baseline), **b)** after exposure to a 100 fM IgG solution, **c)** after exposure to a 100 zM IgM solution **d)** after exposure to a 100 aM IgM solution **e)** after exposure to a 100 fM IgM solution **f)** after exposure to a 100 pM IgM solution. Equation 2.6 is used to fit each dataset and extract the corresponding V_{CNP} . The solids lines represent the best-fit curves, with the shadowed area representing the fit uncertainty. **g)** V_{CNP} values obtained from fitting data in panels (a-f)

To define the limit of detection (LOD) of our biosensor and test the reliability of our detection method, a blank experiment has been conducted. It consists of sequential 10 minutes incubation of the anti-IgM functionalized sensing electrode (a newly prepared one) with plain PBS solution. The blank solution does not contain any chemical interferent, therefore the variability of the V_{CNP} values measured in this experiment represent the noise level of our device. Figure 4.5a reports the V_{CNP} estimated after each incubation step. It shows that V_{CNP} varies around an average value of $(14 \pm 11) \text{ mV}$. The sensitivity of our biosensor is then determined, according to the IUPAC definition of the

(LOD), which is the minimum analyte concentration producing a signal greater than the average level of a blank experiment plus three times its standard deviation, that is (14 ± 33) mV for our device. Figure 4.5b summarizes the results from the blank experiment (black dot), along with the LOD (dashed line), the output of three different sensing experiments (red dots) and negative controls (blue dots). Remarkably, the CNP shifts of (-55 ± 15) mV, beyond the noise threshold, after exposure to a PBS solution of IgM at 100 zM concentration. Then V_{CNP} remains almost constant upon exposure to higher analyte concentration. In other words, the biosensor exhibits an ON/OFF-type response with a LOD=100 zM. Also, analogous variations are not observed upon exposure to non-binding antigen, in the negative control experiments, demonstrating the selectivity of the device.

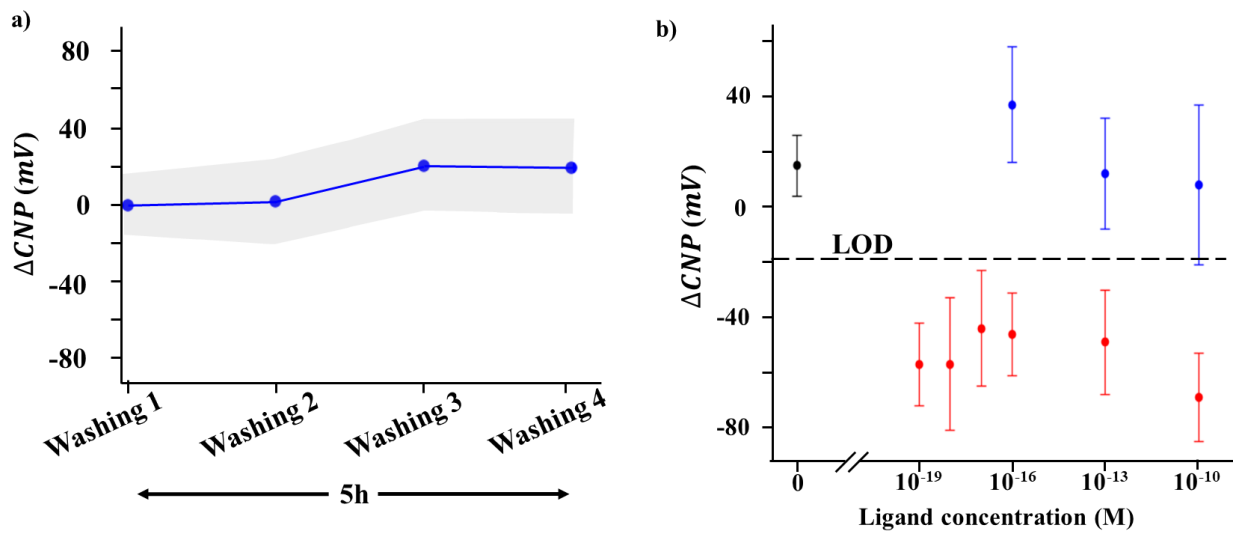


Figure 4.5 IgM detection. CNP shift (ΔC_{NP}) measured after incubation of the sensing electrode with antigen solutions. Red dots represent the ΔC_{NP} averaged over three sensing experiments, after incubation with increasing concentration of binding IgMs. Blue dots represent the ΔC_{NP} measured after incubation with increasing concentration of unbinding IgGs (negative control). The black dot represents the ΔC_{NP} average value after four sequential incubations of the functionalized electrode with plain PBS solution (blank experiment). The LOD (dashed black line) is evaluated as three times the standard deviation of the blank experiment. Taken from [30]

The binding of few IgM at ultra-low concentrations, occurring onto the sensing electrode are effectively probed by the graphene layer and transduced through Raman spectroscopy. Thus, the field-effect tuning of the G-peak in the Raman spectrum of graphene has proven as a suitable strategy to monitor CNP variation for biosensing applications. Moreover, the negative control experiments show the selectivity of the device. Note that IgG has been selected as a non-binding analyte to assess the sensor selectivity as it shares overlapping epitopes and similar molecular structure with capturing anti-IgM. The inspection of the detection performances using complex biological fluids (i.e. human

serum, saliva or urine) has not been performed at this stage, though it would represent the final testbed to establish the robustness of the proposed biosensing approach.

To further assess the transduction mechanism and provide direct evidence of the effect of IgM binding onto the anti-IgM biolayer, Kelvin probe force microscopy (KPFM) experiments have been carried out. KPFM (described in Appendix A) enables the inspection of the local surface potential of the sensing electrode before and after exposure to ligand solution, providing direct evidence of changes as the detection mechanism of our biosensor is based on the surface potential variations at the sensing interface upon affinity bindings.

A sensing electrode bio-functionalized with physisorbed anti-IgM onto the Au surface was prepared following the functionalization protocol described in section 3.1.4. The bio-functionalization method allows the formation of a sharp interface between the pristine Au surface, and the bio-functionalized area. Figure 4.6a features the surface potential (SP) image acquired by KPFM onto a $(90 \times 90) \mu\text{m}^2$ area across the interface showing the anti-IgM biolayer (bright left region) and the bare Au surface (dark right region). The measured values are centred at the average SP of the bare Au surface, used as an internal reference, as the surface potential difference (SPD) between the functionalized and bare regions is the relevant analytical parameter. Then the very same sample is, incubated with 100 μL of a standard PBS solution of IgM affinity antigen at 10^{-19} M concentration for 10 minutes. Afterwards the sample is rinsed, dried and a new SPD image is acquired exactly onto the previously scanned area. Figure 4.6b show the resulting image. It appears clearly that the whole inspected region has undergone a SP variation. For a quantitative analysis, the SPD is addressed through the histogram reported in figure 4.6c. The SP of the biolayer decreases of (66 ± 11) mV upon exposure to IgM solution encompassing ~ 6 antigens.

This protocol has been carried out for the sequential incubation of the electrode with IgM solution at 10^{-17} M and 10^{-16} M. Figure 4.6d reports the SPD variations with respect to the value measured before the incubation with IgM solution, averaged over two replicate samples. To correlate those findings with the response of our graphene biosensor, ΔSPD data are compared to the measured CNP shifts as a function of the analyte concentration. Remarkably, the KPFM data shows a perfect agreement with the CNP shift measured in the graphene biosensor, confirming the hypothesized detection mechanism based on the surface potential variation upon the binding of IgM. KPFM data suggest that the biochemical reaction alters the electrostatic configuration of dipoles across an extended area at the antibody layer, possibly involving conformational changes of the proteins structure or a collective rearrangement of the overall dipole moments within the biolayer. In turn, those variations change the charge distribution within the EDLs and are transferred to the graphene transducer. Moreover, the

response of our graphene-based biosensor is consistent with the detection performance of the SiMoT biosensor. Both relies on the capacitive coupling of a sensing interface with a transducer. Also, the zeptomolar sensitivity reached with both biosensors requires the assumption that an amplification mechanism takes place to propagate the binding information across the large-area sensing interface. The understanding of this phenomenon demands further investigation, as few (~ 6) antibody-antigen biorecognition reactions trigger a surface potential change involving trillions of unbounded antibodies.

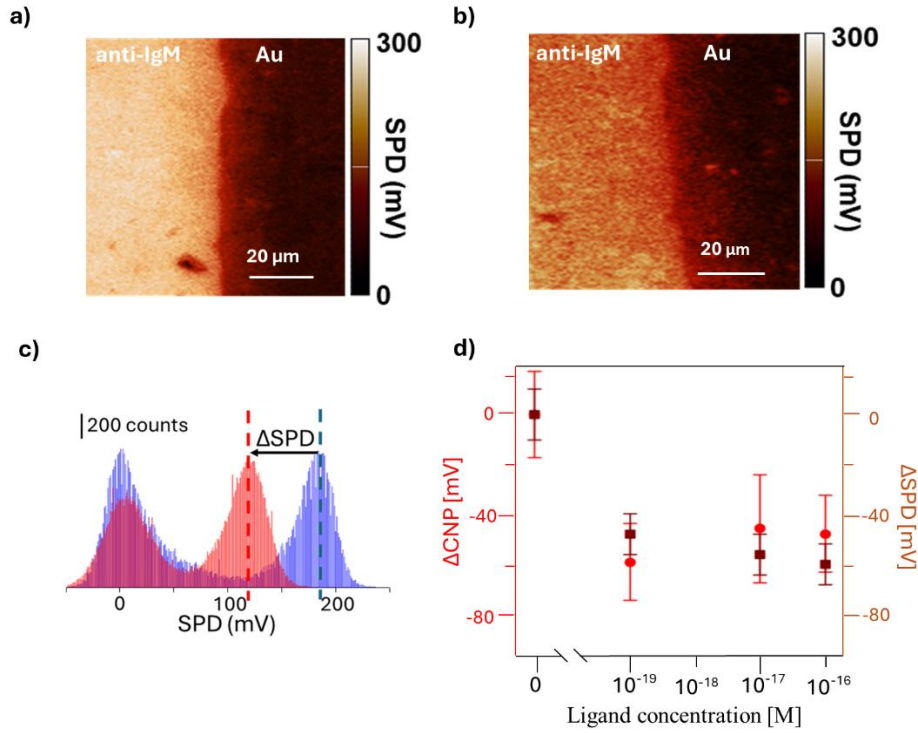


Figure 4.6. Surface potential of the anti-IgM biolayer. **a)** KPFM image of the anti-IgM functionalized electrode at the interface between the bare Au surface (dark right area) and the Au/anti-IgM region (bright left area). **b)** KPFM image of the same area inspected in panel a) after the incubation with a solution of IgM 10^{-19} M. **c)** Comparison of the SPD histograms of the KPFM images from panels a) (blue) and b) (red), showing a decrease of (-66 ± 11) mV in the SPD after incubation with IgM solution. **d)** Comparison between the Δ CNP and the Δ SPD for an Au/anti-IgM electrode subjected to the same sensing protocol. Δ CNP are the same shown in figure 4.5b. Δ SPD values are the average over two replicate experiments. Taken from [30]

4.4 Future perspectives

Our graphene-based biosensor has proven capable of label-free detection of IgM proteins reaching a LOD = 100 zM ($100 \cdot 10^{-21}$ M). The Boolean ON/OFF character of the device response allows

qualitative protein detection, determining the presence or absence of a specific analyte. This class of biosensors are relevant for application in medicine or environmental sciences in detecting marker molecules whose presence inform about a pathological condition, regardless of their concentration, such as metabolites related to the presence of pathogens like as bacteria and virus and biomarkers.⁷

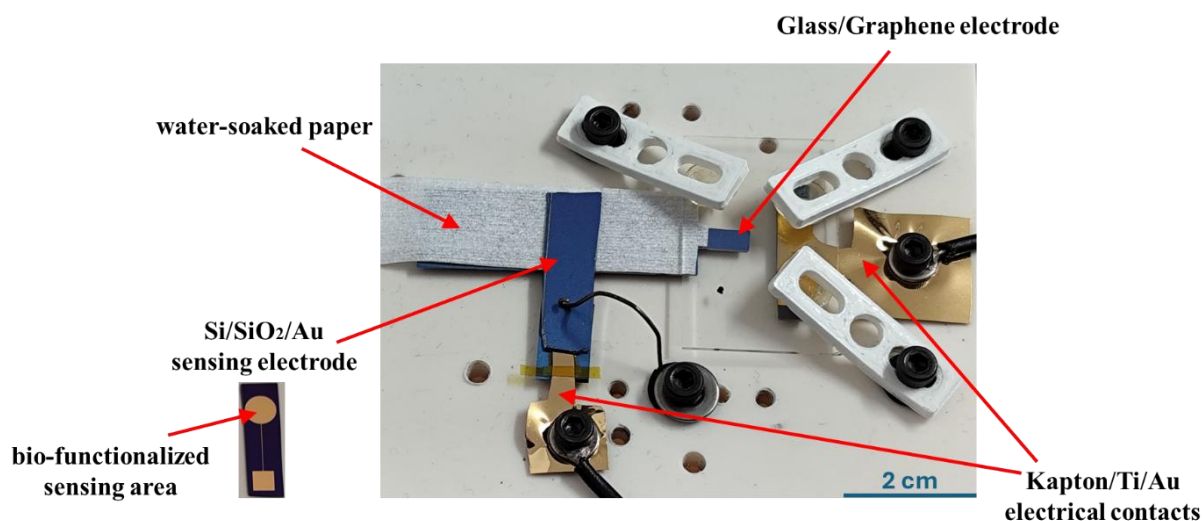


Figure 4.7. Picture of the graphene-based biosensor. It includes a Si/SiO₂/Au sensing electrode (placed upside-down with the circular sensing area toward the paper), a water-soaked paper strip, a CVD single-layer graphene electrode deposited on a glass slab; the electrodes are connected to a voltage generator through Kapton/Ti/Au soft electrodes. The entire setup is secured with clamps and mounted on a plastic holder.

A picture of the real device utilized in the experiments is shown in figure 4.7. The design of the biosensor is that of an electrolyte-gated graphene device, with a lateral-architecture: the sensing electrode, bio-functionalized with a layer of physisorbed capturing antibodies, and the single-layer graphene transducer are connected by water-soaked paper strip. The use of a soaked-paper strip provides a simple means for the coupling of the two electrodes, while maintaining a stable liquid environment. The capacitive coupling sustained by the soaked-paper effectively enables the transduction of the biorecognition reaction taking place onto the sensing electrode into a CNP shift of graphene. The use of Raman spectroscopy to monitor the CNP of graphene constitutes an innovative transduction mechanism in the field of graphene biosensors. The unique optoelectronic properties of graphene enabled ultrasensitive detection performances, almost at the single-molecule limit. The perspective of integrating our device into a compact bench-top system constitutes the base for further development of a precise point-of-care tools for protein biomarker detection. Indeed, portable Raman spectrometer have been developed for in-field application, facilitating the acquisition of spectral data outside research laboratory.

A future advancement of our biosensor could focus on the development of a comprehensive setup, including a customized spectrometer, a laser source and a fiber optic probe directly coupled to the graphene electrode, as schematized in figure 4.8. The biosensor could encompass a disposable cartridge as sensing electrode, while the graphene electrode is secured in a protected box for several use. An electronic control unit and a costumes software could manage the acquisition of Raman spectra and the processing of data, to provide a fast and real-time report of the biosensing results.

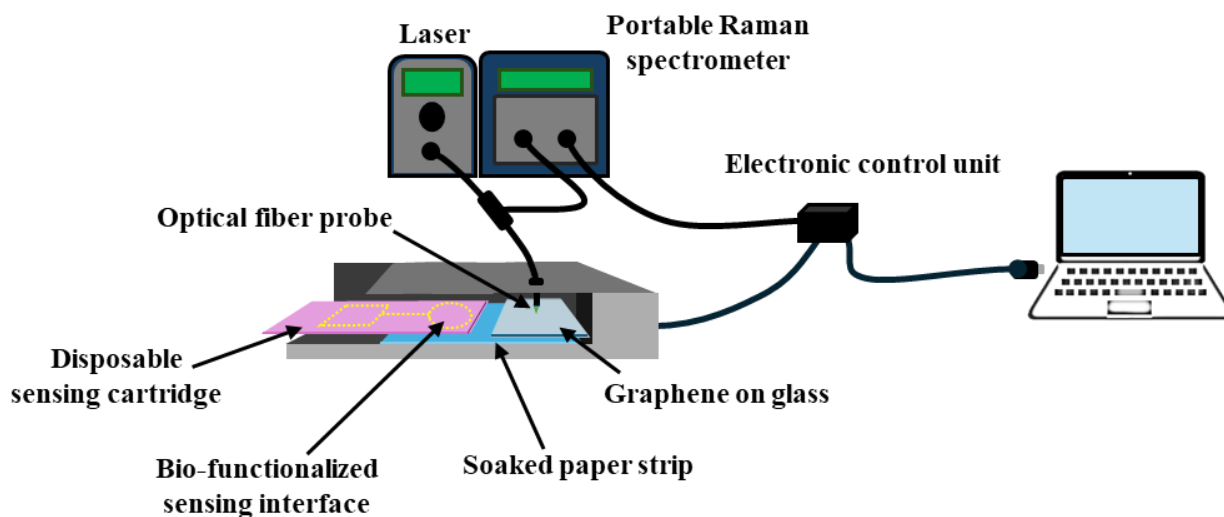


Figure 4.8. Integrated system. Schematic representation of a bench-top system for point-of-care diagnostics employing our graphene-based biosensor. It includes a graphene transducer, a water-soaked paper strip, and a disposable sensing electrode (represented upside-down with the dashed line suggesting that the bio-functionalized Au surface faces the water-soaked paper). An optical fiber Raman probe couples a laser source to the graphene electrode. The Raman spectrum is measured via a portable Raman spectrometer. All components are connected to an electronic unit which controls the device, and manages the reading and transfer of data to a computer for fast processing and real-time response.

Chapter 5

Infrared spectroscopy of bio-functionalized interfaces

5.1 Orientation of β -sheets in monolayers of physisorbed proteins

The bio-functionalization of solid surfaces through the physisorption of antibodies has proven an effective, rapid and low-cost strategy for the fabrication of the sensing interfaces of biosensors for antigen detection. The prevailing notion that physisorbed antibodies have reduced capability of targeting antigens because they form disordered films may be inaccurate. It is conceivable that antibodies, or proteins in general, reach a stable self-organized arrangement upon physical adsorption on solid surfaces. Indeed, the absence of strong covalent bonds with the surface, lend them a certain degree of mobility on the surface. Also, proteins may adapt their conformation to minimize the interaction energy with the surface. Thus, non-random arrangement of proteins may naturally arise upon physisorption.

