Study of drug formulations and development of immunosensing interfaces with membrane model systems

Mestrado em Bioquímica
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Resumo

Grande parte dos processos bioquímicos encontra-se associada às membranas biológicas enquanto estruturas capazes de delimitar variados compartimentos e, simultaneamente, desempenhar funções ao nível da sinalização, comunicação e transmissão de informação, bem como de servir de suporte a inúmeras moléculas essenciais a esses processos bioquímicos. A capacidade de desempenhar todos estes papéis prende-se com o facto de as membranas biológicas apresentarem uma complexa estrutura, possuindo na sua composição, para além de inúmeras proteínas, um vasto leque de moléculas lipídicas, conferindo-lhes diversas propriedades físicas características. Por si só, os lípidos são caracterizados por uma elevada diversidade estrutural, quer ao nível da região hidrófila, quer ao nível das cadeias alifáticas, que podem diferir no seu comprimento, no grau de insaturação hidroxilação, etc. Assim, no sentido de perceber toda esta complexidade, diferentes sistemas modelo capazes de mimetizar as membranas biológicas têm surgido, tendo como ponto de partida a estrutura da bicamada lipídica proposta por Gorter e Grendel, em 1925.

Estes modelos, cujo tamanho, geometria e composição podem ser adaptados face aos objectivos, têm sido amplamente utilizados no estudo de processos tão variados como o comportamento de diferentes fases lipídicas ou mesmo a fusão de membranas. Os sistemas modelo de biomembranas vão desde sistemas de bicamadas lipídicas em suspensão (lipossomas) com diferentes tamanhos e diferente número de bicamadas, a bicamadas lipídicas suportadas (SLBs). Nos estudos com sistemas modelo, uma vasta gama de técnicas de caracterização biofísicas pode ser aplicada, incluindo diferentes espectroscopias, como a espectroscopia de fluorescência e a espectroscopia de infravermelho com transformada de Fourier (FTIR), e diferentes técnicas de caracterização de superfície, tais como a microscopia de força atómica (AFM), a elipsometria, ainda que pouco utilizada, e a ressonância do plasmão de superfície. A interacção de pequenas moléculas com interesse biológico com biomembranas tem sido um tópico amplamente estudado recorrendo aos sistemas modelo. Os sistemas compostos por monocamadas lipídicas suportadas foram ainda utilizados, neste trabalho, na criação e desenvolvimento de imunossensores, os quais podem ser utilizados como importantes ferramentas de diagnóstico de diversas patologias.

Na primeira parte deste trabalho, que se encontra escrita sob a forma de um artigo para publicação, estudou-se a interacção de um derivado fenólico, o m-cresol (3-
metilfenol), com a membrana biológica segundo diferentes perspectivas. Para tal, recorrendo a dois tipos de sistemas modelo membranares, os lipossomas e as SLBs, foram empregues duas técnicas de caracterização, a espectroscopia de fluorescência e a AFM.

O m-cresol é um excipiente frequentemente utilizado em elevadas concentrações em diversas formulações farmacêuticas e, recentemente, tem sido associado a diversos casos de toxicidade após utilização de formulações prescritas para o tratamento da diabetes. No entanto, os efeitos da sua interacção com membranas biológicas encontram-se ainda por decifrar. Utilizando lipossomas constituídos por misturas ternárias de 1-palmitoil-2-oleoil-sn-glicero-3-fosfocolina (POPC) / N-palmitoil-esfingomieliina (PSM) / Colesterol (Chol), nas proporções adequadas para abranger a linha de conjugação que contém a proporção equimolar 1:1:1 do diagrama de fases publicado para esta mistura, em presença de fase líquido desordenado (l_d), líquido ordenado (l_o) e coexistência de ambas. O POPC é um dos glicerofosfolípidos mais abundante nas membranas celulares de organismos eucariotas, a PSM o principal esfingofosfolípido em membranas celulares de mamíferos e o Chol o esterol que se encontra em níveis mais elevados nas mesmas. Esta mistura foi escolhida por representar um modelo bem estabelecido que mimetiza a formação de jangadas lipídicas.

Através de medidas de anisotropia de fluorescência em estado estacionário, de decaimentos de fluorescência e de medidas de transferência de energia por ressonância de Förster (FRET), utilizou-se a fluorescência intrínseca do m-cresol para inferir acerca do seu comportamento na presença de lipossomas (vesículas unilamelares grandes (LUVs)). Para além disso, recorreu-se a sondas de fluorescência de membrana para melhor caracterizar esta interacção: N-(7-nitrobenz-2-oxa-1,3-diazol-4-il)-1,2-dipalmitoil-sn-glicero-3-fosfoetanolamina, sal trietilamónio (NBD-DPPE) e N-(7-nitrobenz-2-oxa-1,3-diazol-4-il)-1,2-dioleoil-sn-glicero-3-fosfoetanolamina (NBD-DOPE), que têm preferência pelas fases l_o e l_d, respectivamente; 4-(2-(6-(dibutilamino)-2-naftalenil)etenil)-1-(3-sulfopropil)-piridínio (di-4-ANEPPS), que permite extrair informação acerca do estado de hidratação da bicamada; ácido trans-parinárico (t-PnA), que traduz o grau de empacotamento das cadeias acilo dos fosfolípidos e N-(lissamina rodamina B sulfonil)-1,2-dioleoil-sn-3-fosfatidiletanolamina (Rhod-DOPE), que, foi usado em medidas de FRET como aceitador com NBD-DPPE enquanto dador, permitem concluir acerca de alterações do tamanho das jangadas lipídicas. Paralelamente, utilizando SLBs depositadas em mica, compostas por misturas ternárias de 1,2-dioleoil-sn-glicero-3-fosfocolina (DOPC)/PSM/Chol na proporção 2:2:1, um sistema que
apresenta coexistência de fases $l_d$ e $l_o$ e mimetiza jangadas lipídicas, foram efectuados estudos de AFM para avaliar em tempo real os efeitos causados pelo $m$-cresol nas jangadas lipídicas.

