Functional and Structural Effects of UV Light on Biomacromolecules

from basic research to therapeutic applications

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BY
ODETE SOFIA LOPES GONÇALVES

DISSERTATION SUBMITTED 2019

AALBORG UNIVERSITY
DENMARK

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Dissertation submitted: October 2019

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Paper 3. "Novel microfabrication stage allowing for one-photon and multi-photon Light Assisted Molecular Immobilization and for multi-photon microscope". **Odete Gonçalves***, Scott Snider, Ruben Zadoyan, Quoc-Thang Nguyen, Henrik Vorum, Steffen B. Petersen and Maria Teresa Neves-Petersen. Proceedings SPIE 10079, Reporters, Markers, Dyes, Nanoparticles, and Molecular Probes for Biomedical Applications IX, 100790F

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Paper 5. "Detection of cancer markers using light activated Molecular Beacons". Odete Gonçalves*, Guy Wheeler, Tamas Dalmay, Houquan Dai, Miguel Castro, Patrick Castro, Jaime García-Rupérez, Ángela Ruiz-Tórtola, Amadeu Griol, Juan Hurtado, Laurent Bellieres, María José Bañuls, Daniel González, José António Antonio López-Guerrero and Maria Teresa Neves-Petersen (2019). RSC Advances 9, 12766–12783.

Paper 6. "Photonic modulation of EGFR – 280nm low level light arrests cancer cell activation and migration". Cláudia M. Botelho*, Rogério Marques Thiagarajan Viruthachalam, **Odete Gonçalves**, Henrik Vorum, Andreia Castro Gomes and Maria Teresa Neves-Petersen* (2017). Proceedings SPIE 10048, Mechanisms of Photobiomodulation Therapy XII, 100480R.

Paper 7. "Photonic modulation of EGFR halts receptor activation and cancer cell migration". Cláudia Manuela Botelho*, **Odete Gonçalves**, Rogério Marques, Viruthachalam Thiagarajan, Henrik Vorum, Andreia Castro Gomes and Maria Teresa Neves-Petersen* (2018). Journal of Biophotonics, e201700323.

Paper 9. "The repeated 36 amino acid motif of Chlamydia trachomatis Hc2 protein binds to the major groove of DNA" **Odete Sofia Lopes Gonçalves***, Gunna Christiansen, Arne Holm, Bjørn Herrmann, Markus Klintstedt, Steffen B. Petersen, Svend Birkelund. Res Microbiol. Vol.4, No.3, 2019. PMID: 31419583

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Paper 8. "The therapeutic window of light: modulating receptor proteins and metabolic pathways with light and its importance for disease control" **Odete Gonçalves***, Andreia Castro Gomes and Maria Teresa Neves-Petersen

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Submitted:

Paper 2. “Structural and functional analysis of broad pH and thermal stable protease from Penicillium aurantiogriseum URM 4622” José Manoel Wanderley Duarte Neto*, Jônatas de Carvalho Silva, Flávia Sousa, Bruno Sarmento, **Odete Sofia Lopes Gonçalves**, Maria Carolina de Albuquerque Wanderley, Carolina de Albuquerque Lima, Maria Teresa Neves-Petersen, Ana Lúcia Figueiredo Porto. Submitted.

CV Brief Summary

Odete Sofia Lopes Gonçalves

2019

Born June 1988
BSc in Biology 2010, University of Porto, Portugal
Msc in Molecular Genetics 2012, University of Minho, Portugal
PhD Student since 2015, University of Aalborg, Denmark
11 peer-reviewed international publications
10 national and international conference poster presentations
10 oral communications

Odete Gonçalves holds a BSc in Biology from the University of Porto (2010) during which she performed an internship as a research trainee at CIBIO – Research Center in Biodiversity and Genetic Resources in Vairão, Portugal (October 2009-June 2010). In 2012, Odete Gonçalves obtained her MSc in Molecular Genetics in the School of Sciences of the University of Minho in Braga, Portugal. During her masters, she performed biophysical characterization and in vitro studies of Monoolein based liposomes and lipoplexes as non-viral methods for DNA delivery.

Odete Gonçalves became a researcher, in 2013, for the SPIN-OFF company "Nanodelivery-I&D em Bionanotecnologia, Lda." located at the Department of Biology, University of Minho, where she worked on the characterization and development of nanoparticles for cosmetic applications until September 2014. From November 2014 till March 2015, Odete Gonçalves worked in a research project entitled "Nanotherapeutics: development of bionanosystems for drug delivery" in the Centre of Physics of University of Minho, where she performed drug encapsulation and release studies in a liposomal system.

Since September 2015, Odete Gonçalves is a PhD Fellow in the Department of Health Science and Technology in Aalborg University, Denmark, where she carries out research in biophotonics, immobilization of biomolecules for

medical applications and the study UVB induced effect on proteins. Her PhD studies were funded by Horizon2020 EU project PHOCNOSIS.

Odete Gonçalves has 8 published articles in international peer-reviewed journals, 3 peer-reviewed conference papers, 10 panel communications and 10 oral communications.

PREFACE

The work presented in this PhD thesis represents the work carried out during the doctoral program at Aalborg University. The PhD fellowship was funded by the European project H2020- 634013-2-PHOCNOSIS.

Most of the research work carried out during the PhD was performed at the Medical Photonics laboratory, at the Department of Health Science and Technology, Aalborg University. Other activities were performed in collaboration with prof. Maria-José Bañuls from IDM, Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico, Departamento de Química, Universitat Politècnica de València, with Dr. Jaime García-Rupérez from Nanophotonics Technology Center, Universitat Politècnica de València, with Tamas Dalmay from University of East Anglia, UK, and with other consortium partners from the European project H2020- 634013-2-PHOCNOSIS and the European project H2020-644242 – SAPHELY. Additional collaborations were also established with Prof. Andreia Gomes from Centre of Molecular and Environmental Biology (CBMA), Universidade do Minho, Braga, Portugal and with Cláudia Botelho from Centre of Biological Engineering and Centre of Molecular and Environmental Biology (CBMA), Universidade do Minho, Braga, Portugal.

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There is a long list of people that I would like to acknowledge but in no way will I be able to express in this text all the gratitude I feel for all the people that have helped me in one way or another throughout this journey on this faraway land that now feels like home.

First of all, I would like to thank my supervisors Steffen Petersen and Teresa Neves for their guidance, help and patience. It was an unusual journey but somehow we made it through. Thank you for the opportunity to work with you, for making me aware that light is all around and that it affects us in many different ways. I would also like to express my gratitude to Svend Birkelund and Gunna Christiansen for all the kind help and good discussions we had. It was a pleasure to collaborate with each one of you.

I would like to thank also Cláudia Botelho for our great collaboration and prof. Andreia Gomes for your kind words, patience and guidance. I am very grateful for our collaboration and it was a pleasure to work alongside you in the lab.

I would also like to express my gratitude to the partners from the EU projects PHOCNOSIS and SAPHELY, especially Jaime Garcia-Ruperez and Maria-José Bañuls, for the good collaboration we had. It was a pleasure to work with all of you!

To my office mates, Anne Mørkholt and Kenneth Kasteniegaard, thank you for the comradery and nice laughs we shared. Kenneth, keep blasting your music and spreading your good humor! I also would like to thank my colleagues from HST and Biomedicine which helped me during my PhD with my work.

I also would like to thank all the students that came to our lab and shared experiences with me. Britt, Runne, Danielly, Raphaella, Laryssa, Jônatas, José Manoel, Patyanne, Pilar, Andreas, Heidi, Vinu and Rocio, it was amazing to work with each and every one of you! Also to the Portuguese emigrants Rui, Flávia and Inês that crossed paths with me at AAU. Our encounter in Denmark was brief but our friendship is life long lasting. I am so glad our paths crossed, even if so far from home!

To my former housemates, especially Hicham, Bea, Felipe, Pau and Rocco, thank you for all the good times we shared during this journey.

To my family back in Portugal, a huge thank you. You were far away in distance but never far from my heart.

To Mari and Yoli, who became the first members of my Danish family, a warm thank you!

To Juan, Luisi, Gabi, Jorge, Henrik and Sandra, who became my extended Danish family, a big thank you for your friendship and support and for your help in the most difficult times. Gabi, you are the evil big sister I never had and I am very grateful for your friendship. I carry you all in my heart.

And last but not least, I am so grateful to Fabri and Belinha for believing in me and giving me their endless friendship, laughs, patience, advices, support and help during this period. Near or far, I know I can always count on you and I wouldn't have made it this far without you.

Muito obrigada a todos!

"O amor é uma luz que não deixa escurecer a vida. Ninguém é pobre quando ama."

Camilo Castelo Branco

"You are capable of more than you know. Choose a goal that seems right for you and strive to be the best, however hard the path. Aim high. Behave honorably. Prepare to be alone at times, and to endure failure. Persist! The world needs all you can give."

Edward Osborne Wilson

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LIST OF ABBREVIATIONS

UV - Ultraviolet

NMR - Nuclear Magnetic Resonance

CD – Circular Dichroism

CVD – Cardiovascular diseases

SS bond – Disulfide bond

DTT - dithiothreitol

T_m – melting temperature

LAMI – Light Assisted Molecular Immobilization

TEC – Thiol-ene coupling

POC – Point-of-care devices

e^-_{aq} – aqueous electron

PDT – Photodynamic therapy

PTT – Photothermal Therapy

EGFR – Epidermal Growth Factor Receptor

EGF – Epidermal Growth Factor

MB – Molecular Beacon

CRP – C-reactive protein

Kyn - kynurenine

NFK - N-formyl-kynurenine

CFM - Confocal Fluorescence Microscopy

SOI - silicon-on-insulator

$K_2S_2O_8$ – Potassium persulfate

ABSTRACT

Biomolecules such as proteins and peptides are abundant in living organisms, displaying dynamic and diversified roles. Their diversity and importance for biological processes makes them interesting targets of research for a variety of applications in biotechnology, medicine, industry and many other fields. The three-dimensional structure of these biomacromolecules is a key feature for their action and application. The structure and stability of proteins is dependent on a balance between different forces that are influenced by different environmental parameters. Light is one of such parameters.

Light, or more particularly, UV-light is known to induce several photochemical processes in various biomacromolecules. Proteins are especially prone to photo-induced processes due to the sensitivity of various amino acids to UV light excitation, many of them with nefarious effects on protein structure and stability. However, the UV light-induced photo processes are dependent on dosage and time of exposure as well as on each individual protein characteristics.

The pH and temperature induced conformational changes of two proteases were studied using light-based methodologies: Fluorescence spectroscopy and Circular Dichroism. UV light excitation of tryptophan allowed for the monitorization of the protein's intrinsic fluorescence and the formation of photo-induced products was also investigated. These studies are relevant for the characterization of both proteases in order to optimize their activity for relevant applications.

The knowledge of light induced mechanism was also applied for the production of protein microarrays. Light Assisted Molecular Immobilization (LAMI) is a photonic immobilization technique developed at AAU that is based on UV induced disruption of disulfide bonds in proteins leading to their immobilization onto thiol derivatized surfaces via covalent thiol linkage. This photonic technique was applied for the immobilization of antibodies for detection biomarkers for cardiovascular diseases (Troponin I, Troponin T, C-reactive protein and myoglobin) and for immobilization of peptide coupled Molecular Beacons (MB constructs) for detection of

miRNA21, a biomarker for cancer, as part of the development of two Point-of-Care devices for early diagnosis of cardiovascular diseases and cancer.

The use of UV light induced mechanisms was also researched as a potential photonic therapy for cancer. During this PhD, the UV induced inactivation of Epidermal Growth Factor Receptor (EGFR), a transmembrane receptor overexpressed in many cancers and associated with tumor progression was investigated. Small-lung carcinoma cells A549 with GFP tagged EGFR (A549 EGFR-GFP) were irradiated with wavelength specific UV light which resulted in a delay in EGFR activation and cancer cell migration, showing the potential for this photonic cancer therapy.

Additional work performed during this PhD involved the investigation of the DNA binding ability of a 36 amino acid motif of the Hc2 protein from *Chlamydia trachomatis*. The results showed a binding affinity of the peptide to the major grove of DNA.

The work performed during this PhD gives an insight on UV light induced photo-processes on proteins and peptides and their usefulness on researching protein structural and functional changes as well as on the development of new strategies for cancer therapy and protein immobilization for biosensing of target analytes.

RESUMÉ

Der findes talrige biomolekyler, som fx proteiner og peptider, i levende organismer, der har dynamiske og forskelligartede roller. Deres diversitet og vigtighed for biologiske processer gør dem til interessante mål for forskning i anvendelsesmuligheder inden for bioteknologi og medicin samt i industrien og mange andre områder. Den tredimensionelle struktur af disse biomakromolekyler er en nøgleegenskab for deres virkning og anvendelse. Strukturen og stabiliteten af proteiner er afhængig af en balance mellem forskellige kræfter, der påvirkes af forskellige miljømæssige parametre. Lys er et sådant parameter.

Lys, eller mere præcist UV-lys, er kendt for at inducere adskillige fotokemiske processer i forskellige biomakromolekyler. Proteiner er særligt modtagelige over for foto-inducerede processer på grund af forskellige aminosyrers følsomhed over for energitilførsel fra UV-lys; mange af dem med skadelig effekt på proteinstrukturen og -stabiliteten. Dog er UV-inducerede foto-processer afhængige af dosis og varigheden af eksponering såvel som af de individuelle protein-karakteristika.

pH og temperatur-inducerede tilpasningsændringer i to proteaser blev undersøgt ved hjælp af lys-baserede metoder: Fluorescens-spektroskopi og cirkulær dikronisme. UV-exitation af tryptophan tillod overvågning af proteinets indre fluorescens ligesom dannelsen af foto-inducerede produkter også blev undersøgt. Disse studier er relevante for karakteriseringen af begge proteaser for at kunne optimere deres aktivitet til relevante anvendelsesmuligheder.

Viden om den lys-inducerede mekanisme blev også anvendt til produktion af protein-microarrays. Lysassisteret molekylærimmobilisering (LAMI) er en fotonisk immobiliseringsteknik, som er udviklet på AAU. Den er baseret på UV-induceret disruption af disulfidbindinger i proteiner, som fører til immobilisering til thiol-derivate overflader via kovalent thiol-kobling. Denne fotoniske teknik blev anvendt til immobilisering af immunstoffer for detektion af biomarkører for kardiovaskulære lidelser (Troponin I, Troponin T, C-reaktivt protein og myoglobin) og til immobilisering af peptid-forbundne molekylære beacons (MB constructs) til påvisning af miRNA21,

en biomarkør for kræft, som en del af udviklingen af to Point-of-Care-enheder for tidlig diagnosticering af kardiovaskulære lidelser og kræft.