The orientation of adsorbed molecules onto a surface can be assessed by polarized IR spectroscopy techniques. The conventional method^{100–102} to perform the orientation analysis of SAM adsorbed on metal substrates has been discussed in section 3.5.3. The method is based on the comparison of an IRRAS spectrum of SAM with a theoretical IRRAS spectrum of the sample with randomly distributed

TDM. The simulated spectrum is computed using the optical constants of the SAM extracted from an IR transmission spectroscopy measurement of the bulk material. However, this method is useless dealing with proteins, because the optical constants of the protein film cannot be derived from those of a bulk sample. Indeed, proteins are complex macromolecules, whose structure in a bulk sample could differ from that in an adsorbed monolayer, therefore the optical parameters could be different as well. To overcome this issue and an alternative method has been developed to conduct the orientation analysis of a monolayer of proteins physisorbed on gold, based on PM-IRRAS and ATR spectroscopy.¹⁰⁵

The analysis has been performed for BSA and human anti-IgG proteins, which are selected because of their different tertiary and secondary structure, thus allowing comparison of results for two model molecules belonging to two different protein classes (see section 2.2 and 2.3). The PM-IRRAS substrates were bio-functionalized as described in the experimental section 3.1., with a monolayer of proteins physisorbed on gold. Figure 5.1 shows the experimental PM-IRRAS spectra of the anti-IgG and BSA samples, in the amide I and amide II spectral range (after conversion of the PM-IRRAS signal into absorbance units, as discussed in section 3.3.2). Both spectra feature a broad and rounded amide I band peaked at $\sim 1670 \text{ cm}^{-1}$. Notably, the amide I maximum seems insensitive to the different secondary structure composition of BSA and anti-IgG, contrary to what is observed in the ATR spectra of the bulk (drop-casted) BSA and anti-IgG samples, showed in section 3.5.2, which feature the amide I band peaked at the characteristic frequency according to the secondary structure content of the two proteins. This is because the absorbance measured by PM-IRRAS spectroscopy (A_{film}) on gold, is influenced by the orientation of the TDM of a given vibrational mode (μ), with respect to the normal direction to the substrate (z-direction), according to the equation

$$A_{film} \propto \mu^2 E_p^2 \cos^2(\theta) \quad (5.1)$$

where E_p is the component of the electric field vector polarized perpendicular to the gold surface and θ is the angle between the TDM and the z-direction.

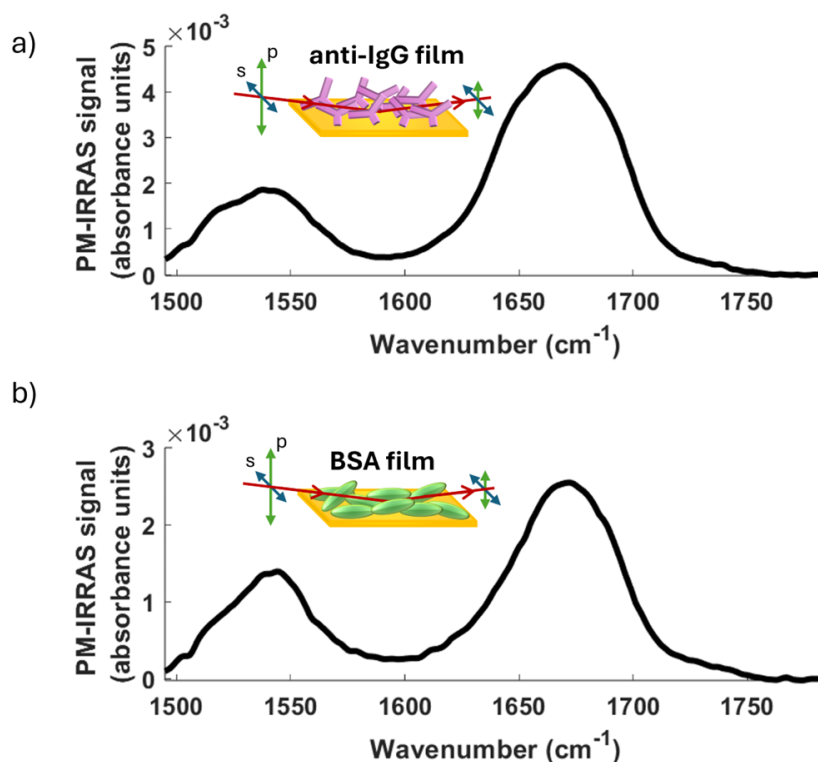


Figure 5.1. PM-IRRAS spectra of anti-IgG and BSA monolayers. PM-IRRAS spectrum of anti-IgG (a) and BSA (b) monolayers physisorbed on gold. Taken from [103]

Accordingly, the lineshape of the PM-IRRAS amide I band depends also on the orientation of amide I dipoles in the physisorbed proteins. The observation that the PM-IRRAS spectra BSA and anti-IgG are peaked at the same wavenumber, despite their different structures, may suggest that both monolayers reach a partially similar arrangement upon physisorption on gold.

To evaluate an average tilt angle $\langle \theta \rangle$ of the TDMs of adsorbed proteins through equation 3.13, the experimental PM-IRRAS absorbance must be compared to a simulated IRRAS absorbance computed for protein monolayer with randomly oriented TDMs. The refractive index $n(\tilde{\nu})$ and of the absorption index $k(\tilde{\nu})$ of the protein layer, in the relevant spectral range, are key quantities to simulate the IRRAS spectrum. Here, we computed $n(\tilde{\nu})$ and $k(\tilde{\nu})$ through the ATR spectra of the BSA and anti-IgG monolayers, obtained by the physisorption onto the ZnSe ATR crystal. The physisorption is achieved following the same protocol used to functionalize the PM-IRRAS gold substrate. Both gold and ZnSe surfaces are negatively charged during the bio-functionalization at pH=7.4 (the isoelectric point of the ZnSe surface is IP=4.9), hence the adsorption is assumed to be the comparable on the two surfaces, leading to a protein monolayer with analogous conformation, thickness and surface coverage.

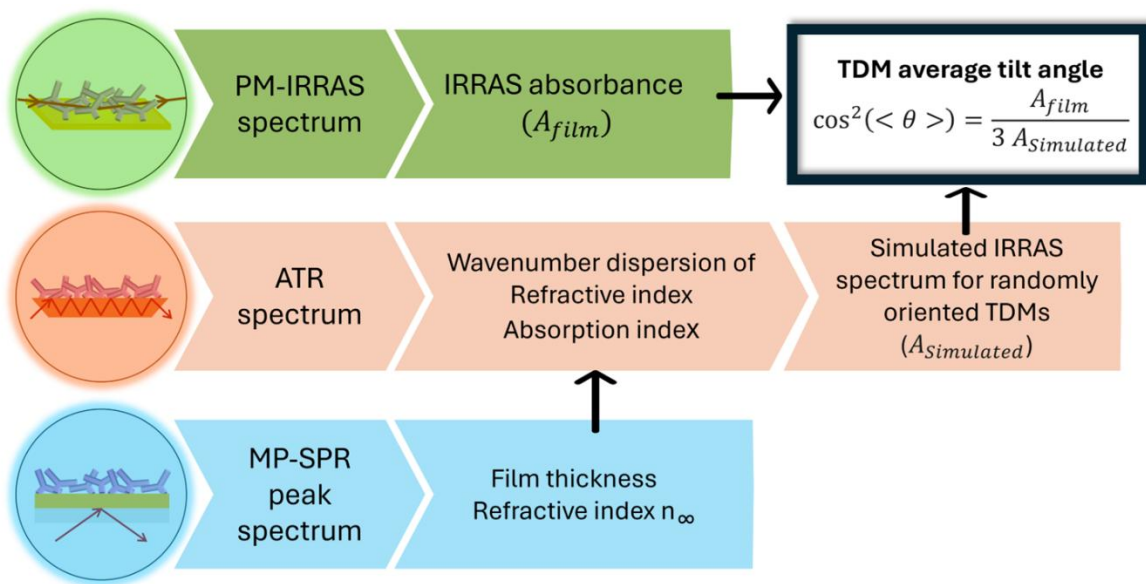


Figure 5.2. Method for the orientation analysis of protein monolayers. The method uses three set of experimental input data. The protein layer thickness and refractive index n_{∞} are measured by SPR. The refractive index and absorption index in the mid-IR relevant spectral range are calculated using the ATR spectrum along with the SPR output. The optical constants are used to simulate a theoretical IRRAS spectrum of the protein monolayer with randomly oriented TDMs. The experimental PM-IRRAS spectrum is converted to IRRAS absorbance units, and compared to the simulated spectrum through equation 3.13 to extract an average tilt angle $\langle \theta \rangle$. Taken from [103]

Our method is schematically summarized in figure 5.2. The orientation analysis is performed out of three experimental inputs: i) the PM-IRRAS spectrum; ii) the ATR spectrum; iii) the SPR spectrum. SPR is used to measure the thickness of the protein layer and evaluate its refractive index in the visible spectral range (n_{∞}). Those data are utilized to compute the refractive index dispersion $n(\tilde{\nu})$ and the absorption index $k(\tilde{\nu})$ in the mid-IR range, through the ATR spectrum. The extracted $n(\tilde{\nu})$ and $k(\tilde{\nu})$ correspond to those of a protein film with randomly oriented TDMs. Then, the IRRAS spectrum of the isotropic protein film is simulated and compared to the corrected PM-IRRAS spectrum for the estimation of the average tilt angle $\langle \theta \rangle$ through equation 3.13.

The ATR spectra of the anti-IgG and BSA films are shown in figure 5.3a-b. The anti-IgG spectrum features an amide I band peaked at 1640 cm^{-1} , whereas it is peaked at 1655 cm^{-1} in the BSA spectrum, as it is expected for proteins with prevalent β -sheet and prevalent α -helix content respectively, in agreement with the position observed in the bulk protein samples (section 3.5.2).

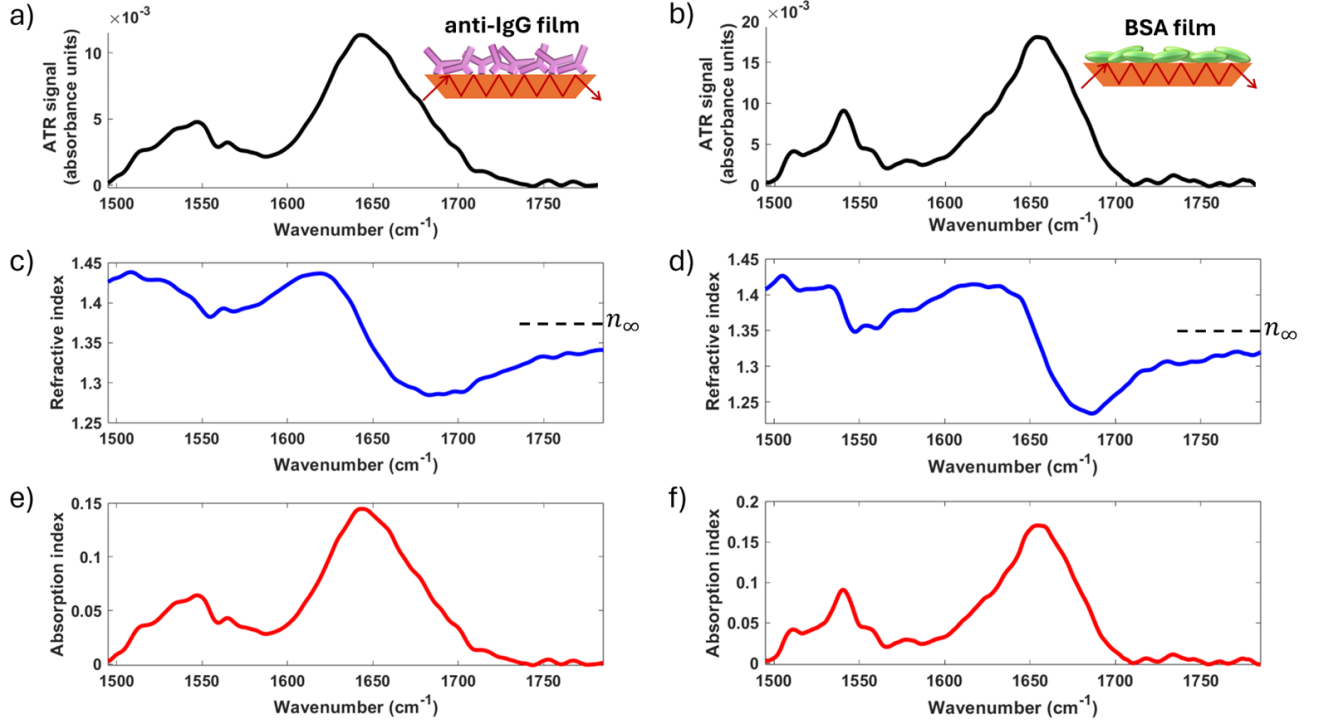


Figure 5.3. ATR spectra and optical constants of anti-IgG and BSA monolayers. a,b) ATR spectra of the anti-IgG and BSA monolayers, physisorbed onto the ZnSe ATR crystal, as depicted in the schematic representations c,d) refractive index dispersion of anti-IgG and BSA monolayers extracted from the ATR spectra in a) and b) e,f) absorption index of anti-IgG and BSA monolayers extracted from the ATR spectra in a) and b). Taken from [103]

The ATR absorbance at each wavenumber can be described by

$$A_{ATR} = -\log_{10} \left(\frac{I_{film}}{I_0} \right) = -\log_{10} \left(\frac{R_s^6 + R_p^6}{2} \right) \quad (5.2)$$

where I_0 and I_{film} are the IR intensity transmitted through the bare ZnSe ATR crystal and after bio-functionalization with the protein layer. The sixth power stands for the 6 internal reflections within the ZnSe waveguide. The I_{film}/I_0 ratio is explicated in terms of the reflectivity at the ZnSe/protein/air interface for the s- (R_s) and p- (R_p) polarized radiation respectively,

$$R_s = 1 - \frac{8\pi d n_{ZnSe} \cos(\theta_i)}{\lambda} \text{Im} \left[\frac{\hat{\epsilon}_{film} - 1}{\hat{\epsilon}_{ZnSe} - 1} \right] \quad (5.3 a)$$

$$R_p = 1 - \frac{8\pi d n_{ZnSe} \cos(\theta_i)}{\lambda} \text{Im} \left\{ \left[\frac{\hat{\epsilon}_{film} - 1}{\hat{\epsilon}_{ZnSe} - 1} \right] \left[\frac{1 - \hat{\epsilon}_{ZnSe}/\hat{\epsilon}_{film} (\hat{\epsilon}_{film} + 1) \sin^2(\theta_i)}{1 - (\hat{\epsilon}_{film} + 1) \sin^2(\theta_i)} \right] \right\} \quad (5.3 b)$$

obtained by equation 3.10a and 3.10b for the ZnSe/protein/air system, using $\widehat{\epsilon}_{air} = 1$ for the relative dielectric constant of air. $\hat{\epsilon}_{film}$ and $\hat{\epsilon}_{ZnSe}$ are the complex dielectric constant of the protein film and of the ZnSe substrate, related to n and k through $\hat{\epsilon} = n^2 - k^2 + i2nk$.

Using $\theta_i = 45^\circ$ for the ATR incidence angle and $n_{ZnSe} = 2.4$, and $k_{ZnSe} = 0$, for the ZnSe into equation 5.3a and 5.3b the reflectivity R_s and R_p became function of the thickness (d) and optical constants of the protein film and the ATR absorbance becomes

$$A_{ATR}(\tilde{\nu}) = -\log_{10} \left(\frac{R_s^6(d, n(\tilde{\nu}), k(\tilde{\nu})) + R_p^6(d, n(\tilde{\nu}), k(\tilde{\nu}))}{2} \right) \quad (5.4)$$

where the dependencies on the film parameters have been explicated.

To compute $n(\tilde{\nu})$ and $k(\tilde{\nu})$ of the protein layer, a custom MATLAB script has been developed, which follows an iterative approach. First, equation 5.4 is solved for $k(\tilde{\nu})$ at each wavenumber in the spectral range between 1500 cm^{-1} and 1780 cm^{-1} . The film thickness is provided by SPR measurements, equals $d_{anti-IgG} = 9 \pm 3 \text{ nm}$ and $d_{BSA} = 7 \pm 1 \text{ nm}$ for anti-IgG and BSA respectively. Also, the refractive index value n_∞ is used as a constant initial guess for $n(\tilde{\nu})$, provided by the SPR measurements equal to $n_\infty = 1.37 \pm 0.01$ for the anti-IgG layer and $n_\infty = 1.35 \pm 0.01$ for the BSA layer.

The obtained values for $k(\tilde{\nu})$ is then used to compute $n(\tilde{\nu})$ by means of the Kramers-Kronig relation (equation 3.15), in the same spectral range $1500\text{-}1780 \text{ cm}^{-1}$. Then, $k(\tilde{\nu})$ is again evaluated using the new $n(\tilde{\nu})$ values and the process is iterated until convergence (4 iterations are typically sufficient). Figure 5.3c-f reports the refractive index, and the absorption index computed for the anti-IgG and BSA monolayers, in the amide I – amide II spectral range.

For the simulation of the IRRAS spectrum, the IRRAS absorbance of a protein film deposited on gold is expressed as

$$A_{Simulated} = -\log_{10} \left(\frac{R_p(d)}{R_p(0)} \right) \approx 0.434 \left(1 - \frac{R_p(d)}{R_p(0)} \right) \quad (5.5)$$

where $R_p(0)$ and $R_p(d)$ are the reflectance of the bare and of the protein-covered gold substrate respectively. The ratio $R_p(d)/R_p(0)$ can be expressed in terms of the optical constants of the protein film and of gold according to the equation (see appendix C)

$$R_p = 1 - \frac{8\pi d \cos(\theta_i)}{\lambda} \text{Im} \left\{ \left[\frac{\widehat{\epsilon}_{film} - \widehat{\epsilon}_{au}}{1 - \widehat{\epsilon}_{au}} \right] \left[\frac{1 - 1/\widehat{\epsilon}_{film}\widehat{\epsilon}_{au}(\widehat{\epsilon}_{film} + \widehat{\epsilon}_{au}) \sin^2(\theta_i)}{1 - (1/\widehat{\epsilon}_{au})(\widehat{\epsilon}_{film} + \widehat{\epsilon}_{au}) \sin^2(\theta_i)} \right] \right\} \quad (5.6)$$

where $\theta_i = 82^\circ$ is the IRRAS incidence angle, $\widehat{\epsilon}_{au}$ is the complex dielectric function of gold (tabulated) and $\widehat{\epsilon}_{film}$ is that of the protein layer, computed by the obtained refractive index and absorption index.

The experimental PM-IRRAS spectrum and the simulated IRRAS spectrum are shown in figure 5.4 for the anti-IgG (a) and BSA (b) layer, in the amide I spectral range. Notably, the amide I lineshape

in the experimental spectrum differs from that in the simulated one for both anti-IgG and BSA. This difference can be ascribed to a partial order of the TDMs within the protein films. Indeed, if the TDMs within the protein films were fully randomly oriented the experimental PM-IRRAS spectrum would show the same lineshape as in the simulated IRRAS one. The different amide I lineshapes are a clear qualitative result supporting the hypothesis that physisorption does not lead to a totally disordered protein layer, both for anti-IgG and BSA.

For a quantitative analysis, the amide I bands are subject to a fit-deconvolution (see section 3.5.2) analysis. A fit-model including 5 Gaussian functions is utilized to account for the contributions of the secondary structure elements: side-chains, β -sheets (B1 and B2 modes), unordered/ α -helix and turns. The starting point for the position of the 5 secondary structure components were chosen according to the literature (table 2.1 [Barth]). A custom MATLAB algorithm (see appendix D) returned a best-fit curves with determination coefficient $R^2 > 0.99$ and a root mean square error < 0.0003 . The results of the fits of the amide I bands in the experimental PM-IRRAS and simulated IRRAS spectra, for anti-IgG and BSA, are shown in figure 5.4, whereas tables 5.1 and 5.2 reports the resulting fit parameters (peak position, FWHM and area) of each gaussian function under the amide I band, respectively.