Os resultados obtidos permitiram concluir que os efeitos do $m$-cresol sobre as bicamadas lipídicas deverão inicialmente estar restritos à sua superfície, sem perturbação das cadeias acilo dos fosfolípidos. Esta interacção superficial deverá fazer-se notar principalmente ao nível das zonas de interface entre as fases $l_d$ e $l_o$, levando ao desaparecimento das jangadas lipídicas com o tempo e com o aumento da concentração de $m$-cresol. Globalmente, observou-se que o $m$-cresol apresenta preferência por fases $l_o$, através da do alinhamento do seu momento dipolar com o dipolo da bicamada lipídica, levando ao desaparecimento das jangadas lipídicas. Como exemplo de uma formulação farmacêutica de insulina humana contendo elevadas concentrações de $m$-cresol, foi utilizada a Humulina®, uma das formulações mais utilizadas no tratamento da diabetes. Foram efectuados os mesmos estudos na presença desta formulação e os efeitos observados são semelhantes aos que se observaram na presença de $m$-cresol, ainda que de uma forma menos acentuada. Isto poderá indicar que, quando inserido em formulações, o $m$-cresol apresenta efeitos adversos, ainda que uma parte deles possa ser atenuada pela interacção com os outros componentes da formulação.

A segunda parte deste trabalho, realizada no âmbito da Sétima Cooperação Científica e Tecnológica Sino-Portuguesa (2013-2015) sob o tema “Construction of Novel Sensitive Biosensing Interfaces for Tumor Markers” foi parcialmente realizada no “Institute of Mechanics, Chinese Academy of Sciences, Beijing”. Recorrendo a modelos membranares depositados em substratos de ouro, teve como objectivo a construção de interfaces para imunossensores estáveis, biomiméticos e que reduzam a adsorção não-específica de proteínas, recorrendo a uma técnica avançada, pouco recorrente e com elevada capacidade de detecção, a elipsometria de imagem de reflexão interna total (TIRIE).

Como ponto de partida, foi utilizada uma abordagem já testada com ditiocarbamatos (DTCs) imobilizados em superfícies de ouro para o biorreconhecimento específico de anticorpos e antigénios. Este método é particularmente útil, na medida em que permite a modificação da superfície do ouro e a imobilização de ligandos, nomeadamente a proteína A (que permite uma orientação correcta do anticorpo que a ela se liga posteriormente) em apenas um passo e, para além disso, não é necessário recorrer à activação dos grupos dos grupos funcionais para a imobilização covalente do anticorpo.
Para além de IgG e Anti-IgG, esta plataforma foi testada com o antigénio específico da próstata (PSA) e anti-PSA por TIRIE. Esta técnica foi utilizada para a detecção e monitorização em tempo real do reconhecimento do anticorpo e respectivo antigénio pelo immunosensor imobilizado no substrato de ouro. Globalmente foi possível mostrar que os DTCs imobilizados em ouro podem ser utilizados no reconhecimento de PSA e Anti-PSA e, portanto, devem ser encarados enquanto possíveis interfaces para a detecção específica de marcadores tumorais.

Ainda no sentido do desenvolvimento de imunossensores, foi testada e optimizada uma nova abordagem recorrendo à deposição de fosfolípidos sobre uma monocamada auto-montada (SAM) depositada num substrato de ouro. Para a formação da SAM utilizou-se 11-amo-1-undecanetiol, que se liga fortemente à superfície do ouro por meio de um grupo tiol. Após activação do grupo carboxilo estrategicamente localizado na extremidade da cadeia acilo do 1-miristoil-2-(14-carboximiristoil)-sn-glicer-3-fosfocolina (DMPC-COOH), este foi depositado sobre a SAM, ligando-se covalentemente ao seu grupo amina, localizado na sua extremidade. Entre as moléculas de DMPC-COOH foram posteriormente inseridas moléculas de ácido 2-hidroxioleico que, contendo também um grupo carboxílico numa extremidade, pode ser ativado para imobilizar proteínas covalentemente. Entre cada um dos passos da construção do imunossensor acima descritos foi utilizada elipsometria convencional para estimar a espessura da superfície, confirmando assim a correcta execução de cada passo de modificação. Com o mesmo objectivo foram também efectuadas experiências de AFM como forma de visualização da topografia da superfície durante a construção do imunossensor. Tal como anteriormente, a técnica de TIRIE foi utilizada para monitorizar o reconhecimento específico dos anticorpos e respectivos antigénios (IgG e Anti-IgG) e, como complemento, foi também utilizada a técnica de SPR. Com este estudo mostrou-se que esta plataforma apresenta algum potencial enquanto imunossensor capaz de evitar interacções não específicas, um dos requisitos necessários para a construção de um biossensor. Para além disto, foi demonstrada a possibilidade da utilização de fosfolípidos modificados para a construção de monocamadas estáveis, que podem ser aplicadas como componentes de biossensores.

Com o trabalho apresentado ao longo desta dissertação mostra-se, uma vez mais, a importância da utilização de sistemas modelo na mimetização das membranas biológicas e enquanto versáteis ferramentas, quer para o estudo de fenómenos associados
ao comportamento das biomembranas no seu contexto natural, quer para o desenvolvimento de importantes plataformas usadas na detecção de patologias.

**Palavras-Chave**

- Sistemas modelo de biomembranas;
- \textit{m}-cresol;
- Espectroscopia de fluorescência.
- Biofuncionalização de superfícies de ouro;
- Construção e desenvolvimento de imunossensores;

Nota: Este resumo encontra-se escrito sem utilização do novo acordo ortográfico.
Abstract

Different model membrane systems have been developed and improved over the years, in order to study the complexity associated with the structure and function of biological membranes. These models retain the lipid bilayer structure, and their size, geometry and composition can be tailored regarding the goals of each specific study. They include free-standing lipid bilayers (liposomes), with several sizes and layers, and supported lipid bilayers (SLBs). The studies employing membrane model systems can be performed with a wide range of biophysical characterization techniques, such as spectroscopies, namely fluorescence spectroscopy, surface characterization techniques, like atomic force microscopy (AFM) and ellipsometry.