Anvendelsen af UV- inducerede mekanismer blev også undersøgt som en potentiel fotonisk terapi for kræft. Den UV-inducerede inaktivering af Epidermal Vækstfaktor Receptor (EGFR), en transmembran-receptor, som er over udtrykt i mange cancertyper og forbundet med tumor-progression, blev undersøgt. Små lunge-carcinoma-celler A549 med GFP-tagget EGFR (A549 EGFR-GRP) blev bestrålet med bølgelængde-specifikt UV-lys, hvilket resulterede i en forsinkelse i EGFR-aktivering og cancercelle-migration og viste dermed potentiale for denne fotoniske cancerterapi.

Endvidere er der under ph.d.-studiet udført undersøgelser af DNA-bindeevnen af et 36-aminosyremotiv i Hc2 proteinet fra Chlamydia trachomatis. Resultaterne viste en bindingsaffinitet af peptidet til den store rille i DNA.

Det arbejde, der er udført under dette ph.d.-studium, giver indsigt i UV-inducerede fotoprocesser på proteiner og peptider og deres brugbarhed i forskning i proteiners strukturelle og funktionelle ændringer såvel som udvikling af nye strategier for cancerterapi og protein-immobilisering for biosensing af målanalytter.

1. INTRODUCTION

Light is a ubiquitous part of our lives. Many processes in nature and life are either controlled by or involve light. Photosynthesis, a process that allows green plants to transform energy from sunlight into chemical energy, is perhaps one of the most important examples of light as an essential part of life. Other processes in life are also light dependent, such as human vision. The photoreceptors present in our eyes that allow us to see the world rely on photochemical processes induced by light. Light is also the basis of many technological advances. Optical instruments that allow us to view other planets but also to view a whole new world on this planet, too small for the naked human eye to see, lasers used for chirurgical interventions, medical devices and even our smartphones, all these things rely on light.

In today's society, the advances in the medical field are important for a prolonged and better quality of life. Proper medical care has high economic and social value leading to a constant effort in researching and improvement of new methods and new equipment in order to maintain the high standard of development. Multiple fields of knowledge are involved in this endeavor, but in more recent years the focus has been increasingly high on the nanoscale. Since most of the processes underlying diseases involve molecules in the nanoscale, such as DNA, RNA and proteins, it is easy to understand the importance of studies on the nanoscale. Many biological and medical applications have been developed, e.g., development of new nanomaterials and nanocarriers for treatment and diagnosis.

Simultaneously, light can be both a tool for the acquisition of knowledge on biological processes and a method to be developed and applied in the industry. Recent technological advances in optical components and laser systems allied to the improvement of more classical techniques such as fluorescence spectroscopy and confocal microscopy allow for new branches of research to emerge, such as bionanophotonics and medical photonics. These concepts are a merger of various disciplines such as nanophotonics, biomedical sciences and technology. Such multidisciplinary advances allow for the research of macromolecular processes, endeavoring for a better understanding of biomacromolecule structure and function. Furthermore,

they allow for the development of existing and new medical and biotechnological applications.

STRUCTURE OF THE THESIS

This chapter introduces light as an important parameter with major influence on various aspects of our lives. The main focus of the work performed during the PhD was the effect of light on structure and function of proteins and potential applications of this knowledge. This study was roughly divided into three main themes:

- UV light induced photochemical processes and their effect on protein structure and function.
- Biosensor microarray production using a photonic immobilization technique
- Wavelength specific UV light as a new potential photonic cancer therapy

In **Section 2**, a brief introduction to protein structure and photochemistry is provided as well as a description of structural changes induced by light and how this knowledge can be applied to follow macromolecular processes for characterization and monitorization of changes in protein structure. Two studies were performed and are described in **paper 1** [1] and **paper 2** [2] (unpublished work).

In **Section 3**, the photochemical mechanisms induced by wavelength specific UV light (280 nm) were explored as the basis for a technique for photonic immobilization of protein based biosensors for biomedical application. LAMI was used as a tool to immobilize various biomolecules in an improved optical setup. The performed studies are described in **paper 3** [3], **paper 4** [4] and **paper 5** [5].

In **Section 4**, the photochemical mechanisms induced by wavelength specific UV light (280 nm) were explored as a new potential cancer therapy. The performed studies are described in **paper 6** [6], **paper 7** [7] and **paper 8** [8]**.**

Additionally, spectroscopy work was performed with a 36 amino acid Hc2 peptide from *Chlamydia trachomatis*, and a scrambled version of that

peptide. In **Section 5**, a brief introduction is provided to the spectrometric studies performed to evaluate the binding capacity of both peptides to DNA. The work is described in **paper 9** [9].

1.1 AIMS OF THE PHD WORK

The content described in this thesis has light as the unifying component. The overall work presented is based on photo induced processes on proteins and how the study and knowledge of these processes can be used for different applications.

The main aims of this PhD were to:

- Perform spectrophotometric studies on conformational changes induced by pH, temperature and UV light on two candidate proteins [**papers 1** [1] **and paper 2** [2] (unpublished work)].
  - Monitor pH and temperature induced changes on secondary structure with circular dichroism.
  - Monitor tryptophan's fluorescence emission as a function of pH and temperature as a measure of changes in protein conformation
  - Monitor the formation of photoproducts upon UV illumination of the proteases as a measure of protein resistance.

- Perform photonic immobilization of proteins and peptide-molecular beacon conjugates using Light Assisted Molecular Immobilization (LAMI) [**paper 3** [3], **paper 4** [4] and **paper 5** [5]].
  - Optimize the illumination procedures on a new optical setup
  - Perform immobilization and bioavailability studies on four specific IgG antibodies for the detection of cardiovascular biomarkers.
  - Perform immobilization of peptide-molecular beacon constructs (MB constructs) for the detection of miRNA21, a biomarker for cancer, and study their performance.

- Investigate the therapeutic window of wavelength specific 280 nm UV light as a potential photonic therapy for cancer [**paper 6** [6], **paper 7** [7] and **paper 8** [8]**].**
  - Study the effect of low dose 280nm UVB illumination on the inhibition of Epidermal Growth Factor Receptor (EGFR) in a model cancer cell line.
  - Screen the effect of different irradiation powers and time on cellular migration and filopodia formation.
  - Compare the effect of UV induced EGFR inhibition with EGFR-signaling inhibitor Tyrphostin.

- Perform spectrophotometric analysis of peptide:DNA interaction [**paper 9** [9]]
  - Analyse DNA-binding capacity of a 36 amino acid Hc2 peptide from *Chlamydia trachomatis* by electrophoresis and electron microscopy and compared with the binding capacity of a scrambled version of that peptide.
  - Perform fluorescence spectroscopy studies using DNA fluorescent probes to determine the binding mechanism of the peptides to DNA (DNA intercalation, binding to major groove of DNA or binding to minor groove of DNA).

2. PROTEINS AND LIGHT INDUCED MECHANISMS

Proteins are very versatile biomolecules with diversified and dynamic roles in living organisms. Proteins are built up by amino acids the sequence of which is encoded by their respective genes. These molecules have various functional roles, from acting as molecular transporters and chaperones, providing structural support, forming receptors and channels in cell membranes, catalyzing biochemical reactions to participating in the regulation of various metabolic pathways. Their diversified functional range has made them good candidates for many therapeutic applications and there has been an extensive effort to develop new drugs and therapies combined with proteins.

In this thesis, light or, more specifically UV light, was used as a tool to study changes in the microenvironment of proteins such as proteases.

2.1 PROTEINS: A BRIEF OVERVIEW ON STRUCTURE AND FUNCTION

First of all, a basic understanding of protein structure and function is necessary for a successful therapeutic or industrial application. Many external factors influence protein structure and, by extension, their function. It is possible to investigate the protein's structure with NMR (Nuclear Magnetic Resonance) spectroscopy and X-Ray Crystallography and with this knowledge create models that experimentally determine the protein's folding patterns and dynamics [10–12]. Fluorescence spectroscopy is also a technique that allows the investigation of certain aspects of protein conformation and function due to the spectral properties of some of their components. For example, amino acids with aromatic moieties such as tryptophan and tyrosine absorb light in the UV range and emit fluorescence [13]. This topic will be further explored in section 1.2 of this thesis.

However, for a protein to be successfully implemented in a particular application, it is also necessary to analyze the conformation and stability in

the specific experimental conditions as well as its specific structural and functional features. Proteins are macromolecules involved in virtually all processes in a biological system. From DNA replication, molecular transport, catalysis, forming a cytoskeletal structure to molecule conversion, proteins present a wide range of biological diversity [14]. Changes in protein structure can lead to the modulation or disruption of their proper function and to a variety of pathologies. For example, specific changes in tumor repressor protein p53 are related to the development of cancer [15]. Many other diseases such as neurodegenerative diseases and diabetes are related to mutation and improper protein function [16–20]. It is no wonder that the knowledge of protein structure and function is also valuable for many fields, such as medicine and industry.

The organization hierarchy of proteins can be divided into four structures: primary, secondary, tertiary and quaternary structure. The protein's primary structure is the sequence of amino acids that composes the polypeptide chain. In Figure 1 is depicted the general structure of an amino acid. They are crystalline in nature and characterized by high boiling and melting temperatures. These properties result from the ionic and dipolar nature of amino acids. According to their side chains (R), amino acids can be classified as polar or non-polar. The polar side chains can be also further classified as neutral, basic or acidic.

Figure 1. Generalized model of amino acids in L-configuration (right) and D-configuration (left), showing the amino group ($NH3^+$), the carboxyl group (COO^-) and the R group (side chain) attached to the α carbon. The D- configuration is rarely found in nature.

Each amino acid is bound to the next by a peptide bond, where the amino group of one amino acid reacts with the carboxyl group of another amino

acid, forming a polypeptide chain [21]. As a consequence, the R groups (free functional groups) shape the chemical reactivity of the polypeptide and play a major role in determining the final conformation adopted by the polypeptide [22]. Though these macromolecules are built based upon the same 20 amino acids (Table 1) the assembly sequence of these amino acids varies from protein to protein, conferring them with diversity both in functionality and 3D assembly. Some proteins display similarities in their primary structure, which leads to the assumption that they are evolutionary related and are classified as homologous. Depending on the level of sequence similarity, the structural homology can be deduced. This is highly dependent on the length of sequence alignment [23].

AA name	Three-letter code	One-letter code
Glycine	Gly	G
Alanine	Ala	A
Serine*	Ser	S
Threonine*	Thr	T
Cysteine	Cys	C
Valine	Val	V
Isoleucine	Ile	I
Leucine	Leu	L
Proline	Pro	P
Phenylalanine	Phe	F
Tyrosine*	Tyr	Y
Methionine	Met	M
Tryptophan*	Trp	W
Asparagine*	Asn	N
Glutamine*	Gln	Q
Histidine*	His	H
Aspartic acid*	Asp	D
Glutamic acid*	Glu	E
Lysine*	Lys	K
Arginine*	Arg	R

Table 1. The 20 amino acids and some of their individual characteristics. Blue: small amino acid; *: May form salt bridge or sidechain hydrogen bond; Red: hydrophobic amino acid; Green: polar amino acid; Orange: negatively charged, acidic amino acid; Purple: positively charged, basic amino acid (adapted from [21]).

Some proteins also display modified amino acids in addition to the above mentioned 20 amino acids. These modified amino acids are usually the result of post-translational modification reactions. Some of the most common modifications are glycosylation, proteolytic processing and phosphorylation, although many other modifications may occur. Normally these modifications are linked to the structural stability or biological activity of the polypeptide [22].

The secondary structure of the protein relates to local spatial conformation of the polypeptide chain and it presents as major elements α-helix, β-strands, turns or unordered structure. The primary structure determines, to some extent, the secondary structure of the proteins in some local regions of the polypeptide chain, i.e., some amino acid sequences favor certain conformations such as α-helices, β-sheets. Some proteins display only one of these structures (e.g. fibrous proteins) while others display several stretches with different conformations (e.g. globular proteins) [22],

The α-helices are the best known and more recognizable structural motif in proteins. In this structural motif, the carbonyl (C=O) of one amino acid acts as a hydrogen bond acceptor for the amino H (N-H) found four residues away. This type of bonding leads to the formation of a helical pattern, where each turn of the helix contains 3.6 amino acids, the side chains are displayed outwards and the polypeptide backbone constitutes the inner core. Most commonly, the helix displays a right-hand rotation due to its more energetically favorable conformation. However, the left-hand rotation can also be found. Furthermore, some amino acids are more favorable to the formation of the α-helix (e.g. methionine, leucine, glutamate and alanine) than others. For example, the presence of proline and other amino acids with bulky side groups (e.g. aromatic side groups) are not conducive to the formation of an α-helix [22, 24].

The β-sheets are, just like α-helices, stabilized by hydrogen bonds. They are composed of two or more segments of a polypeptide chain, adopting a zig-zag conformation, where the amino acid side chains protrude below and above the plane of the sheet. The β-strands that compose the β-sheet may or may not be present in the same polypeptide. When the β-strands are oriented in the same direction (the orientation of the N-terminus and C-terminus of each strand match), the β-sheet is described as parallel and when the

orientation is in alternate directions (the N-terminus of one strand is positioned next to the C-terminus of another strand), it is described as antiparallel. It is also possible to find both orientations in the same β-sheet, which is thus described as a mixed sheet [22].

Frequently, many proteins display loop regions which separate different segments of β-sheets or/and α-helices. These regions are usually located on the surface, are flexible and display an abundance of polar/charged amino acid residues. Loop regions are often important not only for connecting different secondary structural elements but also for the biological function of the protein [22].

The tertiary structure corresponds to the overall three- dimensional structure of the polypeptide, relating to the special arrangement of the amino acid residues that constituting the protein. Larger proteins (≥ 150 residues) may also be organized into more than one structural unit, forming domains. This structure arises from various interactions that stabilize the proteins structure [24]. These forces will be mentioned in section 2.1.1 on protein folding and stability.