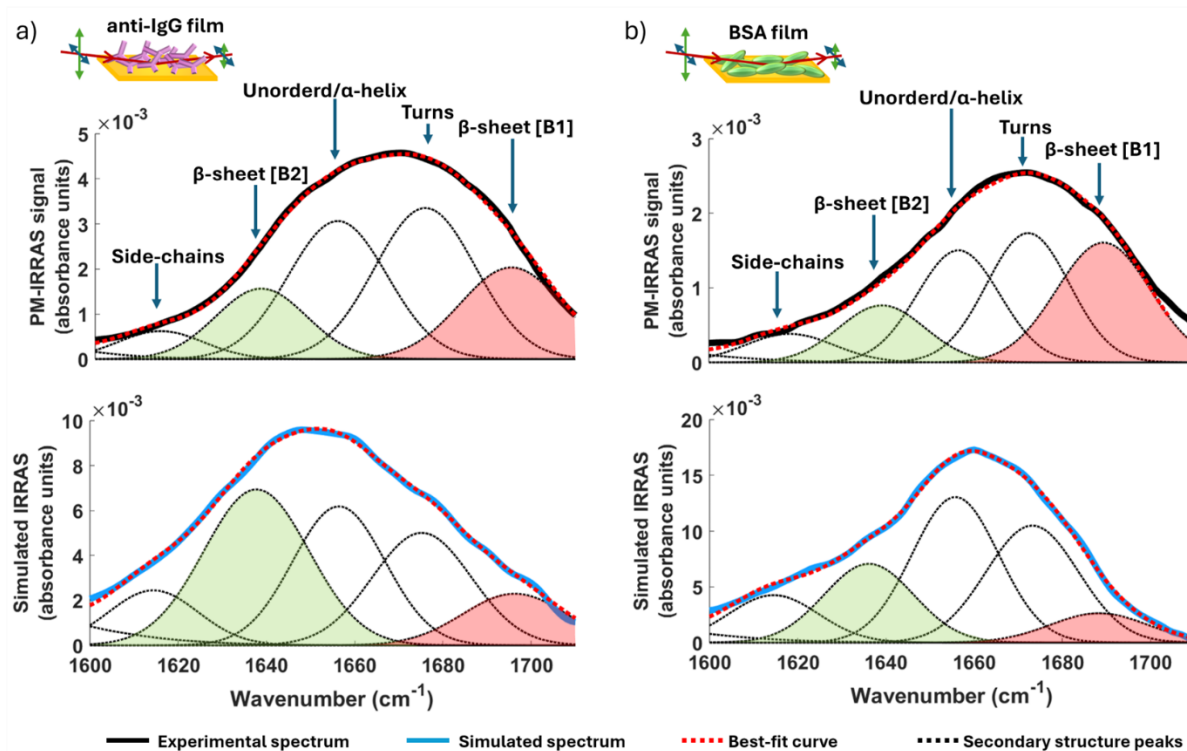


Figure 5.4. Amide I fit deconvolution of PM-IRRAS and simulated spectra. Amide I band of the experimental PM-IRRAS spectra of the anti-IgG (a) and BSA (b) protein monolayers on gold. The dashed red lines represent the fit-curve obtained using a fit-model including 5 Gaussian functions to account for side-chains, β -sheets (B1 and B2 modes), unordered/ α -helix and turns. The highlighted green and red areas corresponding to β -sheets B2 and β -sheets B1 TDMs are used to estimate the average orientation of the β -sheets. Taken from [103]

The area subtended the single amide I components of the PM-IRRAS spectra can be compared to the corresponding area in the simulated spectra and utilized to evaluate an average tilt angle $\langle \theta \rangle$ of the TDMs from the various secondary structure elements. To correlate $\langle \theta \rangle$ with the actual orientation of the polypeptide chain, it is crucial to know the orientation of the TDMs vector within the molecular coordinate system. The orientation of TDMs within β -sheets and α -helix have been discussed in section 2.1.2, highlighting the existence of two orthogonal TDMs associated to the amide I vibrations. For turns and unordered structure this information is lacking. Moreover, the analysis of α -helix is further complicated as its contribution to the amide I band is largely overlapping that of unordered regions, preventing a clear identification. Therefore, only the amide I components originated from β -sheets remain available for the orientational analysis.

Figure 5.5a reports a schematic representation of a β -sheet with the B1 and B2 TDMs oriented with respect to the normal direction to the substrate with tilt angles θ_{B1} and θ_{B2} . The area under the β -sheet-B1 and β -sheet-B2 components of the amide I band in the PM-IRRAS spectra depends on the average angles $\langle \theta_{B1} \rangle$ and $\langle \theta_{B2} \rangle$.

We found $\langle \theta_{B1} \rangle \geq 57.1^\circ \pm 0.8^\circ$ and $\langle \theta_{B2} \rangle \geq 75.3^\circ \pm 0.4^\circ$ for anti-IgG layer, whereas $\langle \theta_{B1} \rangle \geq 63.7^\circ \pm 0.6^\circ$ and $\langle \theta_{B2} \rangle \geq 79.3^\circ \pm 0.2^\circ$ for the BSA layer.

Table 5.2. Amid I fit-deconvolution of anti-IgG monolayer. Peaks position, FWHM and areas of individual secondary structure components extracted from the fit of amide I band in the experimental PM-IRRAS spectrum and in the simulated IRRAS spectrum of anti-IgG physisorbed layer.

	Experimental PM-IRRAS			Simulated IRRAS		
	$\tilde{\nu} (cm^{-1})$	FWHM(cm^{-1})	Area (10^{-3})	$\tilde{\nu} (cm^{-1})$	FWHM(cm^{-1})	Area (10^{-3})
Side-chains	1615	23	15.3	1614	24	62.1
β -sheet [B2]	1638	25	40.8	1638	28	206.8
Unordered / α -helix	1656	27	87	1656	25	162.1
Turns	1677	27	97.2	1675	26	140.7
β -sheet [B1]	1695	28	59.7	1696	28	67.9

Table 5.2. Amid I fit-deconvolution of BSA monolayer. Peaks position, FWHM and areas of individual secondary structure components extracted from the fit of Amide I band in the

experimental PM-IRRAS spectrum and in the simulated IRRAS spectrum of BSA physisorbed layer.

	Experimental PM-IRRAS			Simulated IRRAS		
	$\tilde{\nu} (cm^{-1})$	FWHM(cm^{-1})	Area (10^{-3})	$\tilde{\nu} (cm^{-1})$	FWHM(cm^{-1})	Area (10^{-3})
Side-chains	1618	24	97	1615	24	108.4
β -sheet [B2]	1639	23	18.7	1636	24	180
Unordered / α -helix	1657	22	35.3	1656	23	322
Turns	1672	22	40.7	1673	24	275.2
β -sheet [B1]	1689	25	42.8	1688	26	72.8

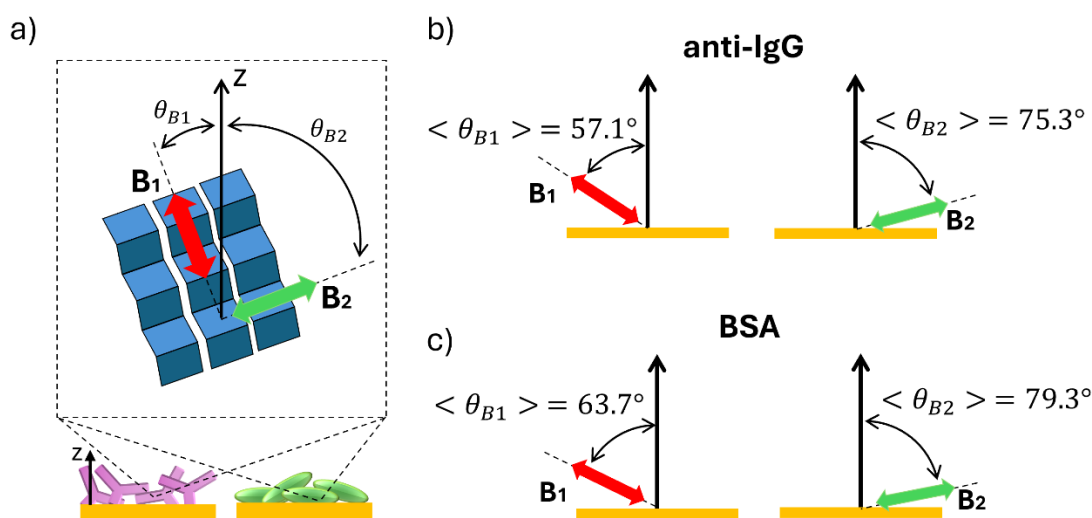


Figure 5.5. β -sheet orientation. **a)** Schematic representation of a β -sheets in anti-IgG and BSA film featuring the TDM associated to the B1(red) and B2(green) amide I mode, and their tilt angle with respect to the normal direction to the substrate **b, c)** Schematic representation of the average tilt angle of the B1 (red arrow) and B2 (green arrow) TDM of the amide I mode in β -sheets for the anti-IgG (b) and BSA (c) films physisorbed on gold. Taken from [103]

Figure 5.5b-c depict the TDMs of the β -sheets in the anti-IgG and BSA layers, oriented according to the obtained results. The findings are remarkably similar for the anti-IgG and BSA physisorbed layers. In both cases, the average orientation of the TDMs corresponds to a partial order of the β -sheets tilted toward the gold substrate. Those results challenge the common belief that physisorbed antibodies adopt a fully random configuration on the substrate, lacking any order thus being less reactive for the binding of antigens. Rather, the lineshape of lineshape of the Amide I band measured in PM-IRRAS spectrum reveals that a certain degree of organization is present within the film. Though the entity of this organization is not assessed through the described experimental method,¹⁰⁶ it is a relevant experimental evidence of the partial protein self-organization on gold. Notably, β -sheets are generally energetically favoured structure upon protein adsorption on solid substrates. For example, the partial unfolding of BSA has been largely demonstrated upon adsorption. This suggests the leading role of

β -sheets in defining the arrangement of physisorbed proteins and support the picture of a partial order in spontaneously physisorbed protein layers.

Comment on the fit-deconvolution of the amide I bands

Note that the fit-deconvolution procedure for the amide I bands analysis is influenced by the choice of the initial parameters of the fit parameters. In particular, for the previous analysis we used literature results to set the starting frequency positions of the secondary structure components. Another possibility is to utilize the second derivative of the PM-IRRAS spectra to identify the position of the sub-band in the amide I range and use those values as starting point for fit parameters. Figure 5.6 reports the fit results obtained in this way. The obtained secondary structure components under amide I are slightly shifted with respect to those previously shown. Table 5.4 and 5.5 reports the resulting fit parameters.

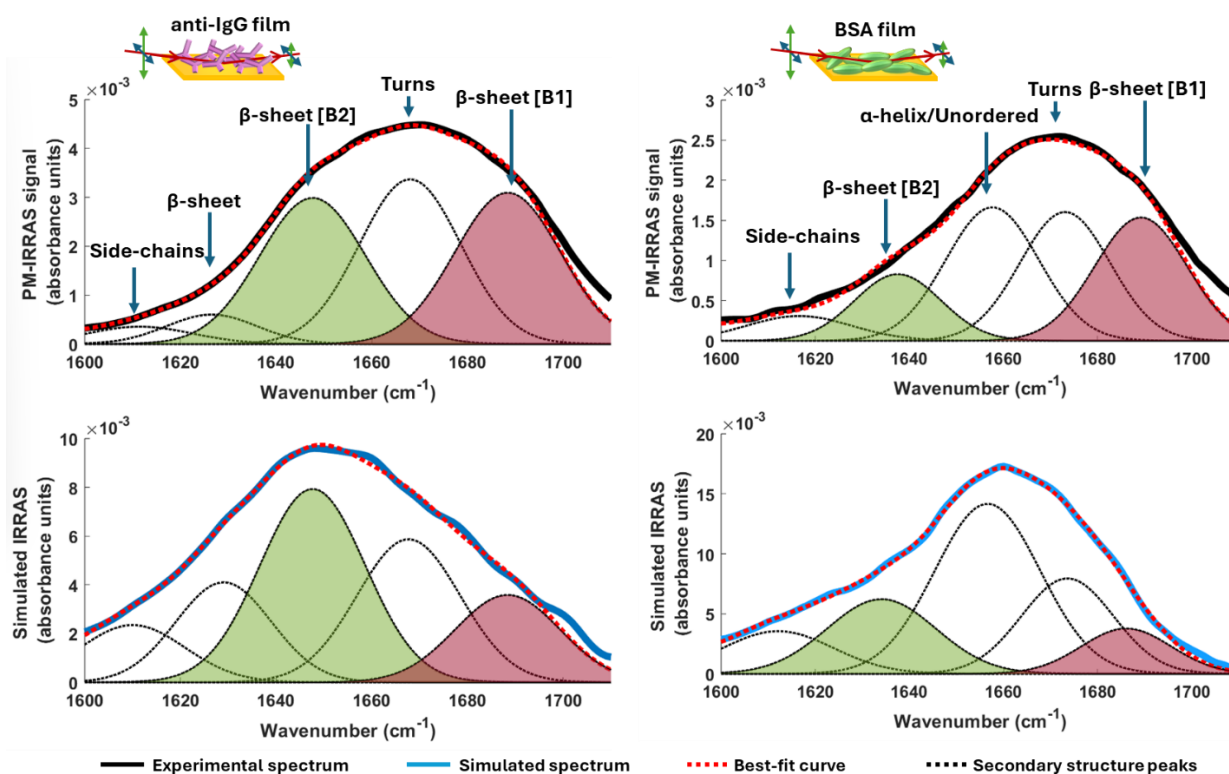


Figure 5.6. Amide I fit deconvolution of PM-IRRAS and simulated spectra. Amide I band of the experimental PM-IRRAS spectra of the anti-IgG (a) and BSA (b) protein monolayers on gold. The dashed red lines represent the fit-curve obtained using a fit-model including 5 Gaussian functions. The main components are resolved by the second derivative spectra, used as a guide to set the starting position of the fit parameters. The amide I components of anti-IgG spectra are ascribed to side-chains, β -sheets (additional contribution, possibly arising from intramolecular β -sheets or from β -sheets units having different geometry with respect to the predominant ones), β -

sheets (B1 and B2 modes) and turns. The amide I components of BSA spectra are ascribed to side-chains, β -sheets (B1 and B2 modes), α -helix/unordered, and turns. The highlighted green and red areas corresponding to β -sheets B2 and β -sheets B1 TDMs are used to estimate the average orientation of the β -sheets.

Also, the second derivative spectra suggest a different assignment if the bands to various secondary structure, as reported in the tables 5.3 and 5.4. Specifically, the main amide I components in the anti-IgG spectrum is ascribed to β -sheet-B2 vibrations falls at 1648 cm^{-1} . The smaller band at 1627 cm^{-1} is still ascribed to β -sheet, but could be related to intermolecular structures, which forms upon physisorption. Indeed, the contribution of intermolecular β -sheet is expected at lower frequency with respect to that from native β -sheet structures. As for BSA the band assignment is not changed.

Table 5.3. Amide I fit-deconvolution of anti-IgG monolayer. Peaks position, FWHM and areas of individual secondary structure components extracted from the fit of amide I band in the experimental PM-IRRAS spectrum and in the simulated IRRAS spectrum of anti-IgG physisorbed layer using the second derivative spectra to set the fit starting parameters.

	Experimental PM-IRRAS			Simulated IRRAS		
	$\tilde{\nu}\text{ (cm}^{-1}\text{)}$	FWHM(cm^{-1})	Area (10^{-3})	$\tilde{\nu}\text{ (cm}^{-1}\text{)}$	FWHM(cm^{-1})	Area (10^{-3})
Side-chains	1611	26	9.8	1619	26	64.4
Intramolec. β -sheet	1627	22	14.2	1629	23	102.4
β -sheet [B2]	1648	26	82.5	1648	26	218.1
Turns	1668	25	89.5	1668	25	158.7
β -sheet [B1]	1688	26	85.7	1688	26	98.1

Table 5.4. Amide I fit-deconvolution of anti-IgG monolayer. Peaks position, FWHM and areas of individual secondary structure components extracted from the fit of Amide I band in the experimental PM-IRRAS spectrum and in the simulated IRRAS spectrum of BSA physisorbed layer using the second derivative spectra to set the fit starting parameters.

	Experimental PM-IRRAS			Simulated IRRAS		
	$\tilde{\nu}\text{ (cm}^{-1}\text{)}$	FWHM(cm^{-1})	Area (10^{-3})	$\tilde{\nu}\text{ (cm}^{-1}\text{)}$	FWHM(cm^{-1})	Area (10^{-3})
Side-chains	1617	26	8.6	1612	28	104.5
β -sheet [B2]	1638	22	19	1634	27	179.5
α -helix /Unordered	1658	23	41.2	1657	26	398.1
Turns	1673	23	38.4	1674	23	194.6
β -sheet [B1]	1689	23	37.6	1686	22	90

Notably, the results of the orientation analysis are not strongly influenced by the different fit deconvolutions.as the following average tilt angle are found: $\langle \theta_{B1} \rangle \geq 56.8^\circ \pm 0.6^\circ$ and $\langle \theta_{B2} \rangle \geq 68.8^\circ \pm 0.4^\circ$ for anti-IgG layer, whereas $\langle \theta_{B1} \rangle \geq 68.0^\circ \pm 0.3^\circ$ and $\langle \theta_{B2} \rangle \geq 79.1^\circ \pm 0.1^\circ$ for the BSA layer, corresponding to a partial orientation of the β -sheets toward the substrate.

5.2 Influence of electric field annealing on physisorbed antibodies

The dielectric properties of interfaces bio-functionalized with a monolayer of antibodies, employed as sensing element in biosensors for antigen detection, are defined by the surface dipoles and charges within the biolayer. The polarization of the antibody layer influences the bioelectronic performance of the devices, therefore a bio-functionalization protocol that guarantees high reproducibility of the electrostatic configuration of the adsorbed film is crucial for biosensing applications. Bio-functionalization strategies based on physisorption of antibodies are regarded as irreproducible processes. However, their application in SiMoT biosensors showed sensitive detection performance, precisely based on a reproducible change of the dielectric properties of the biolayer upon antigen binding. In fact, the sensing interface employed in SiMoT biosensors, is subject to an electric-field cycling which stabilize the electronic response of the device, before incubation with the analyte solution. Specifically, the bio-functionalized gate electrode (section 1.5) undergoes 20 cycles of the gate voltage V_G sweeping in the range between 0.1 V and -0.5V. The transfer characteristic of the SiMoT transistor is acquired 20 times, until it is sufficiently reproducible to proceed with the detection.

The electrical cycling works as an annealing process taking the biolayer into a more stable conformation. A systematic study of the influence of the electric-field annealing on gold interfaces bio-functionalized with physisorbed monolayer of Immunoglobulin M antibodies (anti-IgM) has been conducted, by three potentiometric techniques, namely Kelvin probe force microscopy (KPFM), EG-FET threshold voltage analysis, and ζ -potential analysis, along with the structural analysis of the protein layers by PM-IRRAS spectroscopy.⁶³

The polarization P of the biolayer is defined as the overall net dipole per unit volume. Since each antibody has an associated electric dipole (see section 2.1.1), the polarization is influenced by the distribution and orientation of the antibodies upon physisorption, and its variability follows from the variety of possible configurations taken by the proteins on the substrate. The assessment of P is possible by measuring the surface potential of the biolayer, which in the case of a planar surface is given by $SP = Pd/(\epsilon\epsilon_0)$ where d and ϵ are the thickness and the relative dielectric constant of the protein film.