In this work, biomembrane model systems were applied regarding two different perspectives. Firstly, $m$-cresol interaction with biological membranes was studied in liposomes (large unilamellar vesicles (LUVs)) with several fluorescence spectroscopy techniques and SLBs with AFM. $m$-cresol is an excipient widely used in pharmaceutical formulations, like Humulin, a formulation prescribed to diabetes treatment. Recently, several studies have reporting cases of toxicity associated with $m$-cresol usage but, despite that, its effects on biomembranes are not described. The steady state fluorescence anisotropy and fluorescence lifetime results, both of $m$-cresol intrinsic fluorescence and membrane fluorescent probes, in combination with real-time AFM experiments, show that $m$-cresol interacts with lipid bilayers superficially, leading to the bilayer components reorganization. Furthermore, it keeps this ability when integrated in Humulin, but with less intensity.

The purpose of the second part of the work was the building of biosensing interfaces to avoid protein non-specific adsorption using different gold modifications. The previously reported dithiocarbamate (DTC) formation principle on gold substrates was used to test prostate specific antigen (PSA) and anti-PSA, showing that this immunosensor platform can be employed in tumor markers detection. One more complex approach, based on utilization of self-assembled monolayers (SAMs) and modified lipids, was also tested. The covalent immobilization of IgG and Anti-IgG was successfully achieved with this immunosensor interface that may be used to avoid non-specific adsorption. The immunosensors construction was followed by conventional ellipsometry.
and AFM and its performance was monitored using surface plasmon resonance (SPR) and total internal reflection imaging ellipsometry (TIRIE).

**Keywords**

- Model membrane systems;
- \(m\)-cresol;
- Gold surfaces modification;
- Fluorescence spectroscopy;
- Immunosensors building and development.
# List of abbreviations and symbols

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acceptor</td>
<td>A</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>BB</td>
<td>Blocking Buffer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-Coupled-Device</td>
</tr>
<tr>
<td>CDI</td>
<td>Carbonyldiimidazole</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>D</td>
<td>Donor</td>
</tr>
<tr>
<td>DTC</td>
<td>Dithiocarbamate</td>
</tr>
<tr>
<td>di-4-ANEPPS</td>
<td>4-(2-(6-(Dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium inner salt</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPC-COOH</td>
<td>1-myristoyl-2-(14-carboxymyristoyl)-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster’s Resonance Energy Transfer</td>
</tr>
<tr>
<td>HOPG</td>
<td>Highly Ordered Pyrolytic Graphite</td>
</tr>
<tr>
<td>LUV</td>
<td>Large Unilamellar Vesicles</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar Vesicles</td>
</tr>
<tr>
<td>NBD-DOPE</td>
<td>N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
</tbody>
</table>
**NBD-DPPE** N-(7-nitrobenz-2-oxa-1,3-diazol-4-y1)-1,2-dipalmitoyl-\textit{sn}-glycero-3-phosphoethanolamine, triethylammonium salt

**NHS** \(N\)-hydroxysuccinimide

**POPC** 1-palmitoyl-2-dioleoyl-\textit{sn}-glycero-3-phosphocholine

**PSA** Prostate Specific Antigen

**PSM** \(N\)-palmitoyl-\textit{D-erythro}-sphingosylphosphorylcholine

**Rhod-DOPE** N-(lyssamine rhodamine B sulfonyl)-1,2-dioleoyl-\textit{sn}-3-phosphatidylethanolamine ammonium salt

**SAM** Self-assembled Monolayer

**SLB** Supported Lipid Bilayer

**PSM** \(N\)-palmitoyl-sphingomyelin

**SPR** Surface Plasmon Resonance

**SUV** Small Unilamellar Vesicles

**TIRIE** Total Internal Reflection Imaging Ellipsometry

\(t\)-\textit{PnA} \textit{trans}-parinaric acid

**UV** Ultraviolet

\(X\) Molar Fraction

### Symbols

\(d\) Thickness

\(\Lambda\) Ellipsometric phase parameter

\(f_0\) Resonant frequency, Hz

\(k\) Extinction coefficient

\(l_d\) Liquid disordered

\(l_o\) Liquid ordered
\( n \)  Refractive index or number of electrons

\( \tilde{N} \)  Complex refractive index

\( \theta \)  Angle of incidence or propagation

\( \Psi \)  Ellipsometric amplitude parameter

\( t \)  Time, s

\( \langle r \rangle \)  Fluorescence anisotropy

\( \frac{R_p}{R_s} \)  Fresnel coefficients in p and s directions

\( T \)  Absolute temperature, K

\( \bar{\tau} \)  Lifetime weighted quantum yield or amplitude averaged lifetime
Part I

Study of $m$-cresol effects on membrane biophysical properties using model membrane systems
Study of $m$-cresol effects on membrane biophysical properties using model membrane systems

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Abstract

$m$-cresol is a preservative used in numerous pharmaceutical formulations, including parenteral insulin and vaccines. Humulin® is one such form of insulin containing high levels of $m$-cresol. Therefore, we studied the effects of $m$-cresol alone and in Humulin® on membrane model systems containing raft-like liquid ordered domains ($l_o$), in order to understand the effects that this compound, when present in pharmaceutical formulations, can have on human cell membranes, which might in turn explain its toxicological effects.

The intrinsic fluorescence of $m$-cresol was first studied, showing that it interacts with liposomes and furthermore, this interaction is strongly dependent on membrane lipid composition. Liposomes were also labeled with several membrane probes spanning a range of in-depth locations and with preference for distinct lipid domains. The probes located at the bilayer core reported no effect, whereas for the more superficial probes, $m$-cresol induced a significant intensity-weighted fluorescence lifetime decrease and again a membrane composition dependence.

Real-time imaging was carried out by atomic force microscopy (AFM), on ternary supported lipid bilayers (SLB). It was observed that upon addition of $m$-cresol in the $\mu$M range, a small reduction of the $l_o$ phase fraction occurs without changing its thickness. For higher $m$-cresol concentrations, raft-like domains totally disappeared. All the changes observed occur in less than one hour. $m$-cresol contained in Humulin® has qualitatively similar effects, but much less pronounced.