Some proteins are comprised of more than one polypeptide chain, where each polypeptide chain is termed subunit. The interactions between the different subunits in a protein comprise the quaternary structure. The forces involved in these interactions are the same as the ones responsible for the tertiary structure and will be discussed in the next section.

2.1.1 PROTEIN FOLDING AND STABILITY

The biological function and activity of proteins is highly dependent on the three-dimensional structure. A protein in the native state (N) is a functional protein where only amino acids in the surface interact with the solvent and most amino acids interact with each other. Even though a protein in the native state has an overall stable structure, this structure is not rigid, i.e. there is a certain degree of freedom in movement. On the other hand, a protein in a denatured state (U) is not functional and may display an assemblage of more or less folded states [25].

Protein folding is a complex problem: how is the 3D structure of the proteins "encoded" in the amino acid sequences? Protein sequences are encoded in the nucleic acids and this sequential information flow is unilateral. The sequence of amino acids in the primary structure has an important role in determining protein folding due to the chemical properties of each amino acid. For example, hydrophobic residues in small globular proteins are mostly found in the internal core of the protein, bound by Van der Waals forces, whereas charged and polar aa are mostly found in the surface [26]. The intermolecular interactions between the amino acid side chains and the polypeptide backbone's flexibility generally dictate the specific conformation of the protein. Energetic stability is crucial in a proteins native overall conformation and there are four major contributions that influence the free energy of a protein: hydrophobic interactions, hydrogen bonds, electrostatic interactions and conformational entropy as a result of restrictions in the motion of the main polypeptide chain and side chains [26].

Hydrophobic interactions are an extremely important contributor for the stability of protein native structure. In proteins, most non-polar amino acid residues tend to be buried in the interior of the folded protein. This is related to the propensity of non-polar substances to minimize contact with polar solvents and is referred to as the "hydrophobic effect". Hydrogen bonds are also important for protein stability; although these may be non-specific (intramolecular hydrogen bonds in a folded polypeptide can be replaced by hydrogen bonds with the solvent in an unfolded state). Electrostatic interactions such as ionic interactions and salt bridges work in a more specific manner, playing a major role in the overall protein conformational stability [22, 26]. Covalent linkages (e.g. disulfide bonds) are stronger than the hydrogen bonds and hydrophobic interactions and have great influence in the stability and conformation of proteins and especially in peptides due to their smaller size and their tendency to form fewer hydrophobic interactions [27].

Protein stability is usually seen as its resistance to unfolding. Stability is influenced not only by the physicochemical properties of the protein but also by the surrounding environment. Temperature, pH, solvent polarity and ionic strength are some of the factors that have great influence in protein stability [25]. For example, proteins with higher melting temperature (T_m) tend to be

more stable. A study performed with homologous proteins from thermophiles and mesophiles have shown that the T_m for the thermophiles is on average 31.5˚C higher [28].

Protein folding and unfolding is described in the simplest of ways as involving one single cooperative transition in which the native state (N) and unfolded state (U) interconvert. These two states are divided by an activation free energy barrier. The native state of a protein displays a distinct conformation and is characterized by the lowest free energy, whereas the unfolded state is characterized by a high degree of disorder [25, 26]. The folding and unfolding of a protein is not always such a straightforward process, especially for larger proteins. In order to form a completely folded native state, larger protein usually form partially folded intermediate states initially. In protein engineering, some stabilizations strategies are employed in order to increase protein stability. They usually are employed in order to shift the folding-unfolding equilibrium towards the folded form, either by stabilizing the folded form or destabilizing the unfolded form. For proteins that usually unfold in a stepwise manner, i.e. forming metastable intermediates, stabilization must occur in the region or domain that unfolds first. Some of the strategies used for protein stabilization are the restoration of residues conserved in homologous proteins, addition of disulfide links, addition of Proline residues, substitutions near or in flexible regions, improvement of the hydrophobic packing, optimization of the electrostatic interactions and random mutagenesis methods [25]. Successful substitutions made in order to improve protein stability must be made in a way that the binding ability or catalytic activity of the protein is not affected.

Moreover, proteins also display a tendency to spontaneously assemble in certain conditions, e.g. with concentration increase or with changes in solvent conditions, leading to aggregation and agglomeration of proteins [29]. Protein spontaneous assembly does not always involve misfold. Agglomeration refers to protein assembly in a folded state, where native contacts are not lost. However, aggregation is an irreversible process in which unfolded or partially unfolded proteins associate forming insoluble particles [30]. Denaturation of a protein induced by heat is usually irreversible due to aggregation of the unfolded form (e.g. cooking an egg). In diseases like Alzheimer's and Parkinson's disease among others

neurodegenerative diseases, proteins are misfolded and aggregate forming insoluble particles [31, 32]. Aggregation and chemical degradation can also be a major problem in terms of pharmaceutical formulations stability. Administration of aggregates increases the possibility of an immune response [33–35]. While proteins may agglomerate on pharmaceutical formulations reversibly forming small dimers and oligomers, this can be reversed by optimization of solution conditions (dilution, pH and salt concentrations). Aggregation, however, leads to the formation of net-irreversible species that may only be dissociated under certain conditions like high pressure, high temperature and strong chemical denaturants where most of the proteins in the aggregate lose their native conformation [29].

2.1.2 DISULFIDE BRIDGES AND THEIR ROLE IN PROTEIN STABILITY AND FUNCTION

Disulfide (SS) bonds or bridges the most common type of covalent bond in proteins, second only to peptide bonds. These covalent linkages are formed between two sulfur atoms of two cysteine residues (cystine residues). Most SS bonds are formed as the protein folds and their formation is catalyzed by a variety of enzymes (e.g. glutathione peroxidase, protein disulfide isomerase and peroxiredoxin IV) [36]. Oxidative folding occurs mainly in the endoplasmic reticulum but it has also been suggested that it may also occur in the Golgi apparatus. However, the SS bonds found in mature proteins do not remain inert. Cleavage of SS bonds in some proteins is related to protein function control [37].

SS bonds are most commonly found in extracellular proteins as the environment within the cell is predominantly a reducing environment and they are present in around 10% of the proteins in mammalian cells [22, 36, 38, 39]. In the extracellular environment, the SS bonds provide protection for the reactive thiol groups and also contribute to protein function and stability [36]. A predominant view has been that SS bonds are the result of evolution in order to increase stability of proteins in extracellular environments, which are prevalently oxidizing environments and with varying physicochemical properties [37, 40].

SS bonds can be classified as structural and functional. The majority of SS bonds are structural, playing a major role in folding and stabilization of the tertiary and quaternary structure of the proteins. The structural bonds reduce the entropy of the unfolded form of the protein making this state less favorable than the folded state therefore assisting protein folding [37, 41, 42]. Moreover, they protect the proteins from oxidative damage and proteolytic enzymes found in the extracellular environment [37, 38]. SS bonds keep the proteins and peptides in a particular conformation, however, they can accommodate certain conformational changes [38]. The functional bonds regulate protein function. These functional SS bonds can be catalytic (e.g. oxireductases) or allosteric (e.g. CD4 cytokine receptor). Catalytic SS bonds are commonly found in active sites of enzymes that mediate thiol/disulfide exchanges. Allosteric SS bonds control protein function when their oxidation/reduction induces conformational changes [41, 42].

The study of SS bond formation and function can provide important insights on how to improve protein stability for various applications. Various strategies have been employed to study the role of SS bonds. Some of them consist on performing refolding experiments using a folding buffer (e.g. mixture of glutathione) after replacing the selected cysteine pair with alanine, serine residues or aminobutyric acid. Another strategy that has been employed is the performance of reductive unfolding experiments. In these experiments, the kinetics and conformation during unfolding are studied as the protein is exposed to a strong reducing agent such as dithiothreitol (DTT) and the progressive loss of SS bonds upon unfolding is monitored. Monitoring can be performed through X-ray crystallography, fluorescence spectroscopy, circular dichroism, or nuclear magnetic resonance (NMR) [27].

Some studies focus also on the geometry, prevalence and distribution of SS bonds among various proteins [40, 43–45]. The geometry and the interactions of the SS bond with the rest of the protein determine its conformation and stability [40]. Since native SS bridges confer considerable stability to proteins, they are good candidates for protein engineering. The introduction of SS bonds alters the thermodynamic stability of a protein as they contribute to changes in free energy. It is hypothesized that they are more effective when located in flexible regions of the proteins or when

creating a large loop. Furthermore, SS bonds can also disturb the folding and unfolding rates [46]. One hypothesis is that the SS bonds reduce the entropy of the unfolded state by reducing the conformational freedom of the protein in this state. Another explanation is that the unfolded state is destabilized by SS bonds because they hinder the formation of an adequate hydrogen bonding network [27]. However, not all engineered SS bonds lead to an increase in protein stability [47–49] and there are various studies performed in order to study protein dynamics and to improve or modify protein function with engineered SS bonds [46, 50–53].

It has been also shown that SS bonds are highly conserved in nature. Wong et al. [54] performed a study where the conservation of structurally validated SS bonds from 29 eukaryotic genomes found in the Protein Data Bank was analyzed. The study showed an elevated conservation of half-cystines (SS bonded cysteines) in contrast with other unpaired cysteines and other amino acids, including tryptophan (the most conserved amino acid) [54]. Furthermore, another important observation performed by Petersen et al [40] on 131 non-homologous proteins showed that the average number of SS bonds in a protein decreases exponentially with an increase in the length of the amino acid chain. An explanation for this phenomenon might be that, since smaller proteins lack a strong hydrophobic core, SS bonds have a higher contribution in these proteins structural stability. In this study, it was also observed that SS bonds show preferential special neighbors such as leucine, isoleucine, valine methionine (hydrophobic residues) and especially with tryptophan, tyrosine and phenylalanine (aromatic residues). The observations also indicate that this is also a conserved pattern throughout evolution [40].

2.2 PHOTOCHEMISTRY AND LIGHT INDUCED PROCESSES ON PROTEINS

Proteins compose a high percentage of the body's dry height and, therefore, become a major target for oxidative damage, namely photo-oxidation. The exposure to visible or UV light may lead to loss of protein structure and function, depending on the time and intensity of exposure.

Proteins have intrinsic fluorescence, which typically occurs from aromatic molecules [13]. Tryptophan, tyrosine and phenylalanine have aromatic side chains (Figure 2) and therefore, possess fluorescence properties. This property also makes these amino acids major targets for photo-oxidation. Tryptophan is the amino acid with the highest side chain hydrophobic parameter π constant at neutral pH [55], which might explain also why the fluorescent properties of this amino acid are so dependent on the surrounding environment. Additionally, it has the highest molar absorption coefficients, even though it is one of the least abundant amino acids found in proteins. These three amino acids absorb light in the UV light region around 280-305nm (tryptophan), 260-290nm (tyrosine) and 240-270nm (phenylalanine) in aqueous solution [56] and their frequency in proteins is approximately of 1.4%, 3.2% and 4.0% , respectively [24]. Tryptophan is often used as an intrinsic probe to study changes in protein structure due to high sensitivity of its spectral properties to changes in the surrounding environment.

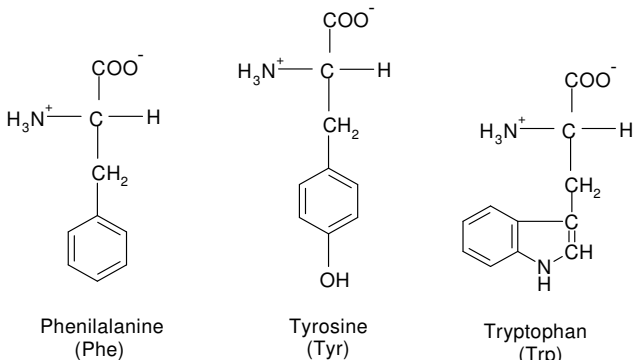

Figure 2. Structure of Phenilalanine, Tyrosine and Tryptophan.

Amino acids that contain aromatic residues as side chains (e.g. tryptophan, tyrosine and phenylalanine) are very susceptible to photochemical induced processes such as degradation by both excited singlet or triplet states and reactive oxygen species (ROS), such as superoxide, peroxide and hydroxyl radicals. Amino acids containing side chains with sulfur residues such as Cysteine (cys) and Cistines (cys-cys) are also prone to photochemical degradation as well histidine and methionine. Upon UV-light absorption, the side-chains of these light absorbing amino acids can undergo direct photo oxidation by generation of singlet or triplet excited states or radicals

resulting from photoionization. This is known as type I oxidation. Numerous factors influence the processes that follow the excitation to higher energy states such as protein structure, pH, polarity, temperature and nearby side chains. After reaching an excited state, one of many processes can occur, such as relaxation to the ground state (e.g. trp fluorescence emission) or formation of a triplet state, ejection of an electron onto the aqueous medium and reactions with oxygen forming free radicals that lead to other photo-oxidation pathways. This more indirect approach, where singlet oxygen species are generated by energy transfer to ground state molecular oxygen are referred to as type II oxidation. The formed singlet oxygen species can undergo further reactions and interact with biomolecules, inducing further oxidation [57]. ROS can be formed as a result of photo-oxidation, but also as a result of various metabolic and biochemical reactions and their effect go beyond protein degradation. Various autoimmune and degenerative human diseases associated with the ROS and their effect oxidative effect on DNA [58–60]. In this thesis, we are going to focus mainly on some of the photochemical processes that occur after UV light exposure of tryptophan, tyrosine and cysteine as they are the basis of the work performed during this PhD.