KPFM is used to assess the relative SP of the anti-IgM film, with respect to that of the bare gold substrate (surface potential difference SPD). Figure 5.7a represents a KPFM patterned sample of anti-

IgM physisorbed on gold, whereas figure 5.7b shows the corresponding SPD image at the Au/anti-IgM interface. The SPD image shows that the surface potential of gold increases with the deposition of anti-IgM of about ~ 250 mV, as a result of the polarization of the biolayer. To evaluate the reproducibility of the polarization of the biolayer, the SPD of 5 anti-IgM sample has been measured. It was found that, the SPD of the as deposited anti-IgM layer on gold, set at an average value of 236 mV with a quite large dispersion of 70 mV (one standard deviation). Then, the effect of the electric field cycling (EF-cycling) on the polarization of the biolayer has been assessed by measuring the SPD of further 5 samples subject to and EF-cycling. The anti-IgM samples were immersed in deionized water (HPLC grade, $\text{pH} \approx 5.5$, $i_s \approx 5 \mu\text{M}$) with an Au counter electrode, as depicted in figure 5.7a, and connected to a potentiostat for the application of a sweeping potential in the range $[0.1\text{V}, -0.5\text{V}]$ at a scan rate of 100 mV s^{-1} for 20 cycles. After the EF-cycling the samples are dried and the KPFM images acquired. The SPD of the five EF-cycled samples set at an average value of 243 mV with a standard deviation of 11 mV, significantly reduced with respect to that found before cycling. The box plot in figure 5.7c summarizes the result obtained by the KPFM analysis. The dispersion of cycled samples is reduced of 84% with respect to that of non-cycled ones. In other words, the reproducibility of the surface potential, and consequently that of the polarization, of the anti-IgM layer is significantly increased upon the EF-cycling. Indeed, upon each V_G cycle, the physisorbed antibodies on the Au surface, experience a cycling electric field varying between $E \approx 5 \cdot 10^4 \text{ V/m}$, when $V_G = 0.1 \text{ V}$ and $E \approx -2.5 \cdot 10^5 \text{ V/m}$, $V_G = -0.5 \text{ V}$ where the positive sign represents E pointing toward the electrolyte, whereas the negative sign represents E pointing toward the substrates. The electric field magnitude is estimated considering a drop of the applied voltage within the $\sim 1 \mu\text{m}$ extended EDLs. At $\text{pH} \approx 5.5$ the anti-IgM (Y-shaped IgG-like antibodies), have an associated electric dipole pointing from the Fc fragment toward the Fab fragment (see section 2.1.1), which renders them responsive to electric fields. Despite there are constrained by protein-surface and protein-protein interactions, within the densely packed biolayer, the electric field may induce little rearrangements leading the antibodies in a more stable and reproducible conformation.

An increased reproducibility upon EF-cycling is also observed for the values of the threshold voltage (V_{th}) of an EG-FET using an anti-IgM biofunctionalized gate electrode. Figure 5.7d depict the schematic of the EG-FET utilized in the measurements, filled with deionized water (HPLC grade, $\text{pH} \approx 5.5$, $i_s \approx 5 \mu\text{M}$) serving as electrolyte. In this case, the EF-cycling of the biolayer is achieved by applying a cyclic gate voltage V_G in the range $[0.1\text{V}, -0.5\text{V}]$, for 20 cycles, and the V_{th} of the EG-FET is extracted from the transfer characteristic of the transistor, after the first cycle and after the 20th

cycle, as represented in figure 5.7e. 10 gate electrodes were prepared with a layer of physisorbed anti-IgM and the V_{th} before and after the EF-cycling is measured for each one. Results are reported in the box plot of figure 5.7f. For non-cycled sample $V_{th} = -200 \text{ mV} \pm 80 \text{ mV}$ whereas after the cycling it becomes $V_{th} = -220 \text{ mV} \pm 40 \text{ mV}$. There is a 50% reduction of the standard deviation of the threshold voltages measured after the EF-cycling, in agreement with the KPFM results. Indeed, the V_{th} depends on the SP of the gate electrode, hence the variability of the SP also influences the overall bioelectronic response of the transistor.

The third potentiometric technique to assess electrostatic configuration of the anti-IgM layer consists in measurements of the ζ -potential of the bio-functionalized interfaces, that depends on the charge on the surface. The ζ -potential is defined as the electrostatic potential generated by the accumulation of charges at a solid surface immersed into an electrolyte, measured at the slipping plane (the ideal plane which separates mobile ions from those that remain attached to the solid surface) in the EDL. Figure 5.7g features the measuring cell, containing the anti-IgM functionalized Au surface, and two Ag/AgCl electrodes immersed in an aqueous solution of KCl electrolyte (pH=5.5 $i_s = 10 \text{ mM}$). The ζ -potential is assessed by measuring the streaming current between the electrodes. 11 anti-IgM samples were prepared, and the ζ -potential measured for each one before and after EF-cycling, as performed for the other potentiometric analysis. It turns out that the $\zeta = -21 \text{ mV} \pm 6 \text{ mV}$ before the EF-cycling and remains quite constant at $\zeta = -22 \text{ mV} \pm 3 \text{ mV}$ after the EF-cycling. Again, a 50% reduction of the data dispersion is measured upon EF-cycling, as shown in figure 5.7i. Also, the ζ -potential measurements enable the evaluation of the isoelectric point of the bio-functionalized interfaces. It corresponds to the pH at which $\zeta=0\text{V}$. The IP of the anti-IgM functionalized interface is found equals $IP = 3 \pm 0.1$, marked in figure 5.7g.

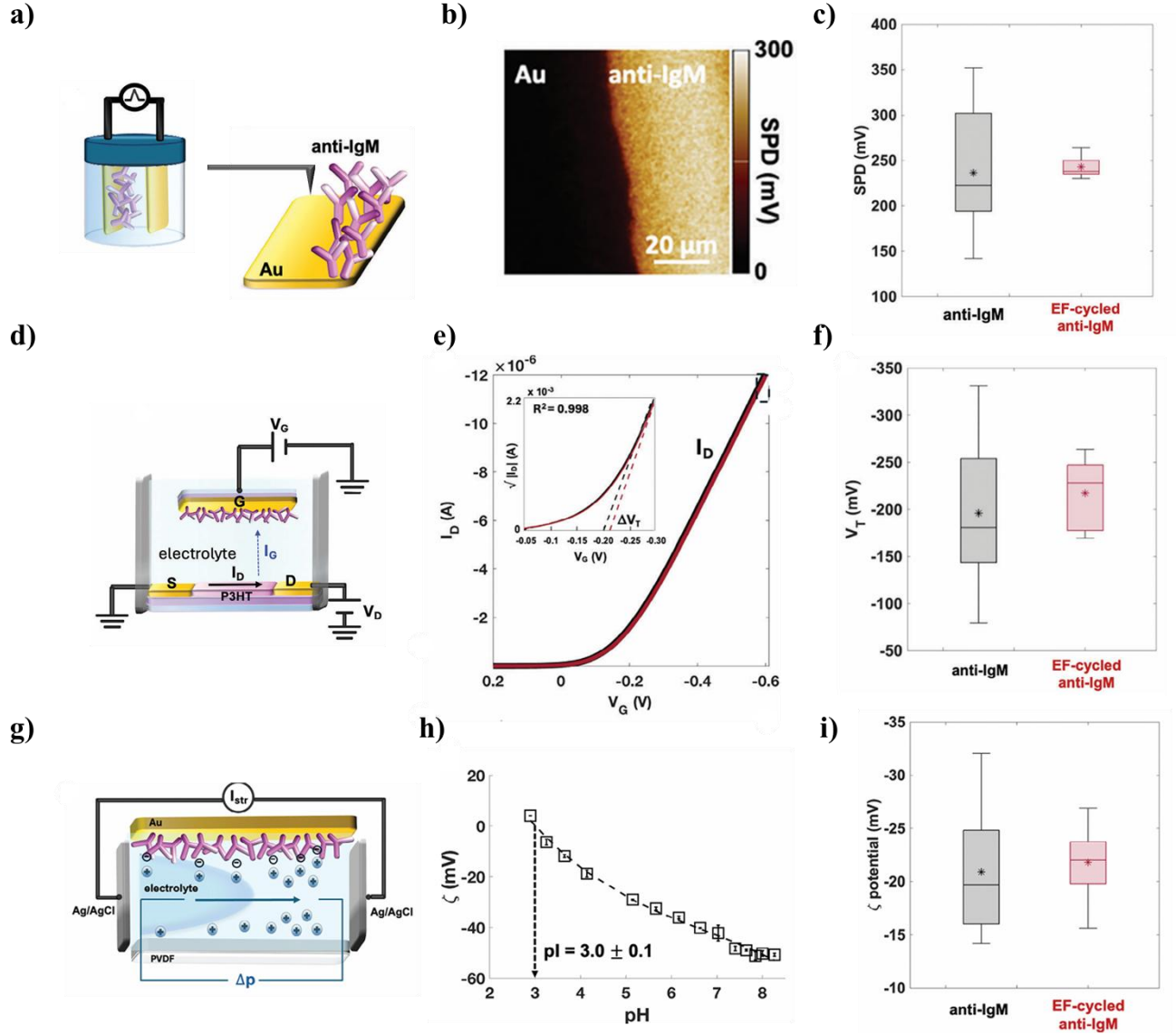


Figure 5.7 Potentiometric analysis of the anti-IgM biolayer upon EF-cycling. **a)** Schematic of the KPFM sample; **b)** SPD image of the patterned Au/anti-IgM surface; **c)** Box plot showing the average SPD assessed over 5 non-cycled samples and 5 EF-cycled samples; **d)** Schematic of the EG-FET with a anti-IgG functionalized gate electrode; **e)** Transfer characteristics of the EG-FET measured for the extraction of the threshold voltage V_{th} ; **f)** Box plot showing the average V_{th} assessed over 10 samples, before and after the EF-cycling; **g)** Schematic of the ζ -potential cell with including an anti-IgM functionalized gold surface; **h)** measured ζ -potential versus pH of the solution of as-deposited anti-IgM film; the arrow marks the isoelectric point at $\text{pH} = 3 \pm 0.1$; **i)** Box plot showing the average ζ -potential assessed over 10 samples before and after the EF-cycling. Taken from [61]

All three experiments feature a significant reduction of the dispersion of the three polarization-related quantities (SP, V_{th} and ζ -potential), following the EF-cycling of the anti-IgM interface. Other papers report the orientation of fragments of antibodies or the whole protein on a substrate, by application

of an electric field. However, in our experiments, the EF-cyclic is not expected to reorient the antibodies, as it switches from positive to negative sign in one cycle. Rather, it acts on the biolayer as an annealing process; the physisorbed protein film is taken out-of-equilibrium by the electric field, allowing its settlement into an new energetically favoured state, which is improbably reached by the “as deposited” layer. The new state could involve conformational changes of the anti-IgM at the secondary or tertiary structure level.

Secondary structure changes have been assessed by PM-IRRAS spectroscopy. The PM-IRRAS spectrum of the anti-IgM layer physisorbed on gold has been measured and compared to that of EF-cycled layers. 5 non-cycled samples and 5 EF-cycled samples have been prepared and analysed. The EF-cycling of the bio-functionalized PM-IRRAS substrate is performed as for the KPFM samples, previously described. Figure 5.8a shows two representative PM-IRRAS spectra of the non-cycled anti-IgM layer and of the EF-cycled one. Both spectra comprise the same spectral features: amide I band ($\sim 1670\text{ cm}^{-1}$) the amide II band ($\sim 1540\text{ cm}^{-1}$), the amide A band ($\sim 3300\text{ cm}^{-1}$) typical of IR spectrum of proteins. All the measured spectra have been analysed looking at their second derivative (see section 3.5.1). Figure 5.8b features the second-derivative spectra of non-cycled sample (blue) and EF-cycled samples (red) in the amide I and amide II spectral range. The minima of the second derivative identify the secondary structure contribution to amide I and amide II bands. Specifically, two main components are resolved in the amide I range, at 1647 cm^{-1} and 1693 cm^{-1} , ascribed to β -sheets, whereas three contributions are resolved in the amide II range, at 1517 cm^{-1} , ascribed to β -sheets, and at 1540 cm^{-1} and 1557 cm^{-1} ascribed to unordered/ α -helix/turns. The second-derivative spectra feature a slight reduction of the intensity of all three β -sheets components in the EF-cycled samples, marked by the arrows in figure 5.8b. A possible interpretation of this difference concerns the average orientation of β -sheets in the anti-IgM layer. Indeed, an higher tilt angle of the β -sheets with respect to the normal direction to the substrate corresponds to a decreased PM-IRRAS intensity. The antibodies may undergo a spatial rearrangement on the substrate upon EF-cycling, leading to an average orientation of β -sheets lying closer to the surface. This hypothesis is consistent with the observed tendency of β -sheets in physisorbed proteins to have high tilt angles, being oriented toward the gold surface, demonstrated in the previous section. Accordingly, the EF-cycling could further favour this prevalent orientation.

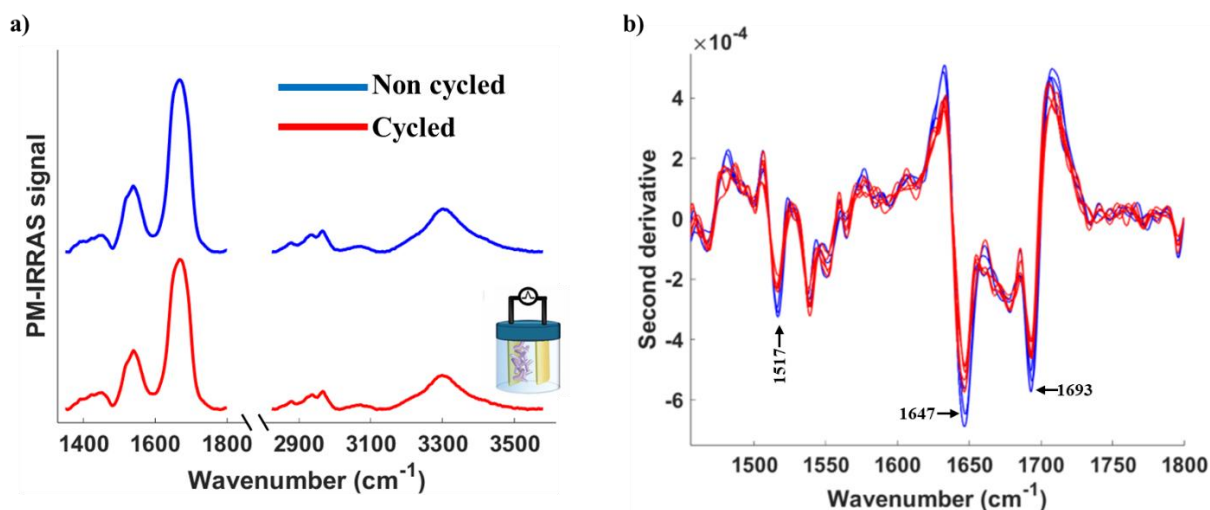


Figure 5.8. PM-IRRAS spectra of anti-IgM upon EF-cycling. **a)** Representative PM-IRRAS spectra of a non-cycled anti-IgM layer (blue) and a EF-cycled one (red), featuring the amide I band ($\sim 1670\text{ cm}^{-1}$), amide II band ($\sim 1540\text{ cm}^{-1}$), amide A band ($\sim 3300\text{ cm}^{-1}$). Also the peaks ascribed to CH stretching vibrations within the proteins appear around $2850\text{--}2950\text{ cm}^{-1}$. **b)** Second derivative spectra of the PM-IRRAS spectra on non-cycled anti-IgM layer (blue) and a EF-cycled one (red), featuring the main secondary structure components under amide I and amide II marked with the black arrows.

The amide I of the PM-IRRAS spectra is further analysed through fit-deconvolution, using a fit-model including 5 gaussian functions accounting for the various secondary structure contribution. Two components are clearly identified by the second derivative spectrum at 1647 cm^{-1} and 1693 cm^{-1} . Other two components are included to account for second-derivative profile in the $1660\text{--}1680\text{ cm}^{-1}$ range, which is consistent with the presence of two not resolved bands. The fifth component is added to account for the PM-IRRAS spectral intensity below 1620 cm^{-1} which is otherwise not accurately predicted by the fit. The following bands are identified by the fit: side-chains (1610 cm^{-1}), intramolecular β -sheets ($1625 \pm 1\text{ cm}^{-1}$), β -sheets ($1648 \pm 1\text{ cm}^{-1}$ and $1691 \pm 1\text{ cm}^{-1}$) and turns ($1670 \pm 1\text{ cm}^{-1}$). For the analysis of secondary structure, the histograms of the PM-IRRAS spectral content derived from the percentage area of the amide I components of non-cycled and EF-cycled samples are compared, in figure 5.9. There is no significant difference between the secondary structure composition of the two set of samples, suggesting that the EF-cycling does not induce the unfolding of anti-IgM antibodies. Moreover, no further spectral differences are observed in other spectral ranges.

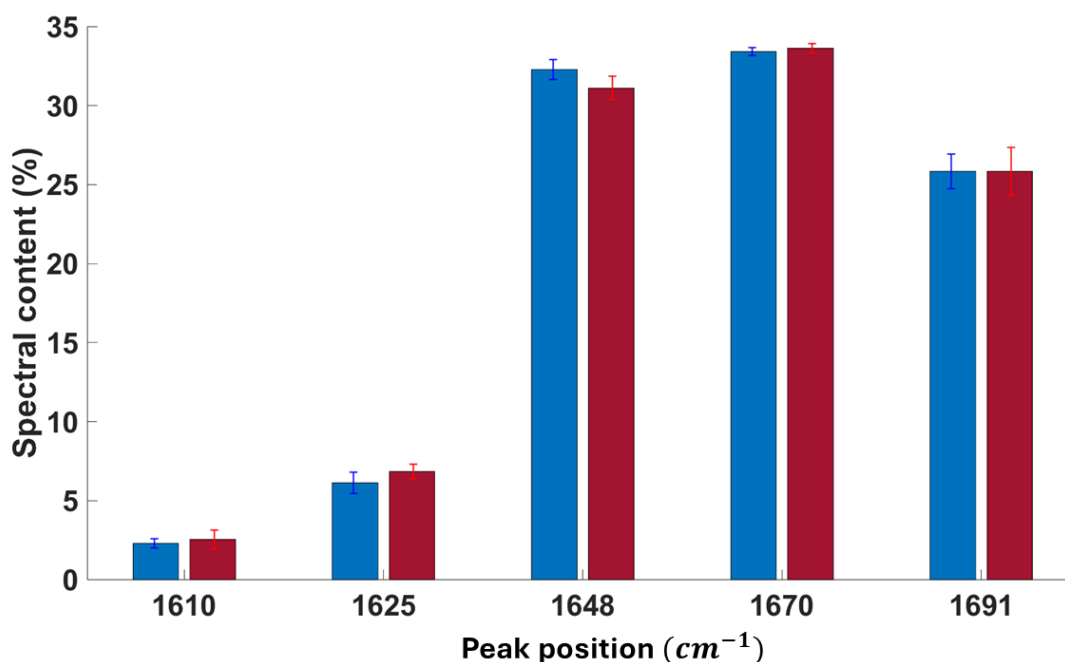


Figure 5.9. Secondary structure analysis of anti-IgM upon EF-cycling. Secondary structure content of the amide I band, assessed through fit-deconvolution, for non-cycled (blue) and EF-cycled (red) samples. The bars represent the percentage area of the secondary structure components under amide I with respect to the total band area, averaged over 5 samples.