As a whole, we show that $m$-cresol interacts with the membrane surface, affecting lipid raft organization. In Humulin®, the presence of other components probably decreases the amount of $m$-cresol available to interact directly with membrane lipids.
1. Introduction

Cresol was for the first time identified in 1851 and the three cresol isomers (o-, m- and p-cresol) in 1896 [1]. Currently, they are used in different areas, such as textiles industries, as bactericide and fungicide in agriculture, in cosmetic industry as antiseptics and fragrances, as industrial solvents in synthetic processes, in pharmaceutical industry and in many others around the world [1-3]. Naturally, they are formed as metabolites from enzymatic oxidation of toluene [2] and menthofuran [3] and are components of foods and drinks, being widely disseminated in nature [1]. For that reason, its toxicity has deserved a great attention and there are several toxicity studies reporting quite a lot of side effects caused by exposure to cresol [2-5]. These effects include severe inflammation of mucous membranes and central nervous system [2] and hepatic injury that leads to death [6]. The toxicity has been well described for the three isomers and, usually, p-cresol is consider the most toxic isomer and its toxicity implicates reactive intermediates formation [2], like reactive oxygen species, contributing to atherosclerosis and thrombosis [3]. Despite that, the degree of cell killing observed using rat liver slices in presence of p-cresol, can be achieved using a 5- to 10-fold higher concentration of either o- or m-cresol. Moreover, oral LD50s of all isomers are very similar [2] and m-cresol (Figure 1) was determined, by the Environmental Protection Agency from U.S. Department of Health and Human Services, as a possible human carcinogen [7].

Dentistry is another field of cresol's application [3, 8]. In conjugation with formaldehyde, they have been used in production of dentistry products, as a component of dental materials and devices, as solvent and as disinfectant. Therefore, exposure to formocresol, via inhalation, has been subject of special concern regarding its mutagenic and carcinogenic potential as well as possible damage of central nervous system [9-11]. According to these studies lipid peroxidation increases after exposure to cresols as a consequence of free radicals production, which may induce changes in membrane fluidity and permeability [10].

Given the ability of phenolic compounds and specially, cresols, to act as antiseptics and stabilizers of protein aggregation [12], they have been widely used in commercial pharmaceutical formulations [13, 14]. Firstly, they were described as preservatives in insulin formulations [14] used to prevent bacterial growth and to stabilize insulin molecules during storage and transport [15]. More recently, m-cresol and other phenolic derivatives were also described as excipients [16], used mainly with the intent of preserve insulin hexameric form and maintain the sterility of solution. Despite the problematic toxic effects known, cresols are used in substantial amounts in these formulations. One example is Humulin that contains m-cresol 2.5 mg/mL and human insulin 100 U/mL, which represents c.a. 38-fold higher concentration of m-cresol. Humulin is a human insulin injection formulation synthesized through rDNA technology and prescribed to improve
glycemic control in patients with type 1 and type 2 diabetes mellitus. It also contains glycerin 16 mg/mL, endogenous zinc (approximately 0.015 mg/100 U) and water. Some of its side effects comprise swelling of hands and feet and heart failure. The most common problem concerning insulin pumps like Humulin are complications associated with continuous subcutaneous insulin infusion. These complications involve skin irritation and local inflammation at the infusion site and seem to be associated to m-cresol presence in large amounts, through an interference with body defense mechanisms [5]. Patients who used insulin formulation containing m-cresol had a significantly higher inflammation rate than those who used insulin with methyl p-hydroxybenzoate [4]. Comparing pure m-cresol with insulin formulations containing m-cresol, the level of toxicity was similar and even a short exposure to m-cresol induces cell death, whereas insulin solution without excipients did not cause cell death [16].

m-cresol interaction with synthetic phospholipid liposomes was studied for the first time by differential scanning calorimetry [8]. It was shown that m-cresol is capable of induce changes in phase transition temperature and in enthalpy of the transition from gel to a liquid-crystalline phase. However, m-cresol effects concerning cell membrane are not well established and there is a lack of information about its fluorescence properties. Thus, the purpose of this work is to present a detailed biophysical characterization of the interaction between m-cresol and lipid bilayers in order to understand the potential effects caused by this compound on cell membrane. Humulin was used as an example of a formulation containing high amounts of this excipient. Its fluorescence properties were evaluated in presence of liposomes containing well characterized binary mixtures and a ternary mixture of the type low main-transition temperature/high main-transition temperature/Chol to mimic lipid rafts [17]. Using atomic force microscopy (AFM) with supported lipid bilayers (SLB), m-cresol and Humulin effects were evaluated in real-time. The chemical structures of m-cresol and lipid molecules used in this work are represented in Figure 1. 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphocholine (POPC) is one of the most abundant glycerophospholipids of eukaryotic organisms’ cell membranes, N-palmitoyl-sphingomyelin (PSM) is the central sphingolipid of the animal cell membranes and Cholesterol (Chol) is the sterol that exists in higher amounts [18].
2. Materials and Methods

2.1. Chemicals

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphocholine (POPC), N-palmitoyl-sphingomyelin (PSM) and N-(lyssamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-3-phosphatidylethanolamine ammonium salt (Rhod-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL); Bovine insulin, Cholesterol (Chol), minimum 99%, and Ludox (colloidal silica diluted to 50 wt% in water) were purchased from Sigma-Aldrich (Barcelona, Spain). trans-parinaric acid (t-PnA), 4-(2-(6-(Dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)-pyridinium inner salt (di-4-ANEPPS), N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-DPPE) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (NBD-DOPE) were purchased from Invitrogen (Madrid, Spain). m-Cresol was obtained from MERCK (Germany) and Humulin® REGULAR 100 UI/mL from Lilly Lda. (Portugal), containing m-cresol 2.5 mg/mL. Other reagents were of the highest purity available. Two buffers were used for hydration of lipid mixtures: i) 10 mM Hepes, 150 mM NaCl, pH 7.4 (HEPES buffer); ii) 2.70 mM K$_2$HPO$_4$, 1.50 mM NaH$_2$PO$_4$, 150 mM NaCl, pH 7.4 (PBS).
2.2. Phospholipid, cholesterol, m-cresol and fluorescent probes quantification

The phospholipid concentration was determined gravimetrically and by inorganic phosphate quantification [19]. Cholesterol quantification was made by gravimetry. m-cresol concentration was determined spectrophotometrically using $\varepsilon (270\text{nm}) = 2.34 \text{ M}^{-1} \text{cm}^{-1}$ [20]. Probe concentrations in stock solutions (spectroscopic grade solvents) were determined spectrophotometrically using $\varepsilon (\text{NBD-DPPE or DOPE, 465 nm, chloroform}) = 21 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}; \varepsilon (\text{Rhod-DOPE, 559 nm, chloroform}) = 95 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}; \varepsilon (\text{t-PnA, 299.4 nm, ethanol}) = 89 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}, \varepsilon (\text{di-4-ANEPPS, 497 nm, methanol}) = 42 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ [21].