Tryptophan is prone to oxidation and this can occur through multiple pathways such as photo-oxidation or by reaction with reactive oxygen species. The pathways and products of tryptophan oxidation have been extensively studied and described in the literature [61–67]. In this review, we are going to focus on some of the main described photo-oxidation processes, though many others may occur. Some of these photochemical processes are also described in **paper 3** [3]. Upon absorption of UV light, tryptophan reaches an excited state that can be followed by a number of processes: relaxation to the ground state with fluorescence emission, relaxation through a non-radiative channel that can lead to electron ejection (e^-_{aq}) and formation of a $Trp^{\bullet+}$ radical cation that promptly deprotonates, yielding a neutral indolyl radical $Trp^{\bullet}$ (schemes 1 and 2) or intersystem crossing with the formation of a triplet state ^3Trp (scheme 3 and 4).

$$Trp + hv \rightarrow Trp^{\bullet+} + e^-_{aq} \quad (1)$$

$$Trp^{\bullet+} \rightarrow Trp^{\bullet} + H^+ \quad (2)$$

$$^1Trp + hv \rightarrow {}^1Trp^* \quad (3)$$

$$^1Trp^* \rightarrow {}^3Trp \quad (4)$$

These transient species can be detected as they present different spectral properties from the parent molecule. For example, the solvated electron (e^-_{aq}) displays a broad absorption peak with its maximum at ≈ 720 nm [68, 69], tryptophan radical cation $Trp^{\bullet+}$, has an absorption maximum at ≈ 560 nm, the neutral indolyl radical $Trp^{\bullet}$ has an absorption maximum at ≈ 510 nm and triplet-state tryptophan ^{3}Trp absorbing at ≈ 450nm [70]. The ejected electron yielding from the relaxation processes can be caged by nearby water molecules and be transferred to nearby disulfide bridges RSSR. Upon electron transfer, a disulfide bridge adduct is formed $RSSR^{\bullet-}$ (absorption maximum at ≈ 420 nm) (scheme 5), ultimately leading to the SS bridge disruption upon adduct dissociation and the formation of free thiol groups RSH (scheme 6 and 7).

$$e^-_{aq} + RSSR \rightarrow RSSR^{\bullet-} \quad (5)$$

$$RSSR^{\bullet-} \leftrightarrow RS^{\bullet} + RS^- \quad (6)$$

$$RSSR^{\bullet} + H^+ \leftrightarrow RS^{\bullet} + RSH \quad (7)$$

This SS bridge adduct can also be formed upon electron transfer from a tryptophan triplet state ^{3}Trp (scheme 8), ultimately leading to SS bridge disruption (scheme and 6 and 7).

$$^3Trp + RSSR \rightarrow Trp^{\bullet-} + RSSR^{\bullet-} \quad (8)$$

The solvated electrons resulting from the processes described above can also react with electrophilic species such as molecular oxygen or undergo fast geminate recombination with the parent molecule. Temperature, pH, ionic strength and polarity are some of the factors that affect the proportion in which these solvated electrons are formed upon UV light absorption [56].

In a more indirect photo-oxidation process, the tryptophan reaction intermediates can also react with ROS species generating various products, among which are kynurenine (Kyn) and N-formyl-kynurenine (NFK). These two photoproducts can act as photosensitizers to visible light and induce

further damage to the proteins, as the maximum absorption wavelengths of both photoproducts (absorption maximum of NFK at 325 nm and of Kyn at 360nm [71]) are at longer wavelengths than 280nm (tryptophan absorption maximum) [56].

Similarly to tryptophan, a varied number of processes can occur for tyrosine upon UV light absorption and excitation to higher energy states. Just as described for tryptophan, photo-excited tyrosine can relax onto the ground state in a radiative (fluorescence emission) or non-radiative manner. The non-radiative relaxation channels involve electron ejection or intersystem crossing to the triplet state [56]. In the triplet state and at neutral pH, ^{3}Tyr can transfer energy to nearby tryptophan or undergo photoionization through a biphotonic process involving the absorption of a second photon, resulting in formation of a solvated electron e^-_{aq} and a phenoxy radical cation (Tyr-$OH^{\bullet+}$) that rapidly deprotonates, yielding the neutral radical (Tyr-$OH^{\bullet}$) (schemes 9 and 10)

$$^3Tyr - OH + hv \rightarrow Tyr - OH^{\bullet+} + e^-_{aq} \quad (9)$$

$$Tyr - OH^{\bullet+} \rightarrow Tyr - OH^{\bullet} + H^+ \quad (10)$$

The OH group of tyrosine side chain deprotonates at alkaline pH, resulting in the formation of tyrosinate (Tyr-$O^{\bullet-}$). Tyrosinate displays a maximum absorption at 240nm and 290nm as opposed to tyrosine which displays a maximum of absorption at 220 nm and 275 nm (red-shift). At high pH, photoionization of Tyr-$O^{\bullet-}$ is monoprotic, resulting in the formation of a tyrosyl neutral radical Tyr-$O^{\bullet}$ and a solvated e^-_{aq} (scheme 11).

$$Tyr - O^{\bullet-} + hv \rightarrow Tyr - O^{\bullet} + e^-_{aq} \quad (11)$$

SS bridges located in the vicinity of tryptophan residues and molecular oxygen promptly quench ^{3}Tyr (scheme 12).

$$^3Tyr - OH + RSSR \rightarrow Tyr - O^{\bullet} + H^+ + RSSR^{\bullet-} \quad (12)$$

This leads to the formation of a disulfide adduct $RSSR^{\bullet-}$. This adduct can also be formed upon capture of the solvated electron by nearby cysteines, ultimately leading to the breakage of the SS bridge (schemes 5, 6 and 7).

Tyrosyl radicals can be produced by many processes. The full photochemical processes are extensively described in the literature [56, 72, 73] and are not the scope of this thesis. However, it is important to mention that these radicals are the main precursors for the formation of dityrosine. Dityrosine results from the crosslink between two tyrosyl radicals that may occur either intra or intermolecularly. As described for the photoproducts resulting from tryptophan degradation, Kyn and NFK, dityrosine formation may also be monitored due to its particular fluorescence properties (λexcitation= 315nm, λemission=410-415nm). Its formation can be a natural phenomenon or a response to environmental and stress factors and it can be used as fluorescent probe to obtain structural and functional information on proteins [74]. The presence of dityrosine cross-linking in proteins has often been used as a biomarker for oxidative stress [75].

Photo-induced protein oxidation can have consequences for protein structure and function, such as protein unfolding, aggregation, fragmentation or inactivation [65]. Many individual factors play a role on these processes and the extent of damage induced is likely dependent on each individual protein's structure and function.

2.2.1 PROTEIN STABILITY AND EXPOSURE TO UV LIGHT

The processes described in the previous section are only some of the multiple processes that may occur during protein photo-oxidation. Other processes can occur both during and after radiation exposure and may lead to altered chemical and physical properties, protein aggregation and fragmentation. All these phenomena can have an impact on a cellular and tissue level leading to dysfunction of cellular processes and emergence of various pathologies [65]. Oxidative damage in proteins is linked with aging, where the affected proteins can lead to degeneration of the cellular processes and eventually cell death. This may be a consequence of reduced efficiency in transcriptional/translational processes leading to a higher number of aberrant proteins [76]. Furthermore, oxidation plays a major role in age-related nuclear (ARN) cataract, where a loss in over 90% of sulfhydryl groups and oxidation of about half of the methionine residues is observed in the most advanced stages [77].

Oxidation can also have great influence in proteins used for industrial applications such as, e.g. the dairy industry, where oxidation of particular amino acids can lead to an off-flavor or affect enzymatic processes fundamental for diverse dairy processes [78]. UV sterilization processes are common in the food industry and may also lead to photo-induced damage [79]. Photo-induced oxidation is also a major issue to consider in the pharmaceutical industry. Therapeutic proteins (e.g. antibodies, insulin) may be adversely affected by natural or artificial light exposure (UV and visible light) from their manufacturing process (e.g. purification process, packaging, storage, handling, etc.) until their administration to patients [56, 80, 81].

Some proteins are susceptible to prolonged UV light exposure, leading to unfolding and conformational changes. The conformation and structure of individual proteins largely dictates the extent of photo-induced damage. In a study performed by Dalsgaard *et al.* [78] on changes induced to milk proteins upon light exposure, it was observed that random coil proteins (e.g. casein) were more prone to damage in individual amino acids than tightly packed globular proteins (e.g. α-lactalbumin). However, changes in secondary and tertiary structure were found to be more pronounced in globular proteins.

Aggregation of proteins is another potential effect of UV light illumination. The formation of aggregates can result from various reactions such as intermolecular dityrosine cross-linking [82, 83], disulfide bond photolysis [56] and indirect oxidation of histidine by ROS formed upon UV excitation [84, 85].

The effect of UV light exposure on enzyme and hormones function has been recognized for many years [56, 86, 87]. Enzymes containing UV sensitive amino acids (e.g. tryptophan, tyrosine and cysteine) in their active sites are more prone to UV light inactivation. As described in section 1.1.2, SS bonds have an important role in maintaining protein structure and, in some cases, are also linked to protein function. Breaking these SS bonds can lead to loss of protein function. This was observed for cutinase in a work performed by Petersen et al. [87], where a 50% loss of lipolytic activity was observed upon UV illumination (295 nm). However, in some cases, SS bond breakage can lead to an increase in proteolytic activity, such as observed for plasminogen

upon low dose UVB excitation of the aromatic residues nearby the SS bond [88].

Understanding the effects of UV light on proteins and the identification of UV triggered mechanisms that induce protein damage is of the outmost importance. It is relevant for safety and quality of food and pharmaceutical products as well as for the identification of photo oxidative pathways in human diseases. The hazardous effects of UV and visible radiation in various biomolecules have been made widely known. UV exposure is widely associated with aging, skin erythema and skin cancer [79]. Even though DNA damage is a major contributor to the development of skin cancer, photo-induced damage to key proteins also plays an important role [89].

Nonetheless, conventional photodynamic therapy (PDT) resorts to UV/Visible light [90]. In **paper 8** [8] the therapeutical application of UV light and the benefits of UVB light are briefly reviewed. The particular application of UV light as a photonic cancer therapy will also be addressed in section 3 and **papers 6, 7 and 8** [6–8].

2.2.2 PROTEIN PHOTOCHEMISTRY AND THE STUDY OF PROTEIN STRUCTURE: TWO PROTEASES CASE STUDIES

Protein structure and stability are important parameters to consider. Various factors have an effect on this stability, which can be translated into effects on protein structure and interaction with various molecules. In the previous section, the importance of light induced changes in proteins in industrial processes was outlined. As described in section 2.2, tryptophan and tyrosine, along with some other amino acids, are prone to degradation by photo-oxidation. The susceptibility of individual proteins to oxidative damage is variable but correctly folded proteins are the most resistant [91]. However, it important to also consider other factors that may influence the individual proteins resistance to damage. As mentioned in section 2.1.1, various factors influencing protein stability. The study of these factors (e.g. pH, temperature and photo-induced damage) are an important feature to measure protein stability when, for example, developing and characterizing proteins for industrial and biotechnological purposes.

Techniques such as fluorescence spectroscopy and Circular Dichroism (CD) spectroscopy are valuable tools to study these effects on protein structure. Fluorescence spectroscopy can be used to perform studies on protein folding [92–94], protein structure [95, 96], protein-ligand interactions [97, 98], etc. These studies can be performed using extrinsic probes (e.g. 8-Anilino-1-naphthalenesulfonate (ANS), Nile Red, among others) [99], natural fluorophores or using intrinsic fluorophores. This technique provides the means to make such protein characterization studies due to the versatility, high sensitivity, low-cost and amount of information that can be extracted from the obtained data [100]. It is a highly sensitive technique that can obtain reproducible signals at low fluorophore concentrations (bellow 1 nM). Moreover, the data obtained can be analyzed in a multitude of ways, according to different aspects of fluorescence. A fluorescence signal can be analyzed in terms of intensity, wavelength, lifetime, rotational freedom (anisotropy or polarization) and energy transfer [101].

Proteins display intrinsic fluorescence due to the presence of tryptophan, tyrosine and phenylalanine, which are fluorescent. Phenylalanine fluorescence is weak and therefore rarely used in protein studies [100]. The fluorescence data obtained can be interpreted due to the relatively rare abundance of these three amino acids in proteins. Tryptophan is the most interesting intrinsic probe due to its high sensitivity to the local environment surrounding the indole ring which leads to spectral changes in emission as a consequence of phenomena such as, e.g., conformational changes, denaturation or changes in polarity. Tryptophan is also very sensitive to collisional quenching and quenching by nearby groups in proteins [13, 100]. Furthermore, the monitorization of tryptophan's intrinsic fluorescence allows for the study of protein structure and function without altering it [102]. For these reasons, many studies use tryptophan's unique spectral characteristics to study changes in protein structure.

Circular Dichroism (CD) is also a good technique to study the secondary structure of proteins [103–105]. Briefly, CD can be described as the different absorption of right-handed and left handed circularly polarized light. In proteins, different structural elements such as α-helix and β-sheets have different CD absorption spectra. Proteins with α-helical structure display band with peaks at 193nm (positive band), 208 nm and 222 nm (negative

bands). In contrast, if the protein displays well-defined β-sheets the CD spectra will exhibit bands at 195 nm (positive bands) and 218 nm (negative bands). Given that the CD spectra of each protein is highly dependent on its structure, this technique is useful to assess changes in conformation induced by various factors such as temperature, pH, mutations, etc. [103, 106].

During this PhD work, both fluorescence spectroscopy and CD spectroscopy were used to characterize two proteases. Fluorescence spectroscopy and CD spectroscopy were used to monitor pH-dependent structural and conformational changes induced by UV light radiation (280nm) and temperature in a protease extracted and purified from *Aspergillus tamarii* URM4634. This protease displayed keratinolytic and collagenolytic activity and the characterization of this protease will provide the knowledge necessary to explore its applications. The work is described in **Paper 1**[1].

Fluorescence spectroscopy and CD spectroscopy were also used to study structural changes another protease extracted from *Penicillium aurantiogriseum URM 4622* induced by pH and temperature. The work is described in **Paper 2** [2] (unpublished work).

2.2.3 APPLICATION OF PHOTOCHEMICAL INDUCED REACTIONS

Photochemical reactions are relevant not only for cellular systems but also for the ecosystem. Amino acids are a source of nitrogen in aquatic systems and photochemical reactions can have an impact on the availability, composition and uptake of free and combined amino acids [107]. The degradation of proteins and/or amino acids also has an impact, especially in the pharmaceutical industry [108]. Chemical modifications due to photo-oxidation can lead to an increase in protein degradation or loss of function, formation of cross-links between proteins leading to an increased aggregation or even development of neoepitopes [109].

The study of photochemical mechanisms and a better understanding of their ramifications are extremely relevant and also a valuable tool in the development of new technologies and therapies. Many therapies rely on the use of organic molecules such as proteins and peptides (e.g. antibodies for immunotherapy) or conjugates of these biomolecules with other drugs. This

knowledge on photochemistry can also be applied for the development of visible-light photoredox catalysis, harnessing the potential to perform selective chemical transformations for modification of biomolecules such as proteins and peptides and incorporate other molecules for therapy, with high selectivity and little side effects. Usually, photocatalysts are able to absorb light, reaching higher excited states and acting as electron donors or acceptors due to their ability to incur into single electron transfers with other organic molecules [110]. Photodynamic therapy is a major example of the application of photo-induced oxidation for therapeutic purposes. This topic will be further explored in Section 4.