The physical adsorption of antibodies on gold is regulated by the interplay of kinetic and thermodynamic factors, which lead to the formation of a monolayer of densely packed antibodies ($\sim 10^{11}$ - 10^{12} cm^{-2}) corresponding to minimum of the Gibbs free energy. However, the free energy landscape in the conformational space of a protein film has a rough and complex structure, presenting several relative minima, each one corresponding to a possible arrangement of the as-deposited film, characterized by its own dielectric properties. Thus, a rather large dispersion of the film polarization results because of the large number of possible metastable relative minima of free energy. This has been assessed through the measurements of surface potential, threshold voltages and ζ -potential, which have shown a $\sim 33\%$ dispersion around the average values. Moreover, it is a known fact that proteins live in metastable states and are subjects to unfolding processes upon interactions with other molecules or upon chemical and physical stimuli. Because of the interaction with the surface, some proteins in the as-deposited film could be trapped into less stable states, also involving conformational changes. The EF-cycling serves as an external stimulus releasing enough energy into the system to take the less stably adsorbed proteins into another lower free-energy minimum. In this way, the film undergoes multiple pathways in the free energy landscapes, upon EF-annealing, finally ending in a lower minimum corresponding to a more stable configuration. Figure 5.10 schematically depicts this process. The PM-IRRAS measurements show that the final state does not involve a significant

unfolding of the antibodies in the film. However, the second derivative of the PM-IRRAS spectra reveal that β -sheet take part to the transition, possibly reaching a higher degree of order, laying closer to the substrate.

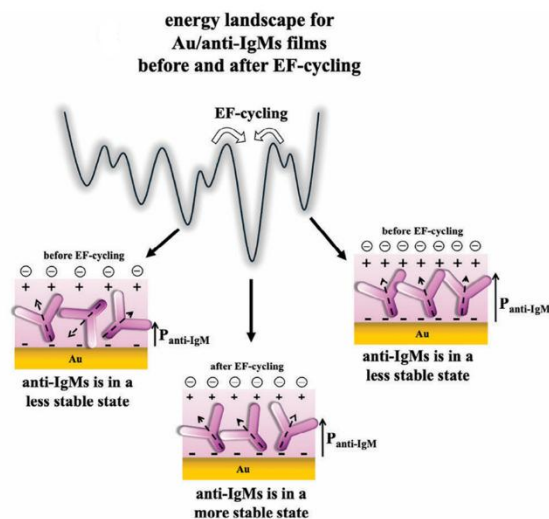


Figure 5.10. Free-energy landscape of the physisorbed protein layer. Schematic representation of the Gibbs free energy landscape for a layer of physisorbed antibodies. EF-cycling acts as an annealing process inducing the transition of the antibody layer into a more stable and energetically favoured state, corresponding to a lower relative minimum. Taken from [61]

5.3 Effect of antigen affinity binding and pH conditioning of the biolayer

Interfaces made of physisorbed antibodies on gold surfaces are implemented as sensing elements in biosensors for antigen detection (see chapter 1), reaching sensitivity at the zepto-molar level (10^{-20} – 10^{-21} M). The high-performance of those devices leverage the changes in the electrostatic configuration of the biolayer upon antibody-antigen affinity bindings.²⁰ The mechanism underlying those modifications is not fully understood and could involve conformational changes of the capturing antibodies, extended across the biolayer, over $\sim(10\text{ }\mu\text{m})^2$ areas. It is known that the antibody-antigen binding triggers allosteric effects, possibly inducing conformational changes or a redistribution of charges within the protein structure.^{61,65,107} However, the understanding of extended structural variation involving multiple proteins and collective electrostatic phenomena propagating over large areas remain elusive.

Here, PM-IRRAS spectroscopy has been utilized to inspect variation of the secondary structure of films of physisorbed antibodies upon binding with antigens.⁴⁷ The assayed sensing interface comprises a monolayer of human Immunoglobulin G antibodies (anti-IgG) physisorbed onto a gold PM-IRRAS substrate, which selectively bind IgG affinity antigens. The bio-functionalization protocol of the PM-IRRAS substrate (described in section 3.1) leads to a protein monolayer having density of 10^{11} - 10^{12} antibodies cm^{-2} , measured by SPR, corresponding to a protein each $\sim(10\text{ nm})^2$. Hence, a PM-IRRAS substrate, 12.5 cm^2 wide, encompasses about $(10^{12}$ - $10^{13})$ anti-IgG antibodies. Two binding regimes can be distinguished concerning the number of targeted antigens with respect to the total number of receptors, namely the “double layer” regime and the “few bindings” regime, schematically depicted in figure 5.11. The former refers to the formation of a high number of antibody-antigen complex, which produce a double-layer of antigens on top of the receptor layer. It is realized by exposure of the bio-functionalized surface to high concentration of analyte, above the nM level. Antigen detection in this regime is facilitated as the detected signal is mainly arising from the increment of mass onto the sensing surface. On the other hand, the few binding regimes refers to very few bound antigens, order of magnitude less numerous than receptors, occurring upon exposure of the sensing interface to extremely diluted analyte solution. It is the regime of operation of the SiMoT biosensors or of the graphene-based biosensor described in chapter 1 and 4 respectively, which detect 1-10 antigens using millimetre-wide bio-functionalized interfaces. In this case, the detection cannot be simply ascribed to an increase of mass onto the sensing surface, as the impact of few antigens is vanishing compared to 10^{12} mostly unbound receptors, rather the occurrence of a

propagation effect triggered by the binding sites and extending throughout a large area must be assumed, which could also involve modification of the structure of multiple proteins.

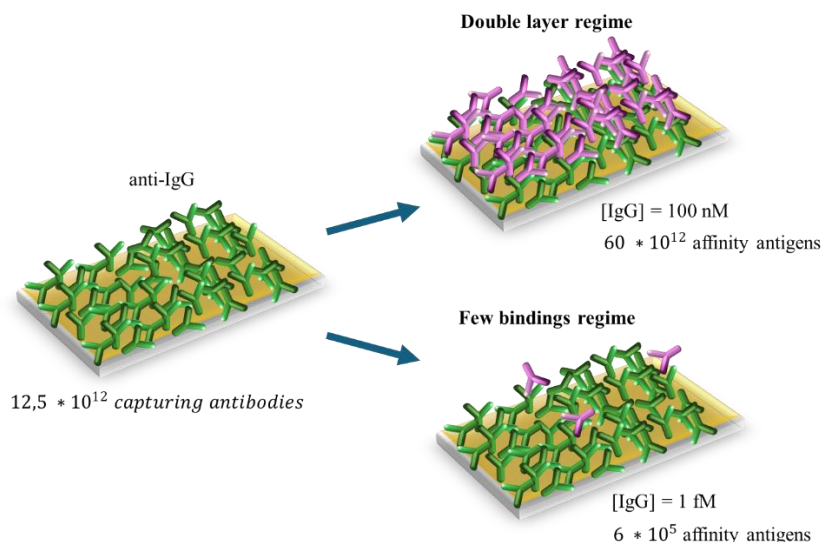


Figure 5.11. Sensing regimes. Schematic description of the double-layer and few-bindings regimes. The PM-IRRAS substrate bio-functionalized with a monolayer of physisorbed anti-IgG is represented on the left. About $12.5 \cdot 10^{12}$ antibodies are immobilized on the surface. In the double-layer regime, the substrate is exposed to high concentration of IgG solution resulting in the formation of an additional antigen layer on top of the anti-IgG ones. This is accomplished by incubating the PM-IRRAS substrate with an IgG solution at 100 nM encompassing $60 \cdot 10^{12}$ antigens. In the few-bindings regime the number of bound antigens is order of magnitude lower than the number of unbound receptors. This is the case obtained by incubating the PM-IRRAS substrate with a IgG solution at 1 fM encompassing $6 \cdot 10^5$ antigens.

Figure 5.12 reports the PM-IRRAS spectra of the anti-IgG sample and that of an analogous sample incubated for 10 minutes with the 100 nM standard solution of IgG antigens at a concentration of 100 nM, in the amide I amide II spectral range. Both anti-IgG and IgG are proteins sharing the typical Y-scape of immunoglobulins, thus characterized by the same IR absorption features. Accordingly, the two spectra feature the same lineshape and the one of the IgG/anti-IgG sample features a 40% increased amide I band, with respect to that of the simple anti-IgG layer, ascribed to the presence of many more absorber on the substrates.

The case of interest for the assessment of structural modifications within the protein film is the few-binding regime. Accordingly, a PM-IRRAS spectroscopy experiment has been conducted to perform a comparative analysis of the spectrum of a monolayer of physisorbed anti-IgG, with respect to that of a monolayer of physisorbed anti-IgG after the incubation with a 1mL IgG solution at 1fM

concentration, encompassing about 10^5 antigens. A set of 54 anti-IgG bio-functionalized gold substrate was prepared and divided into three groups: 18 samples were analysed as-prepared (baselines); 18 samples were incubated with a 1 fM solution of non-binding IgM (negative controls); and 18 samples were incubated with 1 fM solution of affinity IgG (sensing).

To enhance the sensitivity of the layer of capturing antibodies and render it more responsive for antigen detection at low concentrations, the anti-IgG film was conditioned through the exposure of 10 minutes to a more acidic (pH=6) or more alkaline (pH=8) buffer solution with respect to the physiological one utilized for the functionalization and incubation steps (pH=7.4). Indeed, it has been demonstrated that conditioning the biolayer with a pH shift improves the sensing performance of biosensors.⁴⁷ This strategy has been utilized to reach a limit of detection to 10 zM with SiMoT biosensors (corresponding to detecting a single molecule in 100 μ L). The same detection performance has been achieved also by SPR and KPFM biosensors, using pH-conditioned sensing interfaces unveiling the crucial role of pH.

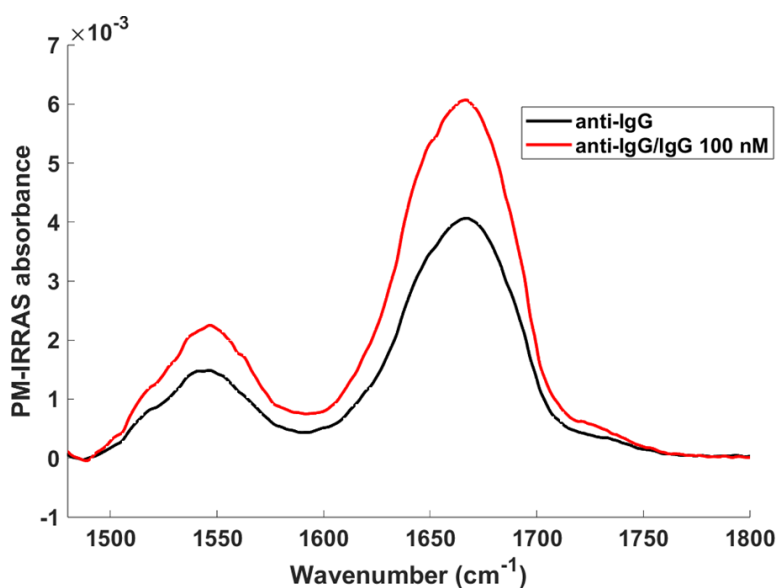


Figure 5.12. PM-IRRAS spectra of the double layer anti-IgG/IgG. PM-IRRAS spectra of the monolayer of anti-IgG physisorbed on gold (black). It features the amide I band ($\sim 1670 \text{ cm}^{-1}$) and the amide II band ($\sim 1540 \text{ cm}^{-1}$). The PM-IRRAS spectra of the double-layer anti-IgG/IgG sample (red), obtained by incubating the anti-IgG biolayer with a IgG solution at 100 nM.

Figure 5.13 depicts the experimental protocol for the preparation of the 54 samples for the PM-IRRAS experiment. The anti-IgG biolayer, physisorbed on gold, is incubated for 30 minutes with a HEPES buffer solution at pH=6 or at pH=8, for pH-conditioning or at pH=7.4 for not conditioned samples. Then the pH is restored by incubation of the biolayer into a HEPES buffer solution at physiological

pH=7.4 for 10 minutes. Then baseline, negative control and sensing samples are prepared as summarized in table 5.3.

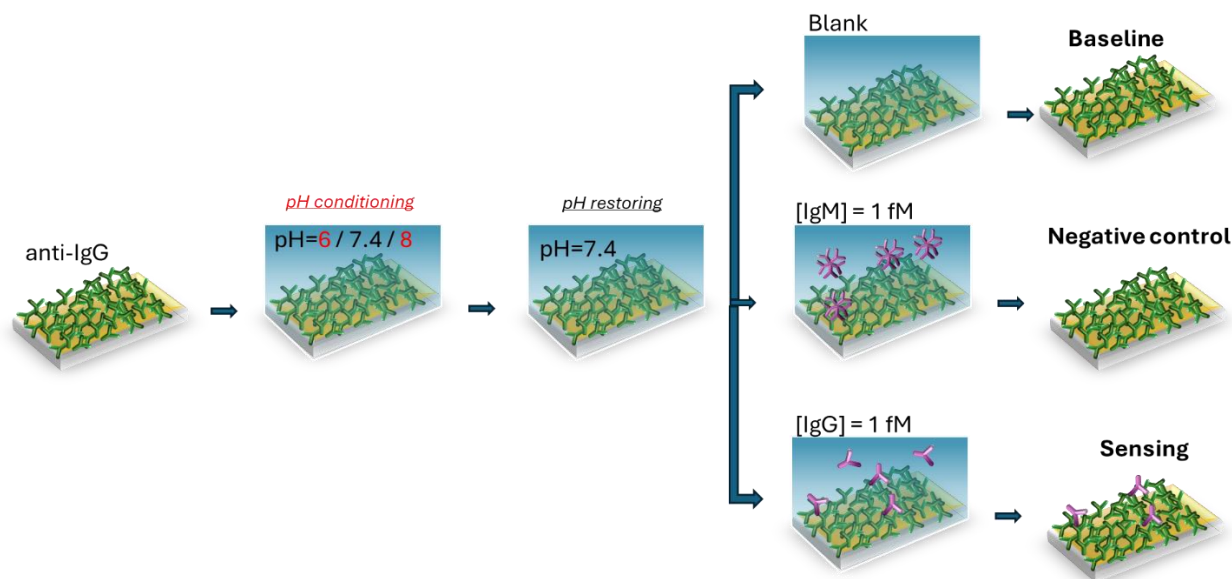


Figure 5.13. PM-IRRAS spectra of the double layer anti-IgG/IgG. Schematic description of the protocol for the preparation of the 54 PM-IRRAS samples, divided in baseline, negative control and sensing. Each group is further divided in non-conditioned (pH=7.4), and pH-conditioned (pH=6 or pH=8) samples.

Table 5.5 PM-IRRAS samples. Summary of the baseline, negative control and sensing PM-IRRAS 54 spectra acquired after the conditioning at pH 6, at pH 8 and with no conditioning

	Baseline (anti-IgG)	Negative control (IgM)	Sensing (IgG)
<i>Non-conditioned (pH=7.4)</i>	5	6	6
<i>Conditioned pH=6</i>	9	6	6
<i>Conditioned pH=8</i>	4	6	6

The PM-IIRAS spectrum of each sample was acquired and analysed in the amide I amide II spectral range between 1450 and 1750 cm^{-1} .

First, the effect of the pH conditioning is assessed. Figure 5.14 features the average PM-IRRAS spectra, obtained for the non-conditioned and conditioned (at pH=6 and pH=8) groups. The amide I vibrational mode is expected to be sensitive to protonation state and to H-bonds involving the carboxyl groups of proteins (see section 2.1.2), whereas the amide II bands is much less sensitive to pH changes. Therefore, amide II it is used as internal reference, and the spectra are normalized with respect to its area. The PM-IRRAS spectra does not show any significant difference between non-

conditioned and pH-conditioned samples, providing that the effect of the pH-shift experienced by the biolayer does not perturb the protein structure and any protonation/deprotonation changes is eventually restored when antibodies are taken back at the physiological pH=7.4.

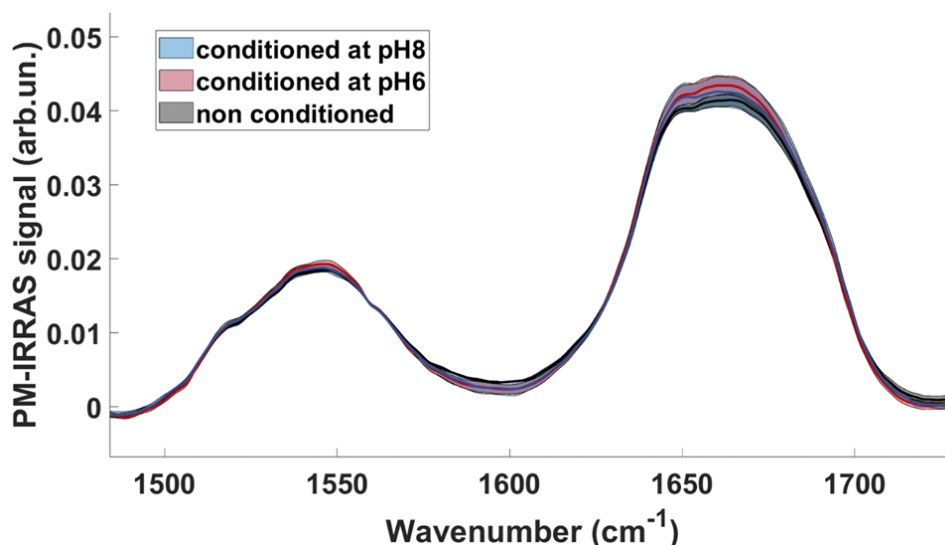


Figure 5.14. PM-IRRAS spectra of non-conditioned and pH-conditioned biolayer. The non-conditioned (black-line) and pH-conditioned biolayers (pH=6, red-lines, pH=8, blue lines) are shown for the anti-IgG biolayer. The lines are the averages while coloured shadings represent one standard deviation over 18 samples. Taken from [45]

Given the quite large set of 54 spectra, the comparative analysis of baseline, negative controls and sensing spectra is performed through a principal component analysis (PCA) (see section 3.5.4). Figure 5.15 reports the result of the PCA. The score plot (figure 5.15a) features the whole dataset, representing each spectrum as a point having (PC1, PC2) coordinates. The first two PCs, accounting for the 98.9 % of the total variance. The physical meaning of the PCs is interpreted looking at the loading plot (figure 5.15b). The loading on PC1 is always positive, following the profile of the PM-IRRAS signal. This means that PC1, which explains most of the dataset variance (95.1%) describes the overall variability of the spectral intensity among spectra. On the other hand, the loadings on PC2 identify two spectral regions in the amide I range, which mainly contribute to the remaining 3.8% variance, around 1640 cm^{-1} and 1670 cm^{-1} .

Notably, baseline spectra (squares) and negative control spectra (crosses) are all clustered in the first and second quarters of the score plot, at positive values of PC2. On the other hand, sensing spectra (circles) are less clustered and located at negative PC2 values. In other words, baseline and negative control spectra are much more reproducible than the sensing ones. Also, the gray line in the score plot suggests a possible border separating the sensing spectra from the other two groups, which corresponds to the evidence that sensing spectra are characterized by an increased PM-IRRAS intensity in the

range between 1660 cm^{-1} and 1680 cm^{-1} . To visualize the difference three spectra have been selected, namely sample S1 for the baseline, sample S2 for the negative control and sample S3 for the sensing group (highlighted in the score plot) and reported in figure 5.15c. The baseline and negative control spectra are perfectly overlapping, whereas the amide I area of the sensing is increased of about 8% with respect to that of the other two spectra. The variation is explicated by the difference spectrum (dotted line) computed between the sensing and the negative control spectra.