2.3. Lipid Mixtures and Multilamellar Vesicles (MLV) Preparation

In AFM studies the ternary mixture DOPC/PSM/Chol 2:2:1 was employed. In fluorescence spectroscopy studies, three different systems were used (POPC/PSM/Chol, POC/Chol and POPC/PSM). For ternary system, the several mixtures prepared had the following molar proportions of each lipid component 80/20/0; 71.8/23.1/5.1; 60/23.1/5.1; 48.5/29.3/22.2; 33.3/33.3/33.3 and 25.2/35.5/39.3. These compositions were selected to span the tie line containing the 1:1:1 equimolar mixture, taken from the ternary phase diagram [17]. For binary system POPC/Chol, the molar proportions of each component were 0/100; 5.1/94.9; 14/86; 22.2/77.8; 33.3/66.7 and 39.9/60.7 and for system POPC/PSM were 0/100; 20/80; 30/70; 40/60; 50/50; 60/40; 70/30; 80/20 and 100/0. Lipid stock solutions and lipid mixtures were prepared with spectroscopic grade chloroform. The solvent was slowly evaporated under a mild flow of nitrogen, followed by overnight vacuum. MLV suspensions were prepared by hydration of the mixtures with either HEPES buffer for small unilamellar vesicles (SUV) and SLB preparation, or PBS for large unilamellar vesicles (LUV) preparation and progressively suspended by vortex stirring and freeze-thaw cycles (T>50°C).

2.4. SLB preparation and interaction with m-cresol

SUV (total lipid concentration 1mM) were obtained by power sonication (Hielscher, UP200S). 150 µL of the resulting suspension with 5mM CaCl$_2$ were deposited on freshly cleaved mica (Veeco) and incubated for 1h at 60°C. After this incubation step, the SLB samples were left at room temperature for 1h to cool and then washed several times with HEPES buffer [22]. The SLB samples were initially covered with 80 µL of HEPES buffer. After AFM imaging in a region of interest, successive additions of higher concentrations of m-cresol and Humulin were performed with at least 2 hours between each one. The same region was imaged several times between and after each addition of m-cresol. Successive additions of HEPES buffer and insulin were also performed as control.
2.5. AFM Imaging

*In situ* AFM measurements were performed at room temperature using a Multimode Nanoscope IIIa Microscope (Digital Instruments, Veeco). Topographic images were taken with a scan rate of ca. 2 Hz in tapping mode. Before each experiment, the glass block holding the cantilever was washed several times with water and ethanol. The cantilevers used were made of silicon nitride (NPS, ca. 0.58 N/m of spring constant, Veeco) with a resonance frequency in liquid of about 9 kHz. In many cases, regions with small defects, and where the lipid domains had distinctive shapes were chosen, in order to easily identify the changes in lateral organization of the membrane. The images presented in this study are representative of each sample (the observations are uniform for the whole sample); at least 3 independent experiments on freshly prepared SLB have been performed. To obtain the thickness values (or thickness differences) for each sample, at least 10 different topographic profiles were drawn, and the median value taken. The most representative profiles were selected for the figures shown. The relative area corresponding to the different types of domains in each image was calculated using the software ImageJ.

2.6. LUV preparation and interaction with m-cresol

Large unilamellar vesicles (LUV) (total lipid concentration 1mM) were prepared by standard procedures [23]. LUV were formed by extrusion (Avanti Mini-Extruder) at 60°C, by forcing the MLV suspension 21 times through polycarbonate filters with pore diameter of 100 nm (Nucleopore, Whatman). The probe/lipid ratio used was 1:500 (mol/mol) for t-PnA, 1:1000 for NBD-DOPE and NBD-DPPE, 1:200 for Rhod-DOPE and 1:500 for di-4-ANEPPS. All probes, except t-PnA and di-4-ANEPPS were incorporated in lipid mixtures with lipid stock solutions. After extrusion, samples were kept overnight at 4°C protected from light. Before the measurements, samples were slowly brought to room temperature and incubated with pure m-cresol 30 µM or Humulin containing m-cresol 100µM and 1mM at least one hour before. In control experiments, measurements were performed with and without the presence of m-cresol, Humulin and insulin.

2.7. Fluorescence Spectroscopy Measurements

Fluorescence measurements were performed at room temperature in 1.0cm x 0.4cm quartz cuvettes using a Spex Fluorolog 3-22/Tau 3 spectrofluorometer (Horiba Jobin Yvon) equipped with double grating monochromators in both excitation and emission light paths. For steady-state measurements, the excitation and emission wavelengths were respectively 275nm and 303nm for m-cresol and Humulin, 303nm and 405nm for t-PnA, 468nm and 536nm for NBD-DPPE and NBD-DOPE, 467nm and 599nm for di-4ANEPPS and 570nm and 593nm for Rhod-DOPE. The steady-state anisotropy \( r \) was calculated according to Equation 1 [24].
\[ r = (I_{VV} - G \times I_{VH})(I_{VV} + 2G \times I_{VH}) \quad Eq. 1 \]

in which G is the instrumental correction factor. Subscripts V and H represent the vertical and horizontal orientations of the polarizers, and the order of the subscripts corresponds to excitation and emission. An adequate blank was subtracted from each intensity reading. The set of the 4 intensity components for each sample was measured 7 times.