The majority of all molecules perform their biochemical reactions in the electronic ground state but many reactions and pathways are possible in the electronically excited states. Many electronic states are accessible and for each of them novel reactions and pathways may be available. Since our general knowledge is rooted in ground state biochemistry, we should expect many surprising reactions if excited state molecules are involved.

3. BIOSENSOR MICROARRAY PRODUCTION

3.1 IMMOBILIZATION TECHNIQUES FOR BIOSENSORS

Biochips containing biomolecules for analytical applications present a wide array of possibilities for both research and medical diagnosis. The production of DNA microarrays is now well established and it has promoted a faster and more detailed analysis of data. The technology developed for DNA microarray production can also be used for the production of protein biochips, enabling a faster profiling of whole proteomes, identification of disease related proteins and study of protein-protein and protein-drug interactions by binding with protein functionalized surfaces. However, the production of protein microarrays is a more complex task due to the sensitive nature of proteins. The chemical treatment necessary for protein immobilization and interactions between the proteins and the surface can lead to changes in protein conformation, mobility and functionality [111, 112]. Thus, the choice of material for the solid surface and the choice of right surface chemistry in order to achieve protein immobilization while maintaining proteins structure and function is one of the main challenges in the production of protein biofunctionalized surfaces [112, 113]. Other important aspects to consider in the production of protein chips are the control over the orientation of the proteins attachment to the surface and the number of steps performed prior to protein immobilization. Selectivity over the functional groups of the protein that interact and/or are attached to the surface is advantageous, as it allows the control over the orientation preferentially adopted by the immobilized proteins on the surface. In line with the importance of achieving protein immobilization with defined surface orientation, a lot of focus has also been given in recent years to the immobilization of proteins in controlled patterns and with micro and nano-scale precision, especially in the production of biosensors [112]. Furthermore, the detection strategy used for protein biochips is of importance. A varied range of techniques is employed and these can be broadly divided into two categories: label-free and labeled detection methods [114]. Surface plasmon resonance, atomic force microscopy and mass

spectrometry based methods are some of the main label-free detection methods used. Labeled detection methods incorporate many strategies such as fluorescence, radioactive and chromogenic labeling. These methods are also used in other classic protein detection methods such as western blotting and enzyme-linked immunosorbent assays (ELISA).

Chemical activation of the chip surface is one of the paramount factors for the production of functional protein biochips [112, 115]. The intended application will have influence on the choice nature of the surfaces. These surfaces usually are adequate for modification with a broad range of chemical moieties in a reproducible manner, present chemical stability and homogeneity and controllable surface features [112]. Commonly used planar chip surfaces are glass, silicon, gold and some polymers [112, 115]. 3D matrixes can also be used to immobilize proteins [116]. In order to functionalize these surfaces, different approaches can be taken. In line with the scope of this thesis, more focus will be given to chemical modification of glass and silicon surfaces.

Silanization is the main method for glass surfaces functionalization. Organofunctional silanes containing a desired functional group are used for this purpose. A variety of functional groups such as carboxy, epoxide, thiol and others can be found in commercial silane reagents. First, reactive silanol groups (Si-OH) can be generated in these surfaces by incubating them in concentrated acid and base solutions in the presence of oxidants, e.g. pre-treatment using a piranha solution[1], oxygen plasma [112]. The alcoxy groups found in the silane reagent with the desired functional group can be hydrolyzed by surface water and react in a covalent manner with the silanol groups available in the surface. At last, the surface goes through thermal curing in order to yield cross-linking of free silanol groups. These chemical reactions result in a surface with a covalently bound silane layer with a reactive group. There is a wide variety of silanization protocols reported in the literature [117–120].

A wide range of strategies have been investigated in order to attach proteins (e.g. enzymes, peptides, antibodies, Fab fragments) to chemically modified surfaces. More conventional methods rely on a non-oriented immobilization

[1] 3:1 mixture of H_2SO_4 and 30% H_2O_2

of proteins to the surface either by non-covalent attachment or covalent attachment. Proteins can adsorb to surfaces by forming ionic bonds, polar and hydrophobic interactions or even through electrostatic interactions. All these interactions are of a non-covalent nature [112]. These interactions will be highly dependent on the surface features of the protein and the nature of the material of the chosen surface. For example, environmental conditions such as pH, temperature and ionic strength will have influence on the adsorption process when driven by electrostatic interactions as the charges on the protein surface and on the material surface can be shielded. Additionally, the layer of adsorbed proteins is likely to be heterogeneous as each molecule is capable of establishing more than one interaction in different orientations both with the surface and with previously adsorbed proteins.

Other strategies used for protein immobilization are chemical crosslinking, enzymatic immobilization, protein modification, affinity peptides, affinity protein [121]. Many of the more classical protein immobilization strategies involve one or more steps of chemical and/or thermochemical treatments or may require the introduction of external chemical groups. Often these immobilization techniques are also strenuous and time consuming [112]. Thus, there is a major interest in the development of new immobilization techniques that can overcome some of these throwbacks. In this sense, photochemical reactions are a good alternative to explore for the development of new immobilization technologies.

Most light-dependent immobilization strategies involve the activation of photosensitive reagents with light at appropriate wavelengths which mediate the immobilization process. The photosensitive reagents are normally coupled to the material surfaces or to the biomolecules prior to illumination. The activation of the photosensitizer triggers photochemical reactions that lead to covalent bonding between the surfaces and the generated photospecies/intermidiates [112, 122].

During the work performed on this PhD a photonic immobilization technique was used to immobilize various biosensor molecules onto thiol derivatized surfaces in a covalent and spatially oriented manner (see section 3.2).

3.2 LIGHT ASSISTED MOLECULAR IMMOBILIZATION (LAMI)

In section 2.2, the photochemical processes undergone by amino acids such as tryptophan and tyrosine were described. Upon UV light absorption, the transient species formed could react with cysteines, leading to the breakage of the SS bridges linking the two cysteins and to the formation of free thiol groups. This UV light induced photochemical reaction is the basis of the technology for protein immobilization named Light Assisted Molecular Immobilization (LAMI). LAMI is a light-dependent immobilization strategy that does not require the present of external photosensitizers as it relies on photochemical processes induced directly on endogenous photosensitive groups on the proteins. LAMI relies on the formation of free thiol groups upon UV excitation of aromatic residues, which become available to bind to a thiol reactive surface. The free thiol groups in the protein bind in a covalent and spatially oriented manner to the free thiol groups in the sensor surface (Figure 3).

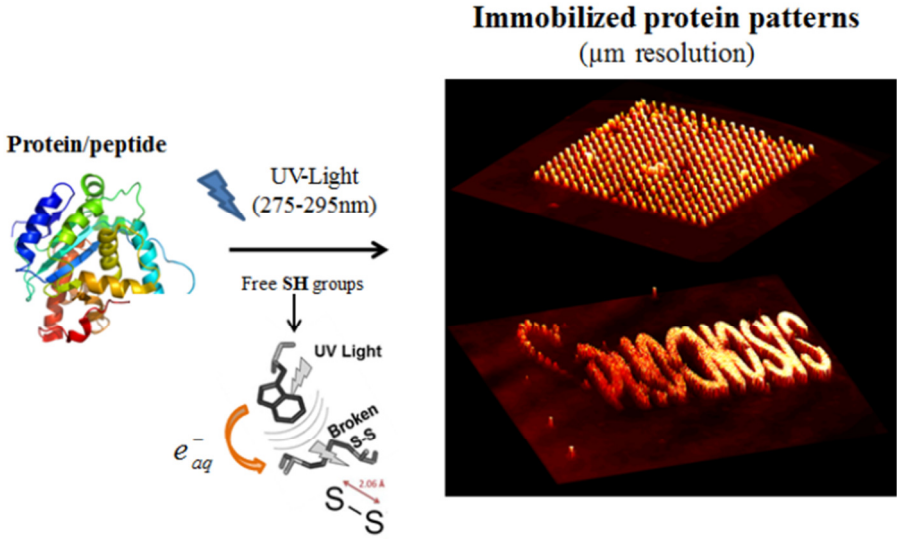

Figure 3. Schematic representation of Light Assisted Molecular Immobilization (LAMI). Upon UV light excitation, the aromatic residues located nearby the disulfide bridges may eject an electron that leads to the disruption of the SS bridges and results in the formation of free thiol groups that can covalently bind to free thiol groups in the support surface.

Immobilization of proteins in a spatially oriented manner is preferred as this will maximize the exposure of the proteins biorecognition site to the analytes while minimizing or limiting the regions of contact of the protein to sites that do not interfere with biorecognition or are involved in conformational changes necessary for biorecognition [121].

Our research group previously demonstrated that the reduction of SS bridges occurs upon UV excitation of models proteins such as cutinase [123], bovine serum albumin (BSA) [124], lysozyme, antibody Fab fragments [123, 125], Prostate specific antigen [126], insulin [127] and plasminogen [88]. The structure of these proteins is known and it was observed that the proteins contain SS bridges with aromatic residues in close spatial proximity. This spatial proximity between aromatic residues and SS bridges is a conserved pattern in nature. Petersen *et al.* [40] investigated 3D environment of 131 non-homologous, single chain proteins concerning cysteines and their contact with the surrounding residues. It was observed that free cysteines (cysteines not bound to other cysteines by SS bridges) are more buried inside the proteins than half cystines (cysteine involved in a SS bond with another cysteine). Furthermore, half cystines were observed to have a high number of contacts with tryptophan. This aromatic residue does not show preference for half-cystines in the sequential analysis but it is the most overrepresented amino acid in the spatial environment around SS bridges.

The thiol reactive surfaces used as support for LAMI were prepared using a silanization protocol. In section 2.1, this process is mentioned as being a commonly used technique for surface chemical activation. For LAMI, the used silane was 3-mercaptopropyl-trimetoxysilane. This silane reagent allowed the introduction of free thiol, groups in the surface (Figure 4). The chemical activation of the surfaces for LAMI is described in various publications [3–5]. The surfaces used for LAMI immobilization during this PhD project were optically flat quartz slides (extremely polished surface with Å precision on one side of the slide) and Silicon on Wafer (SOI) surfaces.

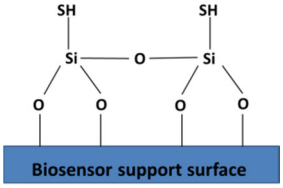

Figure 4. Schematic representation of the thiol reactive surface used for LAMI. The silanization protocol allows the introduction of free thiol groups in the surface that can bind covalently to free thiol groups resulting from the disruption of the disulfide bridges as a consequence of UV light illumination of the nearby aromatic residues.

LAMI is an immobilization technique with already successful development of biosensor microarrays [123–126, 128–131] and thiol-reactive nanoparticles biofunctionalization [124]. In past work, it was demonstrated that immobilization was achieved in a expedite way, with high precision and in a controlled manner [126, 128, 129, 132]. During the work developed throughout this PhD, LAMI was performed in a customized optical setup with various improvements over the previously reported optical setup in earlier works. The description of the new optical setup and the new applications developed are described in **paper 3** [3]. Briefly, the thiol derivatized surface containing the sample is placed on a computer controlled stage, under an UV objective through which 280nm 8MHz femtosecond pulses (100-150 fs) are focused onto the sample. The stage and shutters are controlled by software where the desired illumination pattern is programed and the different exposure parameters are set (e.g. speed of the moving stage, time of exposure, size and shape of the pattern, etc.).

The application of LAMI in this PhD was manly divided into the immobilization of biosensors for cardiovascular diseases (section 3.2.1, **paper 4** [4]) and immobilization of molecular beacon constructs for the detection of miRNA cancer biomarkers (section 3.2.2, **paper 5** [5]). The main aims of this work were: to optimize LAMI procedures on a new optical setup, to achieve biosensor immobilization and to test the bioavailability of the biofunctionalized surfaces.

3.2.1 LAMI IMMOBILIZATION OF BIOSENSORS FOR THE DETECTION OF CARDIOVASCULAR DISEASE BIOMARKERS

An early diagnosis of CVD can help in the reduction of the mortality rate resulting from this disease, which is one of the leading causes of death worldwide. In order to achieve a timely diagnosis and effective treatment, the identification of specific cardiac biomarkers is crucial. A substantial effort has been made over to identify new cardiac biomarkers and to achieve maximum efficiency in their detection by routine tests. Various cardiac biomarkers have been identified for different applications, stemming from screening to diagnosis and some are still under evaluation. However, cardiac troponin still remains the biomarker of choice for early diagnosis of CVD.

The work focused on the immobilization of antibodies on thiol reactive surfaces to perform the recognition of four selected cardiac biomarkers: Troponin I, Troponin T, Myoglobin and C- reactive protein (CRP).

Cardiac troponin is a highly specific biomarker for CVD that is reportedly found in high amount and for a prolonged time in circulation. These characteristics make it still the biomarker of choice for early diagnosis of CVD. Troponin is a complex formed by a total of three subunits: troponin I, troponin C and troponin T. This protein complex plays a major role in the regulation of cardiac muscle relaxation and contraction through Ca^{2+} regulation. Each subunit performs a different role in Ca^{2+} regulation and all subunits display isoforms that are tissue-specific. Of these three isoforms, only troponin I and T are commonly used as CVD biomarkers, as troponin C shares its cardiac isoform with slow-twitch skeletal muscles [133–135].

Myoglobin is another recognized biomarker for CVD diagnosis. It is frequently employed together with other biomarkers. Even though this protein can also be found in skeletal muscle tissue, which means it does not present the specificity found for troponin I and T for cardiac muscle tissue, it is the first protein to be released into the blood stream when damage occurs in myocardial muscle cells [136].

C-reactive protein (CRP) is a known marker for inflammation. This protein is a member of the pentaxin family, has a homopentameric structure and is produced in the liver [137]. The levels of CRP in plasma increase rapidly in

the event of infection or tissue injury [138]. The use of this protein as a biomarker for prediction of early cardiovascular events has been debated in recent years [139], but a definitive link has been established between inflammation and CVD [140].