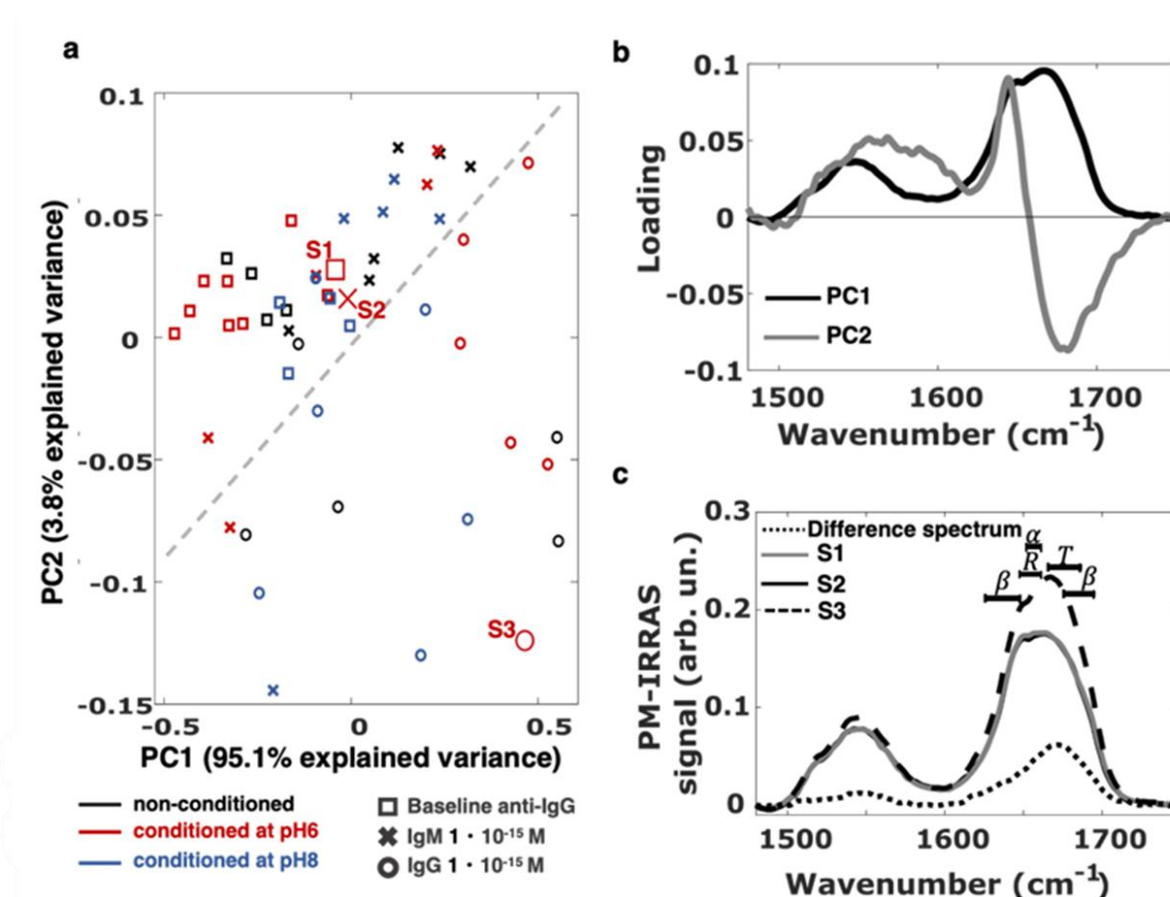


Figure 5.15. PCA of the PM-IRRAS spectra of anti-IgG. **a)** Score-Plot evaluated for the two principal components PC1 and PC2, featuring 17 baseline spectra (squares), 17 negative control spectra (crosses) and 17 sensing experiment spectra (circles). The different colours identify different pH conditioning: pH=6 (red) pH=8 (blue) and non-conditioned (black) **b)** Loading plot, showing the loadings of each original spectral frequency in the whole inspected range ($1480 - 1750\text{ cm}^{-1}$) on PC1 and PC2. **c)** Spectra S1 (baseline), S2 (negative control), S3 (sensing) in the Score-Plot. The black dotted line refers to the difference spectrum obtained as the sensing S3 spectrum subtracted by the negative control S2 spectrum. Taken from [45]

The amide I intensity at frequency in the range $1660\text{--}1680\text{ cm}^{-1}$ is influenced mainly by unordered or turns structures. The increased PM-IRRAS signal can be related to unfolding processes or to reorientation of portion of the antibodies.

To provide a picture of the mechanism underlying the amplified response of biosensors operating at the few-binding regimes, it is necessary to add pieces of information from other experiments. Relevant results have been obtained assessing the degree of hydrophobicity of the antibody layer before and after the binding of few antigens, by measuring the static contact angle (SCA) of samples. The role of pH has been inspected as well in this case. Measurements are performed on a 1 cm² wide gold surface, bio-functionalized with physisorbed anti-IgG. Figure 5.16 shows the SCA results of a triplicate experiment. The anti-IgG biolayer start with a certain hydrophilicity, corresponding to a SCA of about 37°. The pH-conditioning does not influence this value significantly. Also, it stays quite constant upon negative control with the incubation of the sample in a 1fM solution of non-binding IgM. Strikingly, a large variation toward a more hydrophobic character is observed for the sample conditioned at pH=6, after incubation with 100μL solution of affinity IgG antigens at 10 zM concentration. The SCA increases of 18°±7°, meaning that a single antigen binding a surface encompassing 10¹² antibodies modify the degree of hydrophobicity of the biolayer. The same behaviour is not observed for non-conditioned and conditioned at pH=8 samples, suggesting that the acidic environment play a crucial role in this phenomenon.

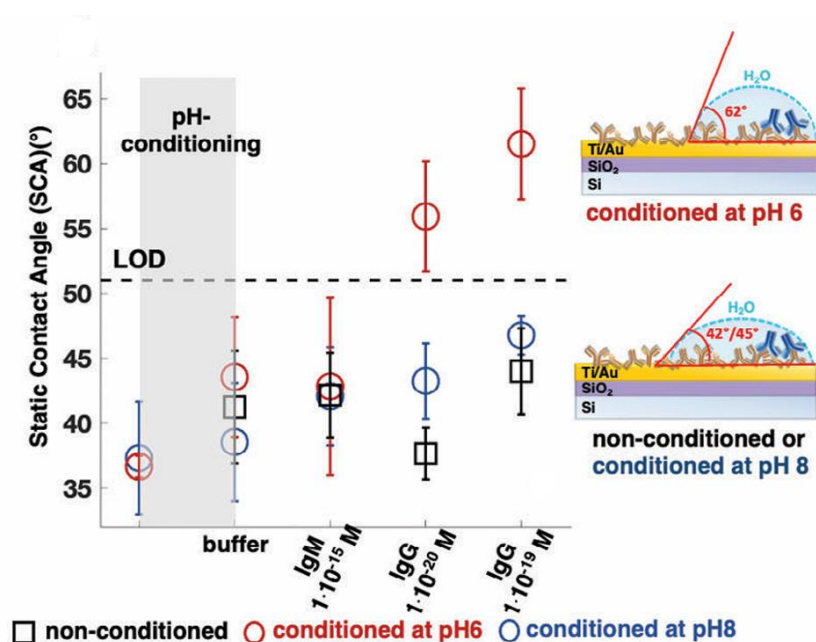


Figure 5.16. Hydrophobic interactions. Average static contact angles (SCA) measured at each sensing step on non-conditioned (black squares), conditioned at pH6 (red circles), or at pH8 (blue circles) anti-IgG biolayers. The first data points (from the left) are relevant to the pristine as deposited anti-IgG layers. Taken from [45]

An additional piece of information comes from the investigation of the nanomechanical properties of the biolayer, through a AFM-Force spectroscopy, which provides the Young's modulus and adhesion force of the biolayer before and after exposure to few affinity antigens. The AFM-Force spectroscopy measurements demonstrate a decrease of 37 ± 8 % of the Young's modulus and an increase of 66 ± 12 % of the adhesion force of the anti-IgG layer, after the incubation antigens (the ratio #antigens/#antibodies was 10^6 in this case). Those measurements are performed rinsing the biolayer with water (pH=5.5) before the incubation step therefore, the biolayer undergoes an acidic pH conditioning in this case too. The AFM results agree with those from the SCA, as hydrophobic surfaces are known to be stickier (increased adhesion) and less stiff (reduced Young's modulus).

A phenomenological mechanism of the surface modification occurring within the biolayer upon single/few antigen bindings is possible. The small variation occurring in the amide I band of PM-IRRAS spectra of anti-IgG biolayer upon antigen binding could be related to a conformational change involving a network of antibodies, leading to the exposure of the hydrophobic residues of the proteins, typically buried within the interior of their structure. Similar processes have been observed for other protein systems, suggesting that partially unfolded proteins undergo self-propagating aggregation, characterized by minor changes in the secondary structure and significant tertiary structure variations. Physisorption leads to a biolayer of partially misfolded proteins, due to the interaction with the substrate and to lateral interaction with other proteins. The hydrophobic forces drive the adsorption on the surface, favouring the aggregation of antibodies through a network of interactions. When antigen binding occurs, for the non-conditioned biolayer, the very local structural variations induced by the binding may propagate through this network affecting the conformation of multiple proteins on the substrate. When the biolayer is previously pH-conditioned this effect is amplified. This is because the pH-shift temporarily take the protein in non-physiological condition, therefore inducing charge redistributions and partial unfolding which further favouring the aggregation process within the film. When a single antibody-antigens binding occurs, it acts as a trigger for a further rearrangement of the network of antibodies which aggregates into a more hydrophobic layer.

Chapter 6

Conclusion and perspectives

Proteins represent the intricate balance of structural complexity and functional specificity. Their key role in countless biological processes is due to their dynamic nature dictated by the interactions with the surrounding environment and other biomolecules. This complexity comes with an inherent instability. Pathologies such as Alzheimer's and Parkinson's neurodegenerative diseases arise from protein misfolding and aggregation, involving a transition into more stable yet functionally detrimental states.^{57,108} Understanding the nature of conformational changes in proteins offers valuable insights into the fundamental principles governing their stability and function. The mechanisms involved in structural variation can be harnessed, in controlled manner, to develop biomedical technologies. The ability of proteins to bind ligands, catalyse reactions and undergo structural transition, makes them highly attractive for analytical applications. "Single-Molecule with a large Transistor– SiMoT" is a relevant example of an advanced an electronic point-of-care (PoC) platform, that it is now at the pre-clinical stage, exploiting the high sensitivity and selectivity of antibodies to target specific antigens.^{1,2,7} The integration of capturing antibodies through physisorption onto gold surface enabled detection performance with the single-molecule sensitivity. The extreme sensitivity of SiMoT biosensor is ascribed to a collective response of the biolayer of capturing antibodies upon binding of a single antigen.

Within this thesis, the properties of monolayers of antibodies physisorbed on gold have been exploited to develop a novel graphene-based biosensing platform. Proof-of-concept label-free detection of IgM is reported. The developed biosensor reached a limit of detection at 100 zM (10^{-19} M), leveraging on the coupling of a large-area bio-functionalized interface with anti-IgM with a bare graphene electrode

via a water-soaked paper strip. The possibility of directly measure changes in graphene phonon frequencies through Raman spectroscopy, modulated by surface potential variation on the bio-functionalized interface, underscores the unique transduction mechanism for rapid optical biomarker detection. Our device surpasses the performance of existing graphene-based biosensors. Moreover, the growing availability of portable Raman spectrometers offers perspectives for future development of compact graphene-based platforms as advanced and high-performance diagnostic tools.

The IR spectroscopy investigation of the biolayer of highly packed proteins highlights the crucial role of β -sheets upon physisorption. Via a comparative analysis of experimental PM-IRRAS and simulated IRRAS spectra we provide compelling evidence that the organization of proteins physisorbed on gold is not random, particularly regarding their secondary structure. Analysis of anti-IgG and BSA biolayer show that β -sheets adopt a partially tilted orientation toward the substrate, suggesting an energetically favourable configuration which enhance the stability of the film. This arrangement may also improve the overall functionality of the protein films serving as bio-interfaces of recognition element in biosensors.

Moreover, a systematic analysis of the dielectric properties of antibodies, anti-IgG, within the biolayer has been conducted to assess the effect of an electric field cycling. Given that partial unfolding occurs during anti-IgG physisorption, some molecules become trapped on less stable conformational states corresponding to local minima in the free-energy landscape. Our findings suggest that electric field cycling acts as an annealing process, guiding proteins toward more stable conformational states, not readily accessible for the as-deposited film.

Additionally, data from potentiometric and surface sensitive techniques shed light on an amplification effect driven by the self-propagation of amorphous aggregates of partially misfolded proteins. This phenomenon is influenced by pH conditioning and affinity binding. PM-IRRAS analysis, coupled to static contact angle and AFM-force spectroscopy measurements, confirms that while the secondary structure remains largely unaffected by pH shift, it significantly impacts on the tertiary structure of the proteins. This discovery has profound implications for understanding of complex free-energy landscape of metastable states, opening new perspectives for the development of advanced biomaterials with potential applications in biosensing technologies.

While these findings provide important insights on the stability and function of physisorbed protein biolayers, further investigation are necessary. A key focus for future research is the partial misfolding of immunoglobulins upon physisorption. Distinguishing the influence of substrate from that of neighbouring proteins in determining the final biolayer configuration could be a crucial challenge.

Also, exploring different proteins physisorbed on conductive and non-conductive substrates may offer valuable insight about those biological interfaces.

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Appendix

A. Kelvin probe force microscopy

Kelvin probe force microscopy is a variant of the atomic-force-microscopy (AFM) used to assess the local surface potential of samples with nanometre resolution. It leverages the contact potential difference (CPD) between the metallized AFM tip and the sample to probe the surface potential contribution of the work function.⁴⁶ Specifically, the CPD equals the difference between the work function of the sample (W_s) with respect to that of the tip (W_{tip}), $CPD = -\frac{1}{e}(W_s - W_{tip})$. Since KPFM measured the CNP, it does not provide an absolute measure of W_s , however it is a powerful analytical technique to monitor work function variations with high spatial resolution.

KPFM measurements were performed by an AFM system (NTEGRA Spectra, NT-MDT, Moscow Russia) equipped with a platinum-iridium coated tip, having an apex size of 35 nm. The measurement is based on a double scan of the sample surface. First, the sample morphology is recorded in a semi-contact mode during the first scan. Then, the tip is lifted at distance $h=250$ nm from the surface and the CPD is measured in the second scan. To measure the CPD the second scan is performed by exiting the tip electrically through the application of an electrostatic potential $V_{tip} = V_{DC} + V_{AC} \sin(2\pi ft)$ between the tip and the sample, as shown in figure A1. The applied voltage is the superposition of a DC component and an AC component, oscillating with a frequency matching the resonance frequency of the AFM cantilever. Because of the CPD between the tip and the sample, their surfaces are charged and act like the plate of a capacitor, vibrating upon electronic excitation according to the following equation

$$F = -\frac{dC}{dz}(V_{DC} - CPD)V_{AC} \sin(2\pi ft) \quad (A.1)$$

where F is the force felt by the tip, C is the equivalent capacitance and z is the tip-sample distance. According to equation A.1, the oscillation is modulated by the DC term. When $V_{DC} = CPD$ the force is nullified. The AFM electronic feedback adjust V_{DC} to nullify the force in every position during the second scan and return the corresponding values of CPD.

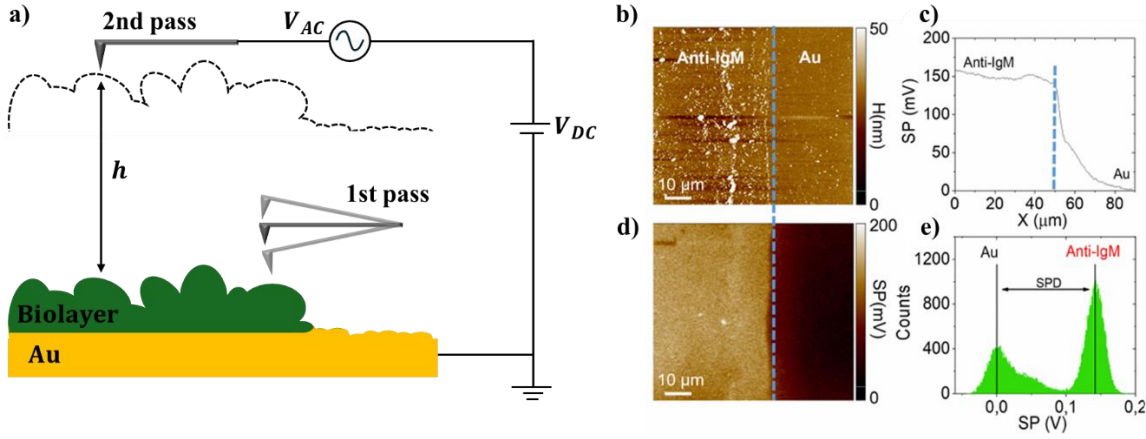


Figure A.1. Schematic of the 2-pass mode KPFM. **a)** Each line is scanned two times: first the morphology is recorded in semi-contact mode, upon mechanical excitation of the tip ; then the CPD is acquired, keeping a constant tip-sample distance $h=250$ nm during the second scan, exiting the tip through an electronic potential $V_{tip} = V_{DC} + V_{AC} \sin(2\pi ft)$. **b)** representative morphology of a patterned sample with anti-IgM (left) and bare Au (right) **c)** KP image of the sample in panel b) **d)** Average SP profile **e)** Histograms of the SP distributions. The dashed blue line marks the protein/gold interface.

In this way high resolution images of the CPD of the sample are obtained, which are interpreted as images sample work function relative to that of the tip.

In general, the work function of a material is defined as the difference between its fermi energy (E_F) and the vacuum level in proximity of the surface (E_V). It arises from the sum of two contributions

$$W = \phi_B + \phi_s \quad (\text{A.2})$$

where $\phi_B = E_{V,\infty} - E_F$ is a bulk potential energy, characteristic of the material and defined by its bulk properties, being $E_{V,\infty}$ the vacuum energy at infinite distance from the surface), whereas $\phi_s = E_V - E_{V,\infty}$ is a surface potential energy, defined by the surface atomic layers and adsorbed species. An intrinsic contribution to ϕ_s arises from a layer of surface dipoles originated by the asymmetric electron distribution at the material surface, and additional contributions depend on the presence of deposited molecules such as contaminants, water from the ambient atmosphere or immobilized antibodies.

To assess the surface properties of protein monolayers, the surface potential ($SP = \phi_s/e$) of biofunctionalized Au electrode has been measured, in air. The bio-functionalized sample are prepared as described in chapter 3, to obtain a patterned sample exhibiting a sharp interface between the protein bio-functionalized area and the bare Au surface area. The KPFM image is acquired on a $90\text{ }\mu\text{m} \times 90\text{ }\mu\text{m}$ area centred at the interface so that each scan includes both the bare Au and protein coated surface. This approach has the advantage of having the bare Au surface constantly accessible as a reference standard. Figure A1b and A1d feature the morphology and the SP image of a representative patterned sample of anti-IgM physisorbed on gold. The KPFM images are analysed by extraction of the CPD values corresponding to the maxima of the CPD distributions for both the bare substrate and for the bio-functionalized area, namely CPD_{Au} and $CPD_{anti-IgM}$. Using CPD_{Au} as a zero reference, it is possible to evaluate the surface potential difference (SPD) between the bare Au and functionalized area

$$SPD = CPD_{protein} - CPD_{Au} = -(SP_{protein} - SP_{Au}) \quad (A.3)$$

which is the relevant quantity for physical interpretations, as it is intrinsically sensitive to the bilayer electrostatic properties.