The fluorescence intensity decays were obtained by the single photon counting technique using a nanoLED N-320 (Horiba Jobin-Yvon) for the excitation of r-Pna and emission wavelength was 405nm. For the excitation of di-4-AEPPS and NBD-DPPE and NBD-DOPE Nano-LED N-460 was used and emission wavelength was 599nm and 536nm, respectively. For Rhod-DOPE, nanoLED N-560 was used for the excitation and emission wavelength was 593nm. Ludox scattering was used to obtain the instrumental response function and the background (obtained with the black sample) was subtracted from the decay. Data analysis was performed using the software TRFA v1.4 (Scientific Software Technologies Center, Minsk, Belarus). Fluorescence decays were analyzed by fitting a sum of exponentials as shown in Equation 2,

\[ I(t) = \sum_{i=1}^{n} \alpha_i \exp\left( -\frac{t}{\tau_i} \right) \quad Eq. 2 \]

where \( \alpha_i \) and \( \tau_i \) are the normalized amplitude and lifetime of the component \( i \), respectively. The mean intensity-weighted fluorescence lifetime \( <\tau> \) was obtained through Equation 3,

\[ <\tau> = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i \quad Eq. 3 \]

The quality of the fit was judged by a reduced \( \chi^2 \) value close to 1 and random distribution of weighted residuals and residuals autocorrelation.

All the data represents the mean ± standard deviation of at least 3 independent samples.

### 2.8. FRET Measurements

NBD-DPPE/Rhod-DOPE (Donor (D)/Acceptor (A)) pair with lipid ratio 1:1000/1:200 was chosen for FRET between \( l_o \) and \( l_d \) phases [25]. FRET efficiency, E, was obtained from the time-resolved fluorescence intensity curves, for each sample, through Equation 4,

\[ E = 1 - \frac{\bar{\tau}_{DA}}{\bar{\tau}_D} \quad Eq. 4 \]

Where \( \bar{\tau} \) is the amplitude-weighted fluorescence lifetime of the probe in the presence of the donor only (\( \bar{\tau}_D \)) and with both donor and acceptor (\( \bar{\tau}_{DA} \)).
3. Results

3.1. Interactions of m-cresol with biomembranes evaluated through intrinsic fluorescence properties of m-cresol and insulin

m-cresol interactions with lipid bilayers mimicking the outer leaflet of mammalian plasma membrane were evaluated through its intrinsic fluorescence properties. m-cresol maximum fluorescence excitation occurs at 270 nm and maximum of fluorescence emission at 302 nm (Figure 2). The excitation and emission spectra measured (Figure 2) are in agreement with previously reported data [26].

![Figure 2](image-url)

**Figure 2.** Normalized excitation (black line) and emission (gray line) spectra of m-cresol at room temperature. The represented spectra are the median of at least 3 independent experiments.

This excipient is a phenol derivative [1] and displays absorption and emission in the UV region of the electromagnetic spectrum [27], similarly to other phenol derivatives such as the aromatic amino acid tyrosine. Thus we used this feature of m-cresol to assess directly its adsorption to lipid bilayers and their consequences without the need of using external probes and still taking advantage of the high sensitivity of fluorescence spectroscopy. The system POPC/PSM/Chol was chosen as a lipid raft model with different lipid proportions accordingly with the tie-line that contains the mixture 1:1:1 in the phase diagram [17]. Using these proportions, m-cresol effects were evaluated in presence of a liquid ordered (l0) and a liquid disordered phase (lα) and in coexistence of both, since phase boundaries are well established with very small uncertainty. The steady-state fluorescence anisotropy of m-cresol (Figure 3- A) increased in presence of liposomes, suggesting that its rotational dynamic is reduced. The intensity-weighted average fluorescence lifetime (Figure 3 - B), that is proportional to the steady-state fluorescence intensity, follows the same trend, confirming that m-cresol mobility is in fact reduced as a result of the interaction with liposomes and that the microenvironment of the molecule is changed significantly. Furthermore, this interaction depends on membrane lipid composition, since the values tend to decrease as the content in PSM/Chol becomes higher. This could indicate that m-
cresol may interact more profoundly with disordered domains. However, the shape of both curves suggests that \( m \)-cresol interaction with ordered domains should be preferred, because small fractions of \( I_o \) are sufficient to induce a noticeable change in the photophysical parameters of \( m \)-cresol.

Regarding Humulin, since it is composed by two species with intrinsic fluorescence properties, insulin and \( m \)-cresol, anisotropy values obtained should be the weighted sum of the individual anisotropies of these two fluorophores, by anisotropy additivity law [24]:

\[
 r_{Humulin} = f_{Insulin}r_{Insulin} + f_{m\text{-}cresol}r_{m\text{-}cresol} \quad Eq. 5
\]

where \( r \) is the anisotropy value and \( f \) is the fractional intensity. The fractional intensity of each specie depends on its molar fraction and fluorescence quantum yield. Since insulin fluorescence emission is mainly due to the four tyrosine residues, an amino acid residue with aromatic group, \([28]\) and \( m \)-cresol is a phenolic compound, quantum yield of each specie should be similar. Thus, the difference between \( m \)-cresol and insulin contribution to Humulin anisotropy is mainly due to their molar fraction. It is known that in Humulin formulation, \( m \)-cresol molar concentration is ca. 38-fold higher than insulin concentration (Eli Lilly and Company) which explains why anisotropy values obtained for \( m \)-cresol are comparable to the ones obtained for Humulin (Figure 3 – C). Insulin anisotropy value in presence of liposomes is much larger than \( m \)-cresol anisotropy values (c.a. 0.06), and it does not change (data not shown) regarding the lipid composition.