LAMI was successfully used to immobilize antibodies onto SOI surfaces for the recognition of these four biosensors. The immobilization of anti-troponin I and anti-myoglobin antibodies was demonstrated in **paper 4** [4]). The immobilization of anti-troponin-T and anti-CRP antibodies was also achieved (unpublished work).

3.2.2 LAMI IMMOBILIZATION OF MOLECULAR BEACON CONSTRUCTS FOR THE DETECTION OF miRNA CANCER MARKERS

LAMI technology was also used for another biomedical purpose: the immobilization of biosensors for the detection of miRNA cancer specific biomarkers [5].

Early detection of cancer can lead to a higher effectiveness of treatment and a reduction of cancer related deaths. MicroRNAs (miRNAs) have emerged in recent years as a tool with great potential to achieve this goal.

miRNAs are a class of small (approximately 22 nucleotides long) non-coding RNAs involved in post-transcriptional regulation of gene expression. These molecules are transcribed from DNA by RNA polymerase II, resulting in a primary miRNA transcript. This primary transcript is then processed, creating precursor miRNA and ultimately mature miRNA. These mature miRNAs target messenger RNAs (mRNAs) by binding to the 3' untranslated region (3' UTR) or, in some cases and to a less extent, to 5' UTR, gene promoters and coding sequences. This miRNA-mRNA interaction induces mRNA translational repression and degradation [141]. Different mRNAs can potentially be targeted by a miRNA molecule due to the small length of the binding site (6 to 8 base pairs) [142].

There is a high number of identified miRNAs from various organism available online [143, 144]. These molecules have been shown to have an important role in numerous cellular processes [145]. Due to its regulatory

role, changes in the expression patterns of these molecules can be associated with the development of various diseases, including cancer [146–148]. Different types of cancer were observed to have dysregulated miRNA expression when comparing with normal tissue. This altered expression patterns can also be detected between subtypes of cancer, making miRNAs good candidates for cancer diagnosis. These molecules can be detected in different body fluids (although in different concentrations) [149, 150], which allows for a less invasive approach for diagnosis. miRNAs can be secreted to the extracellular space and transported into circulating body fluids in different ways, e.g. encapsulated in extracellular vesicles, or bound to other molecules such as proteins, making them resistant to degradation by RNAses [151, 152].

Since the first report by Calin *et al.* [153] identifying the deletion of miRNA-15a and miRNA-16-1 in chronic lymphocytic leukemia, extensive efforts have been made in order to document and characterize the expression patterns of different miRNAs in various types of cancer [149, 154, 155] and how they are implicated at the various clinical stages of the disease [156]. One of the first identified miRNAs with an oncogenic mechanism (oncomiRs) was miRNA-21. This oncomR is known to target various tumor suppressor genes [157, 158]. The overexpression of miRNA-21 in mammalian cells is associated with different cancer types, including colorectal [159], breast [160], lung [161] and prostate cancers [162].

Molecular beacons (MB) were the chosen biosensors to identify miRNA specific cancer biomarkers, namely miRNA21. The developed work is described in detail in **paper 5** [5] (section 5.5).

4. APPLICATIONS OF UV LIGHT THERAPY IN MEDICINE

In section 1.2.1, the effects of UV light irradiation on protein stability and function were discussed. The hazardous effects of UV and visible radiation are well known and publicized [163–167]. However, some of these light induced processes on proteins have also been used for research and for medical and industrial applications (see section 2.2.3).

In the field of medical treatment, PDT is one of the most notable examples of the use of light for therapeutic purposes. The more conventional PDT therapies rely on UV/Visible light as radiation sources, but in recent years, Near Infra-Red light (700-1100 nm) has emerged as an excitation source [90]. PDT usually consists on the administration of a photosensitizer (a molecule capable of inducing changes in another molecule through photochemical processes) prior to illumination with a specific light source resulting in the formation of ROS species. Photothermal therapy (PTT) is another form of light therapy which relies on light induced hyperthermia. Agents capable of causing a photothermal effect (e.g. metallic based nanomaterials, organic dyes, etc) under specific sources of light are administered prior to irradiation [168]. A more detailed description on PDT is described in **paper 8**.

UV light has a bad reputation for its hazardous effects, yet the damaging effects are wavelength dependent. UVC radiation (100-280 nm) has the most harmful effect but usually does not reach the earth's surface as it is absorbed by the ozone layer; therefore, people are not usually affected by the short wavelength UV from sun exposure. UVB radiation (280 – 315 nm) and UVA radiation (315 - 400 nm) are the wavelengths that most affect people. UVB radiation is linked to skin burn and erythema and an increased risk of cancer development as a result of a prolonged exposure [169, 170]. UVA radiation is less intense than UVB radiation (longer wavelengths), however, it has a higher prevalence and a higher power of penetration into the skin layer [170]. However, there are many beneficial effects of UV irradiation [171], such as vitamin D production [172], improved rates of cancer survival [173–175] and melanin production (skin tanning) [176, 177]. Time of

exposure, irradiation intensity and wavelength are crucial aspects to take into account when analyzing the dangers and benefits of UV light exposure.

4.1 PHOTONIC THERAPY IN CANCER TREATMENT: THE UV LIGHT THERAPEUTIC WINDOW

Cancer is a major health problem and still one of the leading causes of death worldwide [178]. Phototherapy is normally focused on the treatment of skin related diseases and psoriasis [179, 180] but in recent years its potential has also been recognized in cancer treatment [181]. New strategies have been developed in recent year such as the research of new phototherapy targets and combination therapies using drugs and nanocarriers along with phototherapy as a means to design more effective and less adverse cancer treatments [182].

The Epidermal growth factor receptor (EGFR) and the members of this receptor family have emerged in recent years as good candidates for targeted cancer therapy. This cancer biomarker is usually mutated and overexpressed in a variety of cancers and also displays important roles in epithelial cell physiology [183]. When activated by specific ligands such as epidermal growth factor (EGF), dimerization of the receptor occurs, followed by intracellular tyrosine kinase domain activation. The activation of EGFR is followed by activation of downstream signaling pathways which may lead to cell migration and proliferation. Targeting EGFR and inactivating it could lead to the inhibition of the downstream signaling pathways leading to cell migration and proliferation, thus making it a promising target for cancer treatment.

The extracellular domain of EGFR has an abundance of aromatic residues and SS bonds [184, 185]. Most of the work presented in this thesis was manly focused on a particular wavelength range of UV light capable of exciting aromatic residues such as tryptophan and tyrosine. At 280nm, both these aromatic residues are excited leading to all the photochemical reactions necessary for LAMI, being one of these reactions the breaking of SS bonds in the vicinity of UV excited aromatic residues. The breaking of SS bonds has different effects on individual protein structure and functionality. In EGFR, the conformational stability is highly dependent on the integrity of

these SS bonds. In previous research, the structural changes induced in EGFR induced by low dose 280nm light ($0.08W/m^2$) were investigated, showing formation of photoproducts and structural changes on the receptors binding site [184]. Thus, the possibility of inactivation of EGFR receptors using 280nm irradiation was explored as a therapeutic approach for cancer treatment [6, 7].

The specific wavelength used in this work (280nm) is in the UVB range, which is the range most associated with erythema formation and carcinogenesis. However, at 280nm, the risk for both skin erythema and carcinogenesis is significantly reduced (Figure 1, paper 8[8]) [170]. When using this specific wavelength at lower power concentration, there is a window of opportunity where 280nm light can be applied for therapeutic purposes.

4.1.1 LIGHT INDUCED SWITCH IN TRANSMEMBRANE PROTEIN EGFR: PHOTONIC INACTIVATION OF THE EGFR RECEPTOR WITH LOW POWER 280 NM LIGHT

Paper 8 is a book chapter written on the possibility of using this therapeutic window of light to modulate transmembrane receptor proteins and metabolic pathways as a potential cancer therapy. Overexpression of EGFR is usually correlated with a poor prognosis and it is found in most solid tumors such as head and neck, prostate, non-small cell lung cancer and breast cancer [186, 187]. Various strategies have been designed for EGFR targeting using small molecule tyrosine kinase inhibitors [188–190] and monoclonal antibodies [189–191] that block ligand-binding or receptor dimerization. Preventing the binding of EGF to the receptor can block cell proliferation, angiogenesis, and metastasis and inhibit apoptosis. These phenomena are considered hallmarks of cancer as they are biological capabilities acquired during tumor development [192]

Previous research demonstrated that low dose 280 nm irradiation lead to structural changes and inactivation of EGFR, hindering ligand binding [184]. Furthermore, it was also previously shown that 280nm irradiation results in an arrest of EGFR signaling pathways [193]. The work described in **papers 6 and 7** explores the possibility of applying low dose 280nm irradiation for

inhibition of EGFR activation and cancer cell migration using as a model the non-small cell lung cancer cell line A549 with GFP-tagged EGFR (A549 GFP-EGFR cell line from Sigma-Aldrich).

5. SPECTROPHOTOMETRIC ANALYSIS OF PEPTIDE:DNA INTERACTION

During this PhD, other work was performed involving the study of a 36 amino acid element of Hc2 protein from *Chlamydia trachomatis* and its DNA-binding ability. This study deviates from the main theme of this thesis, the effect of UV light on protein structure and function, but it is still within the study of protein structure and function.

C. trachomatis is an obligate intracellular human bacterium and the most common sexually transmitted pathogen globally. The developmental cycle of this pathogen alternates between an extracellular, metabolically inactive, infectious form (elementary body) and a intracellular, metabolically active, non-infectious form (reticulate body) [194, 195]. The developmental cycle begins when the elementary bodies adhere to the host cells and are internalized and differentiate into the metabolically active reticulate bodies, within the intracellular vesicle (inclusion). After various rounds of replication, the reticulate bodies are transformed into elementary bodies which are then released into the extracellular medium after the inclusion burst, completing the developmental cycle [194]. Upon re-differentiation of the reticulate bodies into elementary bodies, the outer membrane becomes cross-linked by SS bonds, the size of the bodies is reduced from 1µm to 0.3 µm and the nucleoid is condensed.

Hc1 and Hc2 are histone H1-like proteins with the ability to condense DNA and/or RNA and to halt replication and transcription. [196–198]. These two proteins are synthesized late in the developmental cycle, during the transition from reticulate bodies to elementary bodies [194]. The elementary bodies contain both Hc1 and Hc2 proteins, encoded by *hctA* and *hctB* genes, respectively. The *hctB* gene was found present in all the genome sequences available for the *Chlamydiaceae* family, leading to the assumption that Hc2 is ubiquitous in this family. Hc2 displays a size variation (165 – 237 amino acids) among *C. trachomatis* serovars while Hc1 is more stable. The *hctB* gene contains a variation on the number of repeated elements, each consisting of 36 amino acids, which is the cause for the size variation of

Hc2. The repeated element is composed of one hexamer and six pentamers that result in a high number of variants due to amino acid substitutions and deletions. Within the repeat three aminoacids (valine, alanine and threonine) separate the evenly distributed positively charged residues lysine and arginine. Two prolines are also present in the sequence [199].

In **paper 9**, is described the study performed with a synthesized peptide containing the conserved primary sequence of the repeated 36 amino acid element (Hc2rep) and a 36 amino acid peptide with a randomized sequence (Hc2 scrambled) on the binding affinity for DNA.

6. COMMENTS ON THE EXPERIMENTAL DESIGNS

6.1 PHOTOCHEMISTRY AND PROTEIN'S STABILITY AND STRUCTURE

Protein structure and stability is dependent on the equilibrium of various forces. The biophysical characterization of individual proteins is important to understand its mechanism of action and its structure and stability under different physicochemical environments. This knowledge is important to regulate the activity of proteins, especially when applying them for biomedical or industrial purposes.

In **papers 1 and 2**, the effect of pH, temperature and UV light on the stability and photo oxidation processes of two proteases was assessed using fluorescence spectroscopy and CD. In **paper 1**, a protease from *Aspergillus tamarii* URM4634 was investigated [1] and in **paper 2** [2] (unpublished work), a protease from *Penicillium aurantiogriseum* URM4622 was investigated. The purified protease investigated in **paper 1** has shown collagenolytic and keratinolytic activity [1] whereas the alkaline serine peptidase investigated in **paper 2** [2] has been shown to hydrolyze various proteins and used for the production of casein antioxidative peptides [200].

The spectroscopic techniques used to investigate both proteases allowed for the monitoring of structural changes as a function of temperature, pH and UV light irradiation as a measurement of protein stability. The CD data obtained for the protease investigated in **paper 1** displayed features of a protein rich in α-helices at all pH values. After thermal denaturation (heating the protein up to 90˚C followed by cooling back to 20˚C), the CD spectra showed a change in secondary structure with loss of α-helices and emergence of random coil peaks. There seemed to be some refolding at pH 9, where maximum proteolytic activity at 25˚C was observed, but with blue shifts on the two peaks characteristic of α-helices. It was also observed that the proteins structure at pH 9 was stable up to 40˚C. However, the highest T_m was determined to be at pH 6 [1]. A good stability of activity at 40˚C has

been previously reported for this protease [201] and also for proteases isolated from other *Aspergillus* species [202, 203]. CD data obtained for alkaline serine proteases isolated from *Nocardiopsis alba* OM-4 and *Nocardiopsis alba* TATA-13 demonstrated that these proteases were highly stable at 50 ˚C. However these proteases displayed β-sheets as major secondary structure [204]. This suggests that the composition of the structures in the secondary structure is associated with the structural stability of the protein.

In **paper 2** [2], the CD data revealed that the investigated protease displayed β-sheets as the main secondary structures with a low percentage of α-helices. At pH 9, the optimum pH for protease activity, showed the highest percentage of α-helices (unpublished work). Similar ratios of α-helices/ β-sheets were found in a protease from C*onidiobolus brefeldianus* [205], suggesting that the balance of both secondary structures might be linked to the structural stability of this protease. It was also observed while monitoring the changes induced by thermal denaturation that at optimum pH 9, the secondary structure was stable up to 40˚C, with changes detected from 50˚C. These changes are correlated with the protein activity profile. Yet, the highest T_m was also detected at pH 6 (unpublished work). These results are in line with the observed results in **paper 1** [1] for the protease isolated from *Penicillium aurantiogriseum* URM4622.