B. Surface plasmon resonance (SPR)

Surface plasmon resonance is an analytical technique leveraging the generation of a surface plasmon on a metallic thin film to assess the refractive index variation in a nanometre-thin volume in contact with the surface.^{47,81} A schematic SPR apparatus, in the Kretschmann-Raether configuration, is represented in figure B.1. A SPR slide consists of an optical glass covered with a 50 nm Au layer onto a 2 nm Cr adhesion layer. It is allocated into the flow-cell of the SPR apparatus, placed on top of a prism. The flow-cell is filled with a liquid solution containing the sample molecules. A laser ($\lambda=670$ nm) illuminates the Au layer from the back, through the prism, with an incidence angle θ above the critical angle, and the reflected radiation is collected by a photodetector. The apparatus utilized for the measurements reported in this thesis (Multi-Parameter SPR -MP-SPR- Navi 200-L) utilizes two lasers to probe the sample at two locations. Upon incidence at supercritical angle, an evanescent wave is generated which extends for 300-400 nm into the flow-cell. Thin samples, adsorbed on the gold layer are probed by the evanescent field as they modify the reflectance of the system. Specifically, the reflected intensity is measured as a function of the incidence angle, as shown in figure B2a. The signal features a plasmon peak, with a minimum of the reflectance at $\theta = \theta_{SPR}$, corresponding to the generation of surface plasmon on the Au layer. The angle θ_{SPR} is highly sensitive to the refractive index of the surrounding environment probed by the evanescent field. Surface processes, such as the adsorption of proteins on gold, influence θ_{SPR} .

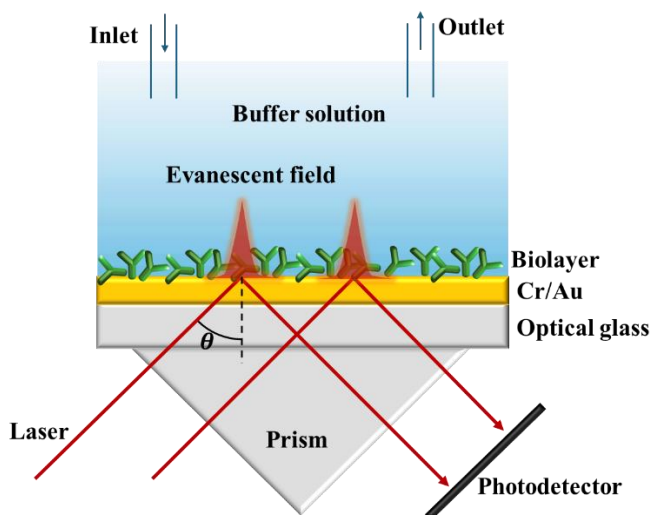


Figure B.1. SPR experimental setup. SPR instrumental setup in the Kretschmann configuration, including a prism, a SPR slide made of glass covered with Cr/Au 2/50 nm, two lasers ($\lambda = 670$ nm) incident on the Au layer at supercritical angles, generating two evanescent waves, and a photodetector. The thin film sample is adsorbed onto the Au surface. The SPR slide is placed in a flow-cell for in-situ real time measurements.

In a SPR measurement, variation of the refractive index of thin film adsorbed onto the SPR slide are probed through θ_{SPR} changes and correlated to the properties such as its thickness, refractive index and density. Figure B.2a features the plasmon peaks measured before (black) and 2.5 hours after (blue) the injection of anti-IgG molecules in the cell. The peaks are shifted of a quantity $\Delta\theta_{SPR}$ which is related to the formation of an adsorbed layer of anti-IgG on gold. The WinSpall 3.02 data analysis software is used to simulate the SPR curves and extract the relevant parameters (d , n_{∞} and k_{∞}).

The surface coverage of the species adsorbed onto the SPR slide is quantitatively assessed by means the de Feijter's equation

$$\Gamma = d (n - n_0) \left(\frac{dn}{dC} \right)^{-1} \quad (B1)$$

where Γ is the surface coverage expressed in ng cm^{-2} , d is the thickness of the deposited biolayer surface, and dn/dC is the refractive index increment of the adsorbed biolayer (taken from literature $dn/dC \approx 0.182 \text{ cm}^3 \text{ g}^{-1}$ for protein layers), and $n - n_0$ is the difference between the refractive index of the biolayer and that of the bulk medium, which is evaluated considering the sensitivity factor (k) of the apparatus to refractive index variation ($k = 1.16 \cdot 10^{-2} \text{ RIU deg}^{-1}$ at $\lambda=670 \text{ nm}$) as $(n - n_0) = \Delta\theta_{SPR} k$.

The flow-cell and the continuous recording of the reflectivity signal enables measurements of the sample *in-situ* and in *real-time*. Figure B.2b features the SPR sensogram ($\Delta\theta_{SPR}$ versus time) showing the adsorption dynamics.

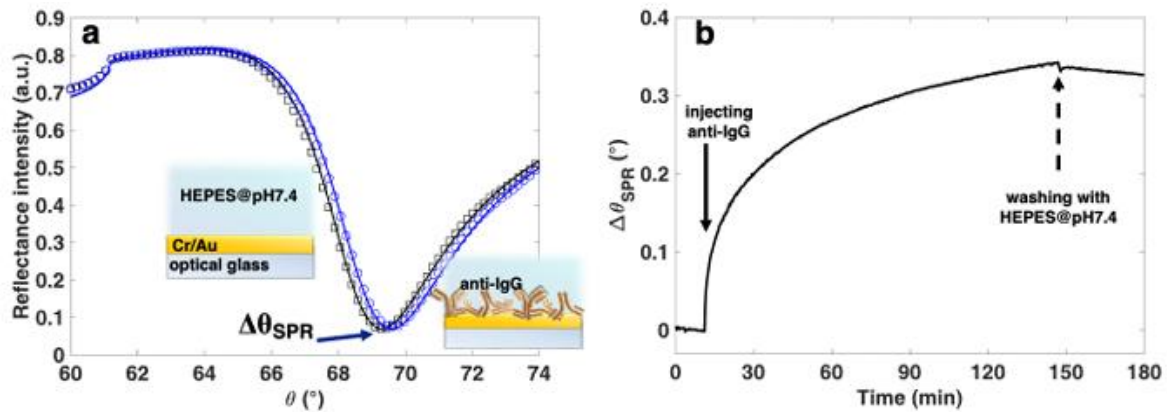


Figure B.2. SPR reflectance signal and sensogram. a) The plasmonic resonance dip for the bare gold SPR slide (black) and for the slide covered by the physisorbed anti-IgG (blue). The solid lines represent the best fit curves. **b)** Sensogram recorded simultaneously to monitor the angle variation of plasmon peak over time, providing information on the adsorption kinetics.

C. IR spectroscopy of thin films

Infrared (IR) spectroscopy represents a powerful analytical method to study the physico-chemical properties of molecular species; it provides insightful information for chemical identification, for the analysis of the structural arrangement of molecules and for studying the influence of external perturbations on samples, on the scale of molecular vibrational modes. Within this thesis, advanced IR spectroscopy approaches have been implemented for the optical characterization of the sensing interfaces constituted by a biolayer of proteins physisorbed on a gold substrate.

In general, an IR spectrum includes the molecular fingerprint of the investigated sample. IR radiation is absorbed at characteristic wavelengths, λ (or wavenumber, $\tilde{\nu} = 1/\lambda$) corresponding to specific normal vibrational modes. For a vibrational mode to be IR-active, it must induce a change in the corresponding *electric dipole moment* vector, $\mathbf{p}$, or in other word, it must result in a non-zero *dynamic dipole moment*, $\boldsymbol{\rho} = \partial \mathbf{p} / \partial q_i$ (where q_i are the normal coordinate of the given vibrational mode). Then, IR radiation is absorbed according to the Fermi's golden rule. The integral intensity of the corresponding absorption band (A), in the IR spectrum, is proportional to the probability of a transition between an initial vibrational state $|i\rangle$ and final state $|f\rangle$. This probability is provided by quantum mechanical calculation, in the framework of time-dependent perturbation theory, resulting proportional to the square of the matrix element of the Hamiltonian $H = -\mathbf{E} \cdot \mathbf{p}$, where $\mathbf{E}$ is the electric field vector. At the first-order approximation (dipole approximation) it results

$$A \propto E^2 | \langle i | \boldsymbol{\rho} | f \rangle |^2 \cos^2 \phi \quad (\text{C.1})$$

being ϕ the angle between $\mathbf{E}$ and $\boldsymbol{\rho}$. The quantity $| \langle i | \boldsymbol{\rho} | f \rangle |$ is the transition dipole moment, TDM associated to the $|i\rangle \rightarrow |f\rangle$ transition.

The microscopic description of the origin of IR absorption bands is essential to interpret experimental spectral features in terms of molecular properties. On the other hand, a macroscopic description is fundamental, as well, for the correct interpretation of the overall IR spectra. Indeed, optical effects specific of the optical geometry utilized to perform experiments could affect the position, intensities and shapes of the absorption bands. Those features depend on the optical constants of the sample and surrounding media, therefore, a theoretical model based on macroscopic Maxwell theory of electromagnetism is necessary, for the description of the interaction of IR radiation with the sample.

For ultrathin samples (thickness $d \ll \lambda$) adsorbed at an interface, the propagation of IR radiation is based on the Maxwell theory. For a linear system in absence of free electrical charges the Maxwell equations read:

$$\nabla \times \mathbf{E} = -\frac{\partial \mathbf{B}}{\partial t} \quad (\text{C.2a})$$

$$\nabla \cdot \mathbf{D} = 0 \quad (\text{C.2b})$$

$$\nabla \times \mathbf{H} = \hat{\sigma} \mathbf{E} + \frac{\partial \mathbf{D}}{\partial t} \quad (\text{C.2c})$$

$$\nabla \cdot \mathbf{B} = 0 \quad (\text{C.2d})$$

being $\mathbf{E}, \mathbf{H}, \mathbf{D} = \hat{\epsilon} \epsilon_0 \mathbf{E}$ and $\mathbf{B} = \hat{\mu} \mu_0 \mathbf{H}$ the electric field vector, the magnetic field vector, the electric displacement vector and the magnetic induction vector respectively, whereas $\hat{\epsilon}$, $\hat{\mu}$ and $\hat{\sigma}$ are the local dielectric function, the magnetic permeability and the electrical conductivity of the medium respectively; ϵ_0 and μ_0 are the permittivity and permeability of free space. In the optical frequency range $\hat{\mu}$ can be approximated equal to unity. Note that, the description in terms of macroscopic Maxwell equations 3.1 is possible considering the biolayer as a continuum medium whose thickness, though small compared to the wavelength, is large enough to define a local permittivity.

For a non-absorbing medium ($\hat{\sigma} = 0$), the solution of the wave equation for the electric field is a plane wave

$$\mathbf{E}(\mathbf{r}, t) = \mathbf{E}_0 e^{i(\omega t - \mathbf{k} \cdot \mathbf{r})} \quad (\text{C.3})$$

propagating with a *phase velocity* $v = \frac{c}{\sqrt{\hat{\epsilon} \hat{\mu}}}$. Here, c is the speed of light in vacuum equal to $\frac{1}{\sqrt{\epsilon_0 \mu_0}}$, ω is the radiation frequency, $\mathbf{r}$ is the position vector and $\mathbf{k}$ is the wavevector, satisfying relation $\mathbf{k}^2 = \frac{\omega^2}{c^2} \sqrt{\hat{\epsilon} \hat{\mu}}$. In this case, a real refractive index of the medium can be defined as $\hat{n} = \frac{c}{v} = \sqrt{\hat{\epsilon} \hat{\mu}}$.

On the other hand, in the case of an absorbing medium, with $\hat{\sigma} \neq 0$, the wave equation has solution if $\hat{\epsilon}$ and $\hat{n}$ become complex quantities:

$$\hat{\epsilon} = \epsilon' - i\epsilon'' \quad (\text{C.4})$$

$$\hat{n} = n - ik \quad (\text{C.5})$$

and the wave equation returns a plane wave with an exponentially decaying profile

$$\mathbf{E} = \mathbf{E}_0 e^{i\left(\omega t - \frac{2\pi}{\lambda} n \cdot \mathbf{r}\right)} e^{-\frac{2\pi}{\lambda} k \cdot \mathbf{r}}$$

being $\mathbf{s}$ the unit vector along the propagation direction. Here, n is the *real refractive index* (from now on called *refractive index*) whereas k is the *absorption index* of the medium. Those are related to the complex dielectric constant as

$$\epsilon' = n^2 - k^2 \quad (\text{C.6})$$

$$\epsilon'' = 2nk \quad (\text{C.7})$$

Also, the electric conductivity and the imaginary part of the dielectric function are related through the following equation

$$\epsilon'' = \frac{\sigma}{\epsilon_0 \omega} \quad (\text{C.8})$$

Equations 3.6 and 3.7 show that the optical properties of a medium are essentially defined by its complex dielectric function $\hat{\epsilon}$ in the relevant spectral range. To complete the picture, it is worth mentioning here two additional fundamental relations, between the real and imaginary part of the optical constants, called the Kramers–Kronig (KK) relations. For the complex refractive index, they are

$$n(\tilde{\nu}) = n_\infty + \frac{2}{\pi} P \int_0^\infty \frac{k(\tilde{\nu}') \tilde{\nu}'}{\tilde{\nu}'^2 - \tilde{\nu}^2} d\tilde{\nu}' \quad (\text{C.9})$$

$$k(\tilde{\nu}) = -\frac{2\tilde{\nu}}{\pi} P \int_0^\infty \frac{n(\tilde{\nu}') - n_\infty}{\tilde{\nu}'^2 - \tilde{\nu}^2} d\tilde{\nu}' \quad (\text{C.10})$$

KK-relations are extremely useful for the calculation of the optical parameters from experimental data.

During an IR spectroscopy experiment, the IR radiation interacts with the sample and is directed toward the detector. The measured spectral features result from the amount of detected radiation at each wavenumber. It depends on the optical properties of the media and on the geometry of the experiment. Specifically, the following description refers to experiment performed in reflection mode, where the detector records radiation reflected by an ultrathin sample, such as nm-thin adsorbed protein layer. Consider a system including three isotropic media: an external medium (1), the sample (2), and a substrate (3), each having its own set of complex optical constants ($\hat{\epsilon}_j = \hat{n}_j^2$ with $j=1,2,3$). Figure 3.1 schematize the three-layer system, with IR radiation incident at an angle θ_i on the sample.

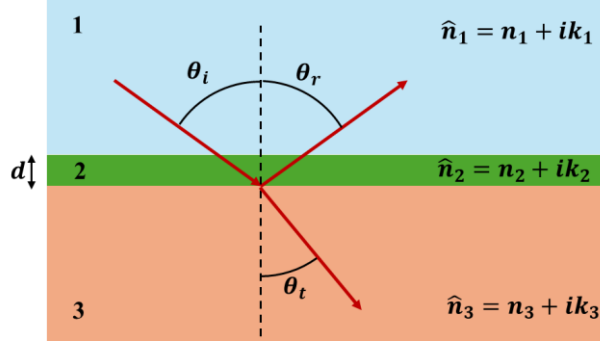


Figure C.1. Schematic representation of the three-layer system involved in an IR spectroscopy measurement. It includes the external medium (1), the sample (2), and a substrate (3). Each medium has its own set of complex optical constants ($\hat{n} = n - ik$). The IR radiation impinges on the sample at angle θ_i and is reflected and transmitted at angles θ_r and θ_t respectively. Multiple reflections and transmissions occur at the interfaces between media 1-2 and 2-3 however, only one reflected beam and one transmitted beam are depicted for the sake of clarity.

In the framework of Maxwell equation for the description of the electromagnetic wave, the reflected electric field at an interface between two media 1 and 2, is determined by the Fresnel coefficients. For absorbing media, they are

$$r_{12}^s = \frac{\xi_1 - \xi_2}{\xi_1 + \xi_2} \quad \text{and} \quad r_{12}^p = \frac{\hat{\epsilon}_2 \xi_1 - \hat{\epsilon}_1 \xi_2}{\hat{\epsilon}_2 \xi_1 + \hat{\epsilon}_1 \xi_2} \quad (\text{C.11})$$

with $\xi_j = \hat{n}_j \cos \varphi_j$ ($j = 1, 2$), for both s- and p-polarized radiation. For the three-layer system in 3.1, which includes the ultrathin sample sandwiched between two semi-infinite media, the Fresnel coefficient can be rewritten in the form:

$$r_{123}^s = \frac{r_{12}^s + r_{23}^s e^{-2i\beta}}{1 + r_{12}^s r_{23}^s e^{-2i\beta}} \quad \text{and} \quad r_{123}^p = \frac{r_{12}^p + r_{23}^p e^{-2i\beta}}{1 + r_{12}^p r_{23}^p e^{-2i\beta}} \quad (\text{C.12})$$

with $\beta = \frac{2\pi}{\lambda} d \sqrt{\hat{\epsilon}_2 - \hat{\epsilon}_1 \sin^2 \varphi_1}$.

Then, the *reflectance*, $R^{s,p}(d)$, is computed as $R_{123}^{s,p}(d) = |r_{123}^{s,p}|^2$. Noteworthy, it can be shown that the reflectance at the interface 1-3, in absence of the sample (2), is provided by equation 3.12 in the limit of $d \rightarrow 0$.

The relevant quantity, assessed in an IR reflection spectroscopy measurement is the *absorbance* (sometimes referred as *reflectance-absorbance*), defined as

$$A = -\log \left(\frac{R}{R_0} \right) \quad (\text{C.13})$$

being R the reflectance measured with the sample coated substrate, and R_0 the reflectance of the bare substrate. An exact analytical expression of the ratio R/R_0 is cumbersome and little informative about the physical meaning of the optical parameters entering the model, therefore the *normalized reflectivity* $\frac{\Delta R}{R} = \frac{R_0 - R}{R_0}$ is often considered, being also proportional to the absorbance as $A \approx \frac{\Delta R}{R} \frac{1}{\ln(10)}$.

For ultrathin layer ($d \ll \lambda$), the normalized reflectivity for s- and p- polarized radiation was derived by McIntyre and Aspens, in the case of a non-absorbing external medium. It is

$$\left(\frac{\Delta R}{R_0}\right)^S = \frac{8\pi d n_1 \cos(\theta_i)}{\lambda} \text{Im} \left[\frac{\hat{\epsilon}_2 - \hat{\epsilon}_3}{\hat{\epsilon}_1 - \hat{\epsilon}_3} \right] \quad (\text{C.14})$$

$$\left(\frac{\Delta R}{R_0}\right)^P = \frac{8\pi d n_1 \cos(\theta_i)}{\lambda} \text{Im} \left\{ \left[\frac{\hat{\epsilon}_2 - \hat{\epsilon}_3}{\hat{\epsilon}_1 - \hat{\epsilon}_3} \right] \left[\frac{1 - \hat{\epsilon}_1/\hat{\epsilon}_2 \hat{\epsilon}_3(\hat{\epsilon}_2 + \hat{\epsilon}_3) \sin^2(\theta_1)}{1 - (1/\hat{\epsilon}_3)(\hat{\epsilon}_1 + \hat{\epsilon}_3) \sin^2(\theta_1)} \right] \right\} \quad (\text{C.15})$$

Equations 3.14 and 3.15 constitute the theoretical background to describe the interaction of IR radiation with ultrathin samples in the framework of a macroscopic continuum model, essential for the understanding of experimental outputs from either external reflection or internal reflection spectroscopy experiments. In particular, this theoretical framework will be used to determine the optical constant of the protein biolayers from the measured IR spectra or, the other way round, to compute simulated spectra in a specific optical geometry, given complex dielectric function of the samples is known.