Additionally, Humulin steady-state anisotropy (Figure 3 – C) and intensity-weighted mean fluorescence lifetimes (Figure 3 – D) exhibit the same behavior than pure \( m \)-cresol in presence of the ternary lipid bilayers (Figure 3 – A, B), increasing with the same dependency on lipid phase. These results show that \( m \)-cresol in Humulin formulation is also able to interact with the membrane. Upon increase of \( m \)-cresol concentration, anisotropy and intensity-weighted mean fluorescence lifetime values slightly decrease, indicating that with the increase in \( m \)-cresol concentration, the system becomes more complex and a simple membrane/water partition of \( m \)-cresol is not sufficient to quantitatively describe the data.
3.2. Interactions of m-cresol with biomembranes evaluated through membrane fluorescent probes

The highly used excipient m-cresol is able to interact with models of the mammalian plasma membrane outer leaflet, both alone, and when present in an insulin formulation used for human diabetes therapy. Thus, it can be anticipated that the interaction of m-cresol, both alone and in the formulation may have effects on membrane lipid organization/biophysical properties. Thus, to fully characterize these interactions, it was necessary to label liposomes with several membrane probes spanning a range of in-depth locations and with preference for distinct lipid domains and biophysical properties (e.g. [29-31]). Two different head-labelled phospholipid analogues localized at the membrane interface [32] were used, NBD-DPPE which partitions preferentially into l_o phase [18, 33] and NBD-DOPE which partitions preferentially into l_d phases [34]. Upon
addition of \( m \)-cresol, both NBD-DPPE (Figure 4 – A) and NBD-DOPE (Figure 4 – B) intensity-weighted average fluorescence lifetimes decreased, indicating that \( m \)-cresol locates preferentially, at least in a first stage, at the membrane surface of both ordered and disordered domains. Moreover, this interaction should preferentially affects disordered domains, since the magnitude of the variation is smaller in the case of NBD-DPPE (Figure 4 – A) and the amplitude-weighted mean fluorescence lifetime values follow the same trend with and without the presence of \( m \)-cresol.

**Figure 4.** Intensity-weighted average fluorescence lifetime of NBD-DPPE in absence (square) and in presence (triangle) of pure \( m \)-cresol 30 µM (A) and \( m \)-cresol 100 µM from Humulin (C); Intensity-weighted average fluorescence lifetime of NBD-DOPE in absence (square) and in presence (triangle) of pure \( m \)-cresol 30µM (B) and \( m \)-cresol 1mM from Humulin (D). Both measurements were performed in presence of LUVs (POPC/SM/Chol ternary mixtures with molar proportions indicated in Materials and Methods) at room temperature. The values are the mean ± standard deviation of at least 3 independent experiments. The dotted lines are merely guides for the eye.

Additionally, the probe di-4-ANEPPS was used to address the hypothesis that \( m \)-cresol interaction with the membrane surface may implicate surface charge effects such as an alteration of membrane dipole potential as a result of an increased hydration. Since it is known that Chol increases membrane dipole potential [35], di-4-ANEPPS is also sensitive to the presence of Chol and Chol-enriched domains [36] and its fluorescence lifetime increases with the amount of Chol,
independently of the bilayer phase [30]. As expected, the intensity-weighted average fluorescence lifetime increases with the conversion of $l_d$ phase in $l_o$ (Figure 5) and its values are not affected by the presence of $m$-cresol. This result shows that $m$-cresol does not induce any changes on membrane hydration or solvent relaxation processes.

**Figure 5.** Intensity-weighted average fluorescence lifetime of di-4-ANEPPS in absence (square) and in presence (triangle) of pure $m$-cresol 30µM in LUVs of POPC/SM/Chol ternary mixtures with molar proportions indicated in Materials and Methods at room temperature. The values are the mean ± standard deviation of at least 3 independent experiments. The dotted lines are merely guides for the eye.

In order to understand if $m$-cresol effects are restricted to the membrane surface or if it also interacts with the hydrophobic core of the lipid bilayer, the intensity-weighted mean fluorescence lifetime of the probe trans-parinaric (t-PnA) was measured in the presence of $m$-cresol. t-PnA is a very useful probe to assess even subtle changes in the organization of lipid rafts and ordered domains, both in membrane model systems [37, 38], and in living cell membranes [30, 39]. In addition, it provides information about the phospholipids acyl chains packing, since its chromophore is integrated inside the bilayer core [40]. The intensity-weighted average fluorescence lifetime of t-PnA (Figure 6) seems not to be significantly affected by the presence of $m$-cresol, even when its concentration increases 10-fold. With conversion of $l_d$ phase into $l_o$, t-PnA intensity-weighted average fluorescence lifetime values are slightly more affected by the presence of $m$-cresol. Interestingly, the intensity-weighted average fluorescence lifetime of t-PnA which is already longer in the $l_o$ [37] becomes slightly longer. Rather than leading to a decreased packing in the more ordered and compact $l_o$ domains, as could be intuitively expected, it seems to have an opposite effect. The major conclusion is, however, that $m$-cresol effects on membrane do not occur at the hydrophobic core level and it only adsorbs to the surface, probably involving H-bonding of the phenol OH group with the lipid headgroups.
Figure 6. Intensity-weighted average fluorescence lifetime of t-PnA in the absence (square) and presence of m-cresol 30µM (circle) and 300 µM (triangle) in LUVs of POPC/SM/Chol ternary mixtures with molar proportions indicated in Materials and Methods at room temperature. The values are the mean ± standard deviation of at least 3 independent experiments. The dotted lines are merely guides for the eye.