Both proteases studied in **paper 1** [1] and **paper 2** [2] (unpublished work) showed a difference between the pH at which the highest Tm was detected and the pH of optimal activity. This discrepancy was also found in an alkaline protease from C*onidiobolus brefeldianus* [205] , where structural resistance to thermal denaturation was higher on acidic-neutral values. A similar effect was also observed for an alkaline serine protease containing a polyproline II fold isolated from *Nocardiopsis sp.* This protease displays an optimum pH of 10 but is more stable in long term storage at pH 5 [206]. Considering a few exceptions, often it is observed that an increase in thermostability can be accompanied by a reduction in activity. The study of these parameters is important in the characterization and application of enzymes, however, some caution needs to be applied when interpreting the results. Assay duration and concentration of protein used determines the optimum temperature of activity, thus, the optimum conditions on

characterization studies may differ from the optimum conditions on large-scale applications [207].

Fluorescence spectroscopy was also used to study the intrinsic fluorescence of tryptophan on both proteases (**paper 1 and 2** [1, 2]) as a function of temperature and pH. Tryptophan residues can be found in most proteins and fluorescence emission of tryptophan is sensitive to solvent polarity and to the presence of fluorescence quenchers. In **paper 1**[1], the fluorescence emission of tryptophan was monitored upon excitation at 280 nm and 295 nm, whereas in **paper 2** [2] it was only monitored upon excitation at 280 nm. Excitation of tryptophan at 295 nm is more selective since both tryptophan and tyrosine are excited at 280 nm. Tryptophan's emission maximum is ≈ 350 nm when completely exposed in a polar aqueous environment while in an hydrophobic environment, a blue shift occurs in the emission maximum, going as low as 310 nm [13].

The fluorescence of these aromatic residues can be quenched by the presence of certain amino acid residues in the vicinity such as histidine, lysine and cysteine due to possible electron transfer from/to various protonated groups such as amides, disulfides or/and carboxyl groups. Moreover, fluorescence energy transfer may occur between tyrosine and tryptophan or between multiple tryptophan residues [13, 102]. Temperature also affects fluorescence emission intensity. At high temperatures, fluorescence emission is quenched due to collisional quenching caused by a higher vibrational state of the molecules in the solvent [13]. Therefore, changes in the structure of each protein and the physicochemical environment will be translated into changes in tryptophan's fluorescence.

The use of extrinsic fluorescence probes can be a good option to complement the information on conformational changes provided by tryptophan's fluorescence emission or even as an alternative for proteins that don't have tryptophan residues [99]. In both **paper 1** [1] and **paper 2** [2] (unpublished work), the fluorescence spectra of both proteases at different values of pH before the thermal treatment showed a decrease in tryptophan's fluorescence emission intensity. The opposite effect was observed for tryptophan's fluorescence emission intensity of a protease isolated from from *Nocardiopsis sp.* In this study, the fluorescent probe 1-Anilinonaphthalene 8-sulphonic acid (ANS) was also used to probe pH

induced conformational changes on the protease. The monitorization of ANS fluorescence provided further information on the exposure of hydrophobic patches in the protease at lower pH [206]. ANS is fluorescent probe binds to hydrophobic regions of the proteins and is widely used to monitor conformational changes in proteins [208]. Fluorescence spectroscopy studies with extrinsic fluorescent probes would be a good strategy to obtain more information on the pH and temperature induced conformational changes on the proteases studied in **paper 1** and **paper 2**.

The spectral calibration of a fluorescence measuring instrument is also an important factor to consider. For example, tryptophan fluorescence spectra measured in different instruments can display slight differences in terms of shape, width and position of the maximum emission peak [100]. Furthermore, even though the use of tryptophan fluorescence allows the study of proteins without altering them [102], this must be done with caution. In section 1.2.1, it was shown that tryptophan is prone to photodegradation upon UV illumination. This phenomenon is highly dependent on the power of illumination used and the time of illumination. The formation of photoproducts will also play a role in the decrease of fluorescence intensity of tryptophan as many of the formed photoproducts display different spectral properties. For example, NFK and Kynurenine are products of tryptophan's photo degradation and absorb light at longer wavelengths which also allows them to act as photosensitizers to visible light [56]. This effect was observed in lysozyme. Irradiation of lysozyme at 295 nm for 2 h with 1 µW led to a decrease in fluorescence emission intensity of 11.5% whereas lower illumination power (0.1 µW) led to a decrease of 2.8% [209].

Even though both spectroscopic techniques were used to monitor conformational changes as a function of pH and temperature, the information obtained from each spectroscopic technique resulted from the monitorization of different parameters. In **paper 1**[1], the T_m for the protease at the different monitored pHs was determined both from the CD data (ellipticity) and from tryptophan's intrinsic fluorescence upon heat treatment. The T_m values obtained from CD spectroscopy were generally higher [1]. The same pattern was also reported when determining the Tm of bevacizumab by fluorescence spectroscopy and CD. The values of Tm obtained from CD data

were higher than the values of Tm obtained from tryptophan's intrinsic fluorescence, even though the values of Tm obtained from both techniques displayed the same tendency [210]. The thermal denaturation of both proteases was found to be irreversible at all the studied pH values upon heating at 90˚C [1]. The study of the thermodynamic parameters and the transition temperatures on the conformational changes of the proteins is an important feature for the determination of the proteins applicability.

The nature and extent of conformational changes induced by UV light will be different for each individual protein, as this is highly dependent on the size, primary structure (amino acid content), intramolecular interactions and overall physicochemical properties (section 1.2.1). Even though UV light illumination is associated with various photochemical processes that may induce changes in protein structure, this is not always translated into a loss of protein function. For example, it was observed that UV light could induce the activation of human plasminogen. This protein presents seven independently folded domains and a reasonable amount of aromatic residues in all domains. It also contains 24 SS bonds which play different roles within the protein. This makes plasminogen a probable target for photo-oxidation. Yet, even though photoproduct formation was observed upon low dose UV irradiation, the native fold was mostly preserved and an increase of plasminogen's proteolytic activity *in vitro* was registered [88].

The data obtained for the proteases in **paper 1** [1] and **paper 2** [2] can only give an insight on the structural changes induced upon the tested experimental conditions. The physical and chemical characteristics of proteases produced by a wide range of fungal strains have been studied previously. These proteases have a major importance in the industry and can be used for a wide range of industrial applications [211]. Proteases also have great applicability in clinical therapy [212]. The study of the physical and chemical parameters of each protease is of great importance for their optimal applicability.

The spectroscopic techniques used in these studies provide global information on protein structure and aromatic residues but it would be relevant to perform further studies to have more detailed insights into their different structural levels. These techniques are a good way to obtain information about conformational changes in a fast way without the

requirement of more sophisticated apparatus, as many laboratories possess the equipment to perform them.

Fluorescence techniques are also widely used for the study of membrane proteins in their native state as they allow for real time structural information as well as a broad range of information on their function, kinetics and thermodynamics. The complexity of these biological systems is not compatible with the experimental procedures of other techniques considered as the golden standard for macromolecular structure determination such as nuclear magnetic resonance (NMR) spectroscopy, cryoelectron microscopy and X-Ray crystallography [101]. The two proteases used in our study are soluble and could be submitted to experimental procedures necessary for these techniques. NMR spectroscopy and X-Ray crystallography are two of the most relevant methods to study the three-dimensional structure of proteins with atomic resolution [213–215]. These techniques could provide a better insight into the structure-function relationship of these proteases under different conditions. The catalytic activity of different serine proteases family has been subject of study for many years. The serine, histidine and aspartate residues present in the catalytic triad of these proteases is generally conserved but there are differences among families in terms of tertiary structure amino acid sequence [216] and NMR and X-Ray crystallography have contributed extensively to a better understanding of their catalytic mechanism [217].

6.2 BIOSENSOR MICROARRAY PRODUCTION

In section 3, LAMI was presented as a new immobilization technique developed at AAU for the immobilization of proteins based on natural photochemical events induced by UV light. LAMI presents some advantages over other immobilization techniques. First of all, it allows for a spatially oriented immobilization of the biosensors in a controlled manner. Spatially oriented immobilization of proteins is a desirable feature as it allows for full exposure of the active site, maximizing the binding affinity and activity of the immobilized biosensor [218]. Furthermore, LAMI does not require biomolecules exposure to any thermal or chemical intermediate steps that might affect protein structure and function, nor does it require the presence of an external photosensitizer.

LAMI proved to be an immobilization technique with good reproducibility. In this thesis, LAMI was used for the immobilization of different biomolecules such as CRP, IgG antibodies and MB constructs (**papers 3, 4 and 5** [3–5]). A novel microfabrication stage was used to achieve immobilization of the biomolecules on thiol derivatized glass and SOI surfaces. The new microfabrication stage is an upgrade of the optical setup previously used for LAMI development, offering the possibility of new applications of LAMI. The new optical setup allows the performance of immobilization through one- and three-photon excitation as well as the performance of two-and three-photon confocal microscopy and is described in **paper 3** [3].

The immobilized biosensors patterns reported in **papers 3, 4 and 5** [3–5] displayed high resolutions in the μm range. However, some variation was observed in terms of line thickness in the immobilized lines pattern, especially in the biofunctionalization of the SOI derivatized surfaces. This was due to the illumination setup installed in the moving stage (described in [3]), which requires a transparent immobilization surface for sample visualization on the CCD camera. When focusing the samples, some slight deviations occurred in the working distance between the sample and the objective. It would be optimal in the future to adapt the illumination setup sample visualization on non-transparent surfaces. Furthermore, the profile of the laser beam light displays a Gaussian distribution. The intensity of the irradiated light will be higher at the center of the beam and lower towards the edge of the beam. This will also influence the drawn pattern.

The various immobilized biosensors proved to retain their bioavailability. However, further studies are relevant in order to understand the molecular mechanisms involved in their immobilization process, namely in terms of SS bond disruption. In **paper 4** [4], LAMI was used to immobilize anti-troponin I IgG and anti-myoglobin IgG antibodies together with another photonic immobilization technique: Thiol-ene coupling (TEC). Both techniques achieved biosensor immobilization, although LAMI was used to immobilize whole IgG antibodies and TEC was used to immobilize Fab fragments of the same IgG antibodies. IgG antibodies are known to have a high number of SS bonds that are evolutionarily conserved [219]. The number of intra-chain and inter-chain SS bonds varies among the four subclasses of IgG

antibodies. Furthermore, the intra-chain SS bonds are more buried and thus less exposed to the solvent than inter-chain SS bonds. This difference in solvent exposure is believed to be linked to the reactivity of the cysteine residues, where more exposed cysteine residues are more reactive [219]. Indeed, the interdomain SS bonds in IgG 1 antibodies were found to be more prone to reduction than the intradomain chains [220]. The immobilization of anti-PSA Fab fragments was previously achieved using LAMI. The immobilized anti-PSA Fab fragments remained bioactive as demonstrated by immunoassays [123, 126]. Analysis of available three-dimensional structures available for IgG Fab fragments show that these structures display a multitude of aromatic residues in close spatial proximity to SS bonds. The interdomain SS bond bridging the light and heavy chains of the Fab fragment is far from the binding site and is solvent accessible whereas the intradomain chains found in CH, VH, CL and VL domains are not solvent exposed. Thus, it is likely that UV illumination of aromatic residues nearby the interdomain SS bond bridging the light and heavy chains of the Fab fragment likely leads to the formation of free thiol groups that can bind to a thiol-derivatized surface [123]. UV illumination is likely to lead to the breakage of the SS bonds in the hinge region, thus immobilizing the antibody without damage or blockage of the active site.

The whole immobilized antibodies were able to recognize the target analytes, thus the illumination process did not hinder their bioavailability [4]. However, there is a need to further investigate the immobilization of the whole antibodies vs the immobilization of Fab fragments of the same antibodies and investigate their binding capabilities. Surface Plasmon Resonance would be a good technique to perform this study. Vashist *et al* performed SPR based immunoassays to test the effect on the analytical performance of an antibody immobilized through different immobilization strategies [221]. This approach could provide information on potential differences on the analytical performance of the immobilized biomolecules.

The limit of detection of myoglobin was studied for TEC immobilized Fab fragments of anti-myoglobin antibody and was reported to be 1ng/mL. The amount of myoglobin detected by LAMI immobilized anti-myoglobin antibodies in this study was 3 nM (50ng/mL) [4]. The sensitivity of the LAMI immobilized anti-myoglobin IgG antibodies is lower than the

sensitivities reached by other POC devices already in the market used for the detection of myoglobin. Some examples of these devices are the Stratus® CS Acute Care™ Diagnostic System dtectes troponin in a range of 1– 900 ng/mL [222] and The Cardiac Reader CARDIACM from Roche, which reportedly has a quantitative measuring range of 30-700 µg/L [223], Abnormal myoglobin values are usually considered to be above 90 µg/mL in serum [224] which are above the reached sensitivity for LAMI immobilized antibodies.

The higher values of myoglobin detected by LAMI immobilized anti-myoglobin antibodies in **paper 4** [4] were due to some difficulties during the experimental procedure. The secondary anti-myoglobin antibodies used in the sandwich assay were labelled using a commercial kit (DyLight® 488 Fast Conjugation Kit) according to the manufactures' instructions. However, the concentration of antibody was lower than the one recommended in the labelling kits instructions. Furthermore, the detection assay used for TEC immobilized Fab fragments was a direct assay where myoglobin was fluorescently labelled. We chose to do a sandwich assay in order to prevent modification of myoglobin during the labelling procedure, as this could cause interference in the antibody-antigen binding. In future assay developments, it would be optimal to first increase the antibody concentration prior to labelling or try to use another labelling kit.

In **paper 4** [4], the detection of two different concentrations of troponin I were reported for LAMI immobilized anti-troponin I antibodies: 4µM and 4nM (100 µg/mL and 100 ng/ml, respectively). The lowest concentration reported for TEC immobilized Fab-fragments of anti-troponin I antibody was 10 ng/mL. A higher value is reported for LAMI immobilized anti-troponin I antibodies (100 ng/mL) [4]. However, in experiments performed after this report (unpublished work), the same sensitivity was reached for LAMI immobilized anti-troponin I antibodies.