D. MATLAB codes

Analysis Raman spectrum of graphene

```
def GrapheneSpectraAnalysis(filename):

    import matplotlib.pyplot as plt
    import numpy as np
    import scipy.constants as pconst
    from scipy.optimize import curve_fit
    import math
    pi=math.pi

    #read data from file
    data = np.loadtxt(filename,delimiter=';',skiprows=3)
    x=[]; y=[];
    for i in range(len(data)):
        for j in range(2):
            if j==0:
                x.append(data[i][j])
            else:
                y.append(data[i][j])
    #create x y data for g-peak
    xG= []; yG=[]; x2D=[]; y2D=[]
    for i in range(len(x)):
        if 1530<x[i]<1645:
            xG.append(x[i]); yG.append(y[i])
        else:
            if 2650<x[i]<2760:
                x2D.append(x[i]); y2D.append(y[i])
    # select reagion to fit baseline
    xblG=[] ; yblG=[]; xbl2D=[]; ybl2D=[]
    for i in range(len(x)):
        if 1500<x[i]<1530 or 1645<x[i]<1680:
            xblG.append(x[i]); yblG.append(y[i])
        else:
            if 2600<x[i]<2650 or 2760<x[i]<2830:
```

```

        xbl2D.append(x[i]);    ybl2D.append(y[i])
#find and plot baseline Gpeak
fitpar_blG = np.polyfit(xblG,yblG,2)
def blG(x):
    return fitpar_blG[0]*np.power(x,2)+fitpar_blG[1]*x+fitpar_blG[2]
baselineG= []
for i in range(len(xG)):
    baselineG.append(blG(xG[i]))
plt.subplot(121)
plt.plot(xG,baselineG,'b--')
#find and plot baseline 2Dpeak
fitpar_bl2D = np.polyfit(xbl2D,ybl2D,3)
def bl2D(x):
    return fitpar_bl2D[0]*np.power(x,3)+fitpar_bl2D[1]*np.power(x,2)+fitpar_bl2D[2]*x+fitpar_bl2D[3]
baseline2D= []
for i in range(len(x2D)):
    baseline2D.append(bl2D(x2D[i]))
plt.subplot(122)
plt.plot(x2D,baseline2D,'b--')
#subtract baseline
for i in range(len(xG)):
    yG[i]=yG[i]-blG(xG[i])
#subtract baseline2D
for i in range(len(x2D)):
    y2D[i]=y2D[i]-bl2D(x2D[i])
plt.figure(3,figsize=(14,6));
plt.subplot(121);    plt.plot(xG,yG,'go')
plt.subplot(122);    plt.plot(x2D,y2D,'go')
# define lorentzian function
def lorentzian(x,xo,g,A,c):
    return c+A*g/(2*pi)*(1/((x-xo)**2+(g/2)**2))
#fit G peak
initial_guessG=[1587,12,5000,0]
fitparG , fitcovG = curve_fit(lorentzian,xG,yG,p0=initial_guessG,absolute_sigma=True)
fitxoG=fitparG[0]; fitgG=fitparG[1]; fitAG=fitparG[2]; fitcG=fitparG[3]
peakintG = fitAG*2/(pi*fitgG)
plt.subplot(121)

```

```

plt.plot(xG,lorentzian(xG,*fitparG),'r--')
errfitparG= np.sqrt(np.diag(fitcovG))
print('G PEAK')
print('central freq = ',fitxoG,' +/- ',errfitparG[0])
print('FWHM = ',fitgG,' +/- ',errfitparG[1])
print('peak area = ',fitAG,' +/- ',errfitparG[2])
print('intensity = ',peakintG)
#fit 2D peak
initial_guess2D=[2685,42,10000,0]
fitpar2D , fitcov2D = curve_fit(lorentzian,x2D,y2D,p0=initial_guess2D,absolute_sigma=True)
fitxo2D=fitpar2D[0]; fitg2D=fitpar2D[1]; fitA2D=fitpar2D[2]; fitc2D=fitpar2D[3]
peakint2D = fitA2D*2/(pi*fitg2D)
plt.subplot(122)
plt.plot(x2D,lorentzian(x2D,fitxo2D,fitg2D,fitA2D,fitc2D),'r--')
errfitpar2D= np.sqrt(np.diag(fitcov2D))
print('2D PEAK')
print('central freq = ',fitxo2D,' +/- ',errfitpar2D[0])
print('FWHM = ',fitg2D,' +/- ',errfitpar2D[1])
print('peak area = ',fitA2D,' +/- ',errfitpar2D[2])
print('intensity = ',peakint2D)

totpar=fitparG,errfitparG,fitpar2D,errfitpar2D
return totpar

```

PM-IRRAS background subtraction

```

function [Corrected] = PMIRRASbg(csv,pol,range,enter)
% Perform the background correction of PM-IRRAS spectrum. The method goes
% through multiple fit of the spectrum with a polynomial function,
% gradually lowering the curve till the lowest spectral intensities.
% Input data are the path to the csv file of the raw spectrum
% The order of the polynomial background
% The spectral range for the evaluation of the background
% The number of points used to compute the first background curve

data=csvread(csv);

freq=data(:,1);
spec=data(:,2);

```

```

if1=find(freq<range(1),1,"last");
if2=find(freq<range(2),1,"last");

freq1=freq(if1:if2);
spec1=spec(if1:if2);

iter=5;

p=polyfit([freq1(1:enter);freq1(end-enter:end)],[spec1(1:enter);spec1(end-
enter:end)],pol);
y=polyval(p,freq1);
ii=find(spec1>y);
spec1(ii)=y(ii);

for i=1:iter
    p=polyfit(freq1,spec1,pol);
    y=polyval(p,freq1);
    ii=find(spec1>y);
    spec1(ii)=y(ii);
end
NewSpec=(spec(if1:if2)./y)-1;
bg=y;

Corrected=[freq1,NewSpec];

end

```

Savitzki-Golay smoothing and second derivative

```

function [Second_Derivative] = DerivativeAnalysis(csv,width_smoothing,width_SecDer)
% Perform the smoothing and compute the second derivative
% of the input spectrum through the Savitzki-Golay algorithm.
% Input data is the path to the csv file of the raw spectrum

data=csvread(csv);

freq=data(:,1);
spec=data(:,2);

%Spectrum interpolation
s=0.5; %
intfreq=[freq(1):s:freq(end)];
intspec=interp1(freq,spec,intfreq);

%Savitzki-Golay smoothing
pol_order=3;
width_smoothing =9;
h=0;
[b,g]=sgolay(pol_order, width_smoothing);
smoothed = zeros(length(intfreq),1);
smoothed(:,1) = conv(intspec, factorial(0)/(-s)^0 * g(:,1), 'same');
intspec=smoothed;

%Savitzki-Golay second derivative
pol_order=3;
width_SecDer =25;

```

```

h=2;
[b,g]=sgolay(pol_order, width_SecDer);

dx = zeros(length(intfreq),3);

for p = 0:h
    dx(:,p+1) = conv(intspec, factorial(p)/(-s)^p * g(:,p+1), 'same');
end

secder=dx(:,3);

Second_Derivative=[intfreq,secder(:,3)];

end

```

Aimde I fit deconvolution

```

function [coeff,sigma] = Amide1DecATR(freq,spec)
% Perform the fit-deconvolution of the Amide I band of protein IR spectrum.

Gauss = @(I,g,w,x) I.*exp(-(x-w).^2/(2*g^2/(log(256))));%Gaussiana
multiGauss5= @(I1,I2,I3,I4,I5,w1,w2,w3,w4,w5,g,Iam2,wam2,gam2,x)
Gauss(Iam2,gam2,wam2,x)+Gauss(I1,g,w1,x)+Gauss(I2,g,w2,x)+Gauss(I3,g,w3,x)+Gauss(I4,g,w
4,x)+Gauss(I5,g,w5,x);
multiGauss4= @(I1,I2,I3,I4,w1,w2,w3,w4,g1,g2,g3,g4,Iam2,wam2,gam2,x)
Gauss(Iam2,gam2,wam2,x)+Gauss(I1,g1,w1,x)+Gauss(I2,g2,w2,x)+Gauss(I3,g3,w3,x)+Gauss(I4,
g4,w4,x);

f1=1610; if1=find(freq<f1,1,"last");
f2=1695; if2=find(freq<f2,1,"last");

w_am2=1590; iw_am2=find(freq<w_am2,1,"last");
w_sc=1613; iw_sc=find(freq<w_sc,1,"last");
w_b0=1620; iw_b0=find(freq<w_sc,1,"last");
w_b1=1639; iw_b1=find(freq<w_b1,1,"last");
w_ra=1655; iw_ra=find(freq<w_ra,1,"last");
w_t=1665; iw_t=find(freq<w_t,1,"last");
w_b2=1687; iw_b2=find(freq<w_b2,1,"last");

fo5=fitoptions('Method','NonlinearLeastSquares',...
'Lower', [0, 0, 0, 0, 0, 0],
w_b0-5,w_b1-2,w_ra-5,w_t-5,w_b2-2, 22, spec(iw_am2)*0.1560,20],...
'Upper', [spec(iw_b0), spec(iw_b1), spec(iw_ra), spec(iw_t), spec(iw_b2),
w_b0+5,w_b1+2,w_ra+5,w_t+5,w_b2+2, 26, abs(spec(iw_am2))*1.1580,40],...

'StartPoint',[spec(iw_b0)*0.5,spec(iw_b1)*0.5,spec(iw_ra)*0.5,spec(iw_t)*0.5,spec(iw_b2
)*0.5, w_b0, w_b1, w_ra, w_t, w_b2 24, spec(iw_am2)*0.2,1570,35]);
f5= fitttype(@(I1,I2,I3,I4,I5,w1,w2,w3,w4,w5,g,Iam2,wam2,gam2,x)
multiGauss5(I1,I2,I3,I4,I5,w1,w2,w3,w4,w5,g,Iam2,wam2,gam2,x),'independent','x','option
s',fo5);

[Amide1,gof]=fit(freq(if1:if2)',spec(if1:if2)',f4)

coeff=coeffvalues(Amide1);
conf95=confint(Amide1);

```

```

sigma=(conf95(2,:)-conf95(1,:))/2;
fitted=Amide1(freq);

figure(400);
set(gcf,'color','w');
hold on;
plot(freq,spec,"-k","LineWidth",5);
plot(freq,Gauss(Amide1.I1,Amide1.g,Amide1.w1,freq),'-k',"LineWidth",2);
plot(freq,Gauss(Amide1.I2,Amide1.g,Amide1.w2,freq),'-',"color",[0.85 0.33 0.10],"LineWidth",2);
plot(freq,Gauss(Amide1.I3,Amide1.g,Amide1.w3,freq),'-',"color",[0.93 0.69 0.13],"LineWidth",2);
plot(freq,Gauss(Amide1.I4,Amide1.g,Amide1.w4,freq),'-',"color",[0.49 0.19 0.56],"LineWidth",2);
plot(freq,Gauss(Amide1.I5,Amide1.g,Amide1.w5,freq),'-',"color",[0.47 0.67 0.19],"LineWidth",2);
plot(freq,Gauss(Amide1.Iam2,Amide1.gam2,Amide1.wam2,freq),':k',"LineWidth",2);

plot(freq,fitted,'--r',"LineWidth",3);
xlim([1600,1720]);
set(gcf,'color','w');
xlabel("Wavenumber (cm-1)");
ylabel("PM-IRRAS signal (arb. units)");
set(gca,'FontSize',15,'FontName','Arial','FontWeight','bold');
end

```

Get the optical constants

```

% Compute the optical constants from the ATR spectrum of a thin film
% Read the ATR spectrum in absorbance
ATR_aIgG=csvread("path/ATR_aIgG.txt");

```

```

%Define a range of wavenumber and a step
f1=1480;
f2=1800;
step=1;
freq=[f1:step:f2];

```

```

%Interpolate data
specAbs=interp1(ATR_aIgG(:,1), ATR_aIgG(:,2),freq);

```

```

%Convert in Transmittance TR=10^(-Abs);
specTR=10.^(-specAbs);

```

```

%Smooth transmittance spectrum
smoot_window_freq=15; %in cm-1
smoot_window=smoot_window_freq/step; %in number of points
[freq,smootTR] = SGsmoothing(freq,specTR,3,smoot_window,"NoFig");

```

```

% Set the refractive index of the ATR crystal
n1=2.4; %Refractive index ZnSe
k1=0;

```

```

% Set the refractive index of environment medium
n3=1; %Refractive index Air
k3=0;

```

```

% Set Incidence angle
teta=45;

% Set the number of iteration
iter =4;

% Set the film parameter
n_inf=1.37           %Refractive index offset of the protein film far from resonance
d=7*10^-7 %         %film thickness in cm

%Initialize the final optical constant matrix
combinazioni_n_d=length(d)*length(n_inf);
opt_const=zeros(1+combinazioni_n_d,2+length(freq));
opt_const(1,3:end)=freq;

for D=1:length(d)
    for N=1:length(n_inf)

n_complex=ThinFilmOpticalConstant(n1,k1,n3,k3,teta,d(D),n_inf(N),freq,smootTR,iter);

        opt_const(1+length(n_inf)*(D-1)+N,1)=d(D);
        opt_const(1+length(n_inf)*(D-1)+N,2)=n_inf(N);
        opt_const(1+length(n_inf)*(D-1)+N,3:end)=n_complex;

    end
end
End

csvwrite("optical_constant_table_aIgG",opt_const); %Create a csv output file

```

Evaluation of the optical constants of thin films

```

function [n_complex] =
ThinFilmOpticalConstant(n1,k1,n3,k3,teta,d,n_inf,freq,ATRtr,iter)

% The following code compute the real and immaginary part of the complex
% refractive index of thin film from a measured ATR spectrum.
% It consider a 3-layer system: Substrate_1 | Film_2 | Environment_3 and uses the
% frenel equations for the reflectivity of very thin films described in McINTYRE
% (1971) under the
% Assumptions: 1) linear approximation valid for d/l <0.03.
%               2) unpolarized incident radiation Is=Ip
%               3) 6 internal reflections
%               4) Works with input ATR spectrum in transmittance TR=10^(-Abs);
%               5) d (film thickness) is in cm

eps= @(n,k) n.^2 - k.^2 + 1i*2*n.*k; %Complex dielectric function

eps1=eps(n1,k1); % Substrate Complex dielectric constant
eps3=eps(n3,k3); %Environment Complex dielectric constant

Rs = @(eps1,n,k,eps3,teta,d,nu) 1-8*pi*d*n1*cosd(teta)*nu.*imag((eps(n,k)-eps3)./(eps1-eps3));
Rp = @(eps1,n,k,eps3,teta,d,nu) 1-8*pi*d*n1*cosd(teta)*nu.*...
    imag(((eps(n,k)-eps3)./(eps1-eps3)).*...
    ((1-(eps1./(eps(n,k).*eps3)).*(eps(n,k)+eps3)*sind(teta)^2)./...

```

```

(1-((eps1+eps3)*sind(teta)^2./(eps3)))));

eq= @(eps1,n,k,eps3,teta,d,nu,tr)
((Rs(eps1,n,k,eps3,teta,d,nu).^6+Rp(eps1,n,k,eps3,teta,d,nu).^6)/2)-tr;

%Initialize the n and k matrix vector for the iterations
n=zeros(iter,length(freq));
k=zeros(iter,length(freq));

%Initial guess for the Refractive index of the protein film
n_guess=n_inf;

syms x;
for i=1:length(freq)
    s=vpasolve(eq(eps1,n_guess,x,eps3,teta,d,freq(i),ATRtr(i))==0,x);
    k(1,i)=s;
end
Rekk=kkrebook(freq,k(1,:),0);
n(1,:)=n_inf+Rekk;

for j=2:iter
    for i=1:length(freq)
        s=vpasolve(eq(eps1,n(j-1,i),x,eps3,teta,d,freq(i),ATRtr(i))==0,x);
        k(j,i)=s;
    end
    Rekk=kkrebook(freq,k(j,:),0);
    n(j,:)=n_inf+Rekk;
end

w=1650; iw=find(freq<w,1,"last");

figure(3);
for i=1:iter
    subplot(1,2,1);hold on;
    name = ['k',num2str(i)];
    plot(freq,k(i,:), "DisplayName",name);
    subplot(1,2,2);hold on;
    plot(i,k(i,iw),"o");
end
subplot(1,2,1);hold on;
xlim([freq(1),freq(end)]);
xlabel("wavenumber");
ylabel("Absorption index");
legend();
set(gca,'FontSize',10,'FontWeight','bold');
set(gcf,'color','w');
subplot(1,2,2);hold on;
xlabel("iteration number");
y_label=['Absorption index @',num2str(w),'cm^{-1}'];
ylabel(y_label);
legend();
set(gca,'FontSize',10,'FontWeight','bold');
set(gcf,'color','w');

% Plot n and k
figure(5);
hold on;

```

```

n_name=['n - ',num2str(n_inf)];
plot(freq,n(iter,:)-n_inf,"-k","LineWidth",2,"DisplayName",n_name);
plot(freq,k(iter,:),"-r","LineWidth",2,"DisplayName","k");
xlabel("wavenumber");
legend();
set(gca,'FontSize',10,'FontWeight','bold');
set(gcf,'color','w');

n_complex= n(iter,:)+1i*k(iter,:);
end

```

Orientation analysis

```

% Evaluate the average tilt angle of transition dipole moments
% from the comparative analysis of the experimental PM-IRRAS spectrum
% and a simulated IRRAS spectrum

Measured_PMIRRAS_aIgG=csvread("Corrected_PMIRRAS_Absorbance");
Tab_IRRASAbs=csvread("Table_IRRASAbs");
Wavenumber = Tab_IRRASAbs(1,3:end);

[coeff_pm,sigma_pm]=Amide1Dec(Wavenumber',Measured_PMIRRAS(2,:),'-k',"pmirras");
Area_pm=coeff_pm(2:6).*coeff_pm(14:18).*sqrt(pi/log(16));

n=18; %18 = number of fit parameters
c=5;  %5 = number of amide 1 components

coeff_Sim=zeros(size(Tab_IRRASAbs,1)-1,n);
sigma_Sim=zeros(size(Tab_IRRASAbs,1)-1,n);
Angles=zeros(size(Tab_IRRASAbs,1)-1,c);
Area_SimaIgG=zeros(size(Tab_IRRASAbs,1),c);

for j=1:size(Tab_IRRASAbs,1)-1
[coeff_Sim(j,:),sigma_Sim(j,:)])=Amide1Dec(Wavenumber',Tab_IRRASAbs(j+1,3:end),'-k',"Simulated");
Area_Sim(j,:)=coeff_Sim(j,2:6).*coeff_Sim(j,14:18).*sqrt(pi/log(16));

Angles(j,:)=acosd(sqrt(Area_pm./(3*Area_Sim(j,:))));

end

```

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List of publications

- 1) **Matteo Piscitelli**, Cinzia Di Franco, Giuseppe Valerio Bianco, Giovanni Bruno, Eleonora Macchia, Luisa Torsi, and Gaetano Scamarcio; Graphene-based opto-electronic platform for ultra-sensitive biomarker detection at zeptomolar concentrations; Small methods; 2025
<https://doi.org/10.1002/smtd.202402026>
- 2) **Matteo Piscitelli**, Cinzia Di Franco, Giuseppe Valerio Bianco, Giovanni Bruno, Eleonora Macchia, Luisa Torsi, and Gaetano Scamarcio; Raman spectroscopy of graphene for ultrasensitive protein detection SPIE 13376, Quantum Sensing and Nano Electronics and Photonics XXI, 1337602 (21 March 2025);
- 3) Eleonora Macchia, Cinzia Di Franco, Cecilia Scandurra, Lucia Sarcina, **Matteo Piscitelli**, Michele Catacchio, Mariapia Caputo, Paolo Bollella, Gaetano Scamarcio, and Luisa Torsi; Plasmonic single-molecule affinity detection at 10^{-20} molar; Advanced Materials; 37, 9, 2025; 2418610
- 4) **Matteo Piscitelli**, Diellza Bajrami, Cinzia Di Franco, Lucia Sarcina, Michele Catacchio, Eleonora Macchia, Luisa Torsi, Boris Mizaikoff, and Gaetano Scamarcio; β -sheets orientation in physisorbed protein layers; Advanced Materials Interfaces; 12, 6, 2025; 2570017
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