3.3. Interactions of m-cresol with lipid rafts evaluated in real-time by AFM

As previously shown, by fluorescence spectroscopy, m-cresol effects on bilayer seem to be restricted to the bilayer surface and dependent on the lipid phase. To clarify these effects, in situ tapping-mode AFM was performed to follow real-time detailed morphological and structural changes induced by m-cresol on SLB. For that purpose, ternary mixtures of DOPC/PSM/Chol with molar ratio of 2:2:1 were used to form SLB containing raft-like liquid ordered domains on mica (Figure 7). According to the phase diagram [41], by using this lipid proportion, at room temperature, two liquid phases are assembled, with both PSM and Chol preferentially localized in l_o phase (thicker) surrounded by DOPC in l_d phase (thinner) [42]. In absence of m-cresol (Figure 7 – A) coexistence of l_o and l_d domains in the predicted proportions [43] is evident (Table 1) and the thickness gap between them is close to 1 nm (Table 1, Figure 7), as previously reported [22]. Upon addition of m-cresol, a gradual decrease in size and number of small l_o domains is observed (Figure 7). Both effects are clearly depicted in the circles drawn in Figure 7, for 300 µM m-cresol concentration. Despite that, l_d phase remains apparently unchanged with no detectable thickness variation (Table 1, Figure 7). For higher m-cresol concentration (successive addition), l_o small domains reduction is even clearer and l_o fraction tends to decrease concomitantly with an increasing of the l_d fraction. Upon ca. two hours exposure of SLB to the highest m-cresol concentration (3 mM), l_o domains totally disappear (Figure 7 - E), meaning that m-cresol effects are both time and concentration-dependent. In fact, these effects on SLB are undoubtedly caused by m-cresol interaction, since scanning the surface repeatedly does not change significantly the domains morphology (Figure S1). The same experiments were performed with addition of Humulin comprising the same range of m-cresol concentrations (Figure 8). On the whole, Humulin effects on SLB are identical to those of m-cresol, promoting a progressive disruption of
lo domains. These effects should be a consequence of an interaction with lo/ld boundaries since that first they lead to the disappearance of small lo domains and then to the entire lo fraction. Interestingly, for each m-cresol concentration, the percentage of lo reduction is lower after addition of Humulin than when pure m-cresol is added. For example, after addition of Humulin containing 300 µM m-cresol, only 8% of lo fraction is lost (Table 1) but, in presence of the same concentration of pure m-cresol lo fraction is reduced by 11%. Moreover, in presence of 3000 µM pure m-cresol, all lo fraction is converted into ld fraction (Table 1) and, in presence of Humulin containing m-cresol in a concentration 10-fold higher (3 mM), lo fraction was reduced only in 46%. These results show that m-cresol, per se, interacts with lipid rafts in a more damaging way than when it is contained in Humulin.

**Figure 7.** m-cresol interaction with a bilayer displaying ld/lo phase separation (lipid rafts) on mica. AFM image of an SLB composed by DOPC/PSM/Chol (2:2:1 mol:mol:mol) deposited on mica, in the absence of cresol (A), 1h (B), 2h (C) after the addition of cresol 300µM and 5min (D), 1h (E), and 2h (F) after the addition of m-cresol 3 mM in Hepes buffer. Circles show changes in lipid rafts shape after addition of m-cresol. The inset shows the topographical profiles corresponding to the colored lines in panels A – D. The images were obtained in a liquid cell at room temperature. The images correspond to an area of 20x20µm². Z = 5nm.
Table 1. Percentage of reduction of the area occupied by \( l_o \) phase on SLB deposited on mica and height difference between \( l_o \) and \( l_d \) phases after addition of both pure \( m \)-cresol and Humulin. \( l_o \), liquid ordered; \( l_d \), liquid disordered. Calculations were performed based on AFM topographic images after 2h exposure to pure \( m \)-cresol and Humulin.

<table>
<thead>
<tr>
<th>[Cresol] (μM)</th>
<th>( l_o ) Area Reduction (%)</th>
<th>Domain Height Difference (nm)</th>
<th>( l_o ) Area Reduction (%)</th>
<th>Domain Height Difference (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.0 ± 0.2</td>
<td>0</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1.0 ± 0.2</td>
<td>0</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>9</td>
<td>1.1 ± 0.1</td>
<td>8</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>300</td>
<td>17</td>
<td>0.9 ± 0.1</td>
<td>8</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>3000</td>
<td>100</td>
<td>-</td>
<td>46</td>
<td>1.3 ± 0.1</td>
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Figure 8. Humulin interaction with a bilayer displaying $l_d/l_o$ phase separation (lipid rafts) on mica. AFM image of an SLB composed by DOPC/PSM/Chol (2:2:1 mol:mol:mol) deposited on mica, in the absence of Humulin (A), 1h (B), 2h (C) after the addition of Humulin containing $m$-cresol 300µM and 5min (D), 1h (E), and 2h (F) after the addition of Humulin containing $m$-cresol 30mM in Hepes buffer. Circles show changes in lipid rafts shape after addition of Humulin. The inset shows the topographical profiles corresponding to the colored lines in panels A – D. The images were obtained in a liquid cell at room temperature. The images correspond to an area of 20x20µm². Z = 7nm.
4. Discussion

The current work presents a detailed study of the effects of an excipient highly used in pharmaceutical formulations, \textit{m}-cresol, on lipid bilayers mimicking the outer leaflet of mammalian plasma membrane. For that purpose, fluorescence spectroscopy and atomic force microscopy techniques were applied to study the effects of this compound on lipid model membranes displaying different lipid phases and lipid raft-forming ternary mixtures. The experiments were performed with pure \textit{m}-cresol and compared with Humulin, an example of a commercial pharmaceutical formulation containing \textit{m}-cresol, showing that when this excipient is incorporated in a formulation, its effects are still detectable.

For the first time, \textit{m}-cresol steady-state fluorescence anisotropy (Figure 3 – A) and intensity-weighted average fluorescence lifetime (Figure 3 – B) were measured. We demonstrated that \textit{m}-cresol intrinsic fluorescence is a valid tool to study \textit{m}-cresol dynamics and its interaction with biomembrane models, namely lipid bilayers. Through these measurements, it was unequivocally shown that \textit{m}-cresol interacts with lipid bilayers. With steady-state fluorescence anisotropy it was shown that, in presence of liposomes, \textit{m}-cresol mobility is more restricted as compared to the aqueous medium. Intensity-weighted average fluorescence lifetime results show a decrease in the number of collisions between \textit{m}-cresol and water molecules or buffer ions and, at the same time, a reduction on polarity of the medium in presence of liposomes which, by itself, may have influence on \textit{m}-cresol fluorescence properties. Moreover, a less polar environment may prevent the formation of dimers or clusters between different \textit{m}-cresol molecules and therefore, the probability of occurrence of self-quenching will be smaller than that in aqueous medium without presence of liposomes.

Another important hallmark of \textit{m}-cresol is the distinct behavior in presence of distinct lipid phases, showing that the biophysical properties of these phases play an important role in the interaction. It seems that \textit{l}_d phase is more affected by \textit{m}-cresol than \textit{l}_o phase, in which steady-state fluorescence anisotropy (Figure 3 – A) and intensity-weighted average fluorescence lifetimes (Figure 3 – B) exhibit smaller values. Nevertheless, this result does not mean that