When testing for lower concentrations of antigen, the fluorescent signal appeared with higher intensity in the edges due to a concentration difference upon sample deposition in the surface. The surface is hydrophobic due to the surface chemistry necessary for LAMI immobilization (thiol reactive surface). When the sample droplets are deposited onto the surface, the sample tends to have a higher concentration around the droplet edges,

creating a "donut effect". The concentration of immobilized antibody is thus higher at the pattern edges. Higher amount of antibodies will be able to bind to higher amount of antigen, leading to a higher fluorescent signal. These observations become more evident when testing the sensibility of the immobilized antibodies. When performing immunoassays with higher concentration of antigen (e.g. troponin I), usually the distribution of fluorescence intensity is more homogeneous across the immobilized pattern (Figures 7 and 8 in **paper 4** [4]).

Other cardiac markers were also considered during these studies. LAMI was also used to immobilize anti-troponin T and anti-CRP IgG antibodies (unpublished results).

In table 2 is presented the sensitivity reached for each of the studied cardiac biomarkers as well as the immunoassay format used.

Target	Sensitivity*	Immunoassay format
CRP	0.4 nM (10ng/mL)	Direct
cTnI	0.4 nM (10ng/mL)	Sandwich
cTnT	30 nM (1µg/mL)	Sandwich
Myo	3 nM (50ng/mL)	Sandwich

Table 2. Summary of the sensitivities achieved for the detection of the 4 studied cardiac biomarkers.

Troponin I and CRP were the cardiac biomarkers detected at lower concentrations. The reached sensitivity for these two cardiac biomarkers is within the detection limits described for other microarray approaches [225, 226]. The detection limit of each immobilized IgG biosensor systems still requires further experimental development of the immunoassays. The detection limit reached for LAMI immobilized whole anti-troponin I and anti-CRP antibodies was sensitive (10 ng/mL), but further optimization of immunoassay conditions might lead to higher sensitivity. However, such sensitivity was not reached for LAMI immobilized anti-myoglobin and anti-

troponin T antibodies. The investigation of antibody cross-reactions in multiplexing experiments also requires additional work.

No cross-reactivity has been reported for assays for detection of troponin I and troponin T, due to the structural differences between the two biomarkers [227]. Furthermore, specificity tests performed on anti-troponin I antibodies immobilized onto silicon nanowires showed no cross-reactivity with CRP [228]. However, in **paper 4** [4] it is reported that the TEC immobilized anti-myoglobin antibody displayed a low level of unspecific recognition of the fluorescently labelled IgG used for troponin I recognition. Such studies were not performed with the LAMI immobilized antibodies. The optimization of the conditions used in the immunoassays could suppress this unspecific recognition and further work should be performed in order to achieve this goal.

Regarding the MB constructs (**paper 5** [5]), the peptide attached to the MB is fairly simple. The structure of the peptide is not important for miRNA biorecognition as the recognition is performed by binding of the MB to the target miRNA, but it would be relevant to perform a study on the induced photochemical processes on the MB construct as a whole. It has been widely reported that UV light induced damage may occur. Besaratinia et al. [229] showed that there is considerable DNA damage induced at 282 nm laser irradiation. The irradiation power and time reported in this study was 0.45 mW and 322 s, respectively. However, the irradiation conditions used for immobilization of the MB constructs in our study were 40uW illumination at 100 µm/s, which is lower. Furthermore, the biofunctionality studies performed on the immobilized MB constructs showed biorecognition of the target miRNA. Photo-induced damage is highly dependent on power and time of exposure. In our study, the biorecognition ability of the biosensors was not hindered by the irradiation conditions used to perform immobilization. However, it would be relevant in the future to study the effect of the used irradiation conditions (280nm pulsed light,) on DNA.

Ruiz-Tórtola *et al.* reported the immobilization of MB thiolated structures using Thio-Ene coupling (TEC) for the detection of target oligonucleotides [230]. This photonic immobilization technique uses shorter wavelength UV light (254 nm) known to also induce oxidative damage to DNA [231]. However, recognition ability was not hindered by the irradiation used for the

immobilization process. UV light induced photoreactions are dose dependent and in both instances, UV irradiation used for TEC and LAMI did not seem to affect the biorecognition ability of the immobilized biomolecules.

The nature of the SOI surfaces also posed some difficulties in terms of pattern detection by CFM. The surfaces present a mirrored nature which allows for higher light scattering. The used confocal microscope as an inverted setup, where the biofunctionalized surface is facing the bottom and the excitation laser used to excite the fluorophores comes from the bottom. Image treatment of the images obtained with CFM with the software ImageJ helped in the analysis of the patterns.

The biosensors immobilized in **paper 4** [4] (anti-myoglobin and anti-troponin I IgG antibodies) and the MB constructs immobilized in **paper 5** [5] are being integrated in two Point-of-Care (POC) devices: PHOCNOSIS [232] and SAPHELY [233]. The IgG biosensors will be used to detect markers for cardiovascular diseases and the MB constructs will be used to detect miRNA cancer biomarkers. In these POC devices, the detection of the biomarkers will be performed with a label-free photonic based detection using nanophotonic sensing structures fabricated onto the SOI surfaces. Thus, the difficulties encountered during the pattern detection by CFM will be circumvented. In **paper 5** [5] are reported some of the results obtained for miRNA 21 detection with LAMI immobilized MB construct using this label-free photonic technique. Most of the experiments reported during this PhD on the bioavailability of LAMI immobilized biosensors used fluorescence based detection methods. However, experiments using this label free detection method are ongoing as the equipment necessary to perform it is located in UPV, Spain. Nonetheless, the nanophotonic sensing structures have already demonstrated high sensitivity for detection of target analytes immobilized with TEC [230].

Label-free detection of biomarkers presents some advantages over the fluorescence detection methods as neither the immobilized biosensors nor the biomarkers need to be fluorescently labeled. More classical protein microarray label-free detection strategies such as Surface Plasmon Resonance (SPR), Mass Spectrometry and Atomic force microscopy usually require specialized equipment, frequently not available in all laboratories

[114]. The development of a label-free detection strategy with high sensitivity in a POC device will bring great benefits to clinical analysis.

6.3 LIGHT INDUCED SWITCH IN TRANSMEMBRANE PROTEIN EGFR: INACTIVATION OF THE EGFR TRANSMEMBRANE RECEPTOR

The aim of the work presented in section 3 was to investigate the effects of wavelength specific UV light illumination on the modulation of EGFR transmembrane receptor as a therapeutic application for cancer treatment. Previous work had already demonstrated the effect of UV light triggered mechanisms on EGFR, particularly disulfide bridge disruption induced by 280nm light on the structure and function of the protein [184]. Moreover, the laser-pulsed UV light induced arrest of the EGFR signaling pathway and induction of apoptosis was previously shown in two cancer cell lines. Western blot results showed that laser-pulsed UV illumination above a certain power threshold lead to no phosphorylation of the EGFR receptor and to the absence of the downstream signaling molecules AKT1 and ERK1/2 were also absent [193]. A downregulation of EGFR expression upon irradiation at 254 nm was also reported by Yamauchi et al. [234] and Kawaguchi et al. [235]. The effect 254 nm has on EGFR activation is likely to be attributed to the disruption of the SS bonds in the receptor, as both 254 nm and 280 nm are known to lead to SS bond disruption.

In the work developed during this PhD, the non-small lung carcinoma A549 GFP-tagged EGFR cell line was used as a model to study the influence of 280 nm UV irradiation on EGFR inactivation and cancer cell migration and proliferation. A549 GFP-EGFR cells express EGFR fused with GFP. In **paper 6 and 7,** different irradiances and illumination times were tested. In **paper 6**, cells were irradiated at 1,8 Wm^{-2} for 15 and 45 minutes [6] and in **paper 7** cells were irradiated at 0.09 Wm^{-2}, 0.27 Wm^{-2} for 15 and 30 minutes and additionally again at 1,8 Wm^{-2} for 15 and 45 minutes for comparison [7]. Both papers focused on observing the changes induced by UV irradiation on cell morphology and migration. The irradiance values used in both studies were supported by previous data on EGFR UV induced changes [184, 193] and on available data on the UVB irradiance levels emitted by sunlight. Irradiance emitted by the sun in the UVB region (< 313 nm) is reported to be

1.75 Wm^{-2} at 39°N in summer time and 0.4 Wm^{-2} in winter time [236]. Furthermore, the lower doses of UV illumination used in these studies (0.09 Wm^{-2}, 0.27 Wm^{-2}) are below the values recommended by the International Electrotechnical Commission (2009) to avoid erythema [237] and below levels known to induce DNA photoionization [238].

Monitorization of GFP fluorescence in A549 GFP-EGFR cells allows for the visualization of EGFR activation upon EGF stimulation. Upon activation, EGFR is redistributed on the cell membrane and subsequently internalized by endocytosis. This is visible under fluorescence spectroscopy by the appearance of fluorescent dots inside the cell. Upon EGF stimulation, the activation and internalization of EGFR was observed [7] as well as changes in cell morphology such as disintegration of cell-cell junctions and filopodia formation [239–241]. The green fluorescence emission intensity of GFP was not affected by 280 nm irradiation. Even though tyrosine absorbs at 280 nm, no effect was observed in the fluorescence of the Ser-Tyr-Gly chromophore in GFP.

In **papers 6 and 7**, UV radiation prior to EGF stimulation at 1,8 Wm^{-2} (highest dose tested), lead to a delay in EGFR activation, especially after 45 minutes irradiation, with a reduction of cell-cell junction disintegration. At lower irradiances (0.09 Wm^{-2}, 0.27 Wm^{-2}), an inhibition of EGFR activation was also observed [6, 7]. In these experiments, the time component proved to be an important factor, as longer irradiation time lead to more pronounced effects on EGFR activation and consequent morphology changes in the cells. The blocking effect on EGFR activation was reversible and its duration was dependent on the power of irradiation and time of irradiation. The observed results in terms of the power of irradiation/time of irradiation balance seem to follow the Bunsen-Roscoe reciprocity law which correlates the cumulative irradiance with the extent of the observed photochemical effects [7].

In terms of cell proliferation, it was observed that EGF concentration does not have a pronounced effect on cell proliferation on both A549 GFP-EGFR cell line and the parental line A549 (**paper 7**) [7], which is in line with previous observations [242]. In **paper 7** it was also observed that irradiation prior to EGF stimulation also did not exert an effect on cell proliferation. However, a functional assay showed a distinct effect in cell mobility. Illumination of the cells at 0.27 Wm^{-2} for 30 minutes prior to EGF

stimulation leads to a reduction of cell migration. This effect was more pronounced than the one observed when using Tyrphostin, an EGFR-signaling specific inhibitor [7].

The results show the therapeutic potential of 280 nm irradiation on the treatment of cancer. This photonic treatment has an advantage over other photonic therapies as it does not require a photosensitizer. Its potential can be developed as a stand-alone therapy or in combination with other cancer therapies. Other photonic therapies have been described in the literature as prominsing strategies in cancer treatment for enhancement of tumor drug delivery [243], increase of immune response against tumors [244] and as a combinational strategy to be applied with other more conventional cancer treatments such as chemotherapy [168] Further work is necessary to optimize the application of this photonic therapy. Parameters such as the irradiation intensity/time require further investigation. Also there is a need to explore other light delivery systems in order to widen the range of applications of this photonic therapy.

Furthermore, it is also relevant to perform studies on the effect of UV irradiation on the cells proteome. Mass spectrometry (MS) is a useful tool for research in proteomics [245]. MS studies would be a relevant in order to obtain information on the effects of UV light in protein expression and modification in cells beyond the effect already observed in EGFR modulation. These studies are in the pipeline for the future development this UV light therapy.

7. CONCLUDING REMARKS AND OUTLOOK

The first eight peer reviewed publications included in this thesis (one being a book chapter and three being conference papers) demonstrate the relevance of UV light induced photochemical processes on protein structure and function and the different approaches in which this knowledge can be applied.

The first part of this thesis focused on the UV light induced photoprocesses and how they can be applied for the monitorization of macromolecular processes. Biophysical characterization of proteins is an important feature for their applicability. The study of two serine proteases isolated from different organisms provided information on conformational changes induced by UV light, pH and temperature on two alkaline serine proteases isolated from different organisms which can help optimize their function.

The second part of this thesis focused on the use of UV induced photochemical processes for protein immobilization onto thiol derivatized surfaces. Light Assisted Molecular Immobilization (LAMI) was used to immobilize various biomolecules onto functional surfaces: IgG antibodies for the detection of cardiovascular biomarkers and peptide-MB conjugates for the detection of specific miRNA21 cancer biomarker. LAMI presents some benefits over other immobilization techniques as it does not require the application of chemical steps prior to immobilization and allows for biofunctionalization of surfaces in a covalent and spatially oriented manner. The studies demonstrated that LAMI was successfully applied for the immobilization of the biomolecules without hindering their bioavailability. The biofunctionalized surfaces were developed for application in two Point-of-care (POC) devices for early diagnosis of cardiovascular diseases and early diagnosis of cancer. Even though the presented results were promising as a proof-of-concept, there is still need for further optimization of the detection assays applied for the biofunctionalized surfaces before they can be used in a POC device.

Light-induced protein switches were another focus of this thesis. The therapeutic window of UV light was investigated as a new potential photonic

cancer therapy. The overexpression of EGFR is linked with cancer progression and our research investigated the use of low-dose wavelength specific UV light in order to modulate EGFR structure and halt the activation of the EGFR signaling pathway, hindering cancer cell migration and proliferation. The results obtained showed a delay on EGFR activation upon low-dose UV light irradiation. These findings need further investigation on the effects caused by UV irradiation on the proteome of the cells. Optimization of the irradiation conditions is also a requirement for a more effective therapeutic result. However, our findings suggest that UV light has the potential to be used as a new stand-alone or combined cancer photonic therapy.

Going out of the scope of the effect of UV light on biomacromolecules, another work was performed on the DNA binding ability of two Hc2 peptides. These two peptides were synthesized according to a conserved primary sequence of the repeated 36 amino acid element (Hc2rep) and a 36 amino acid peptide with a randomized sequence (Hc2 scrambled) of the Hc2 histone H1-like protein found in *Chlamydia trachomatis*. The results showed that the Hc2 peptides bound preferentially to the major groove of DNA. The investigation of the DNA binding ability of the histone-like proteins is important since their ability to condensate DNA is crucial for the developmental cycle of *Chlamydia trachomatis.*

Summarizing, the work presented in this PhD thesis provides an overview on UV light induced photochemical processes and their application on the development of new technological approaches and new therapeutic treatments.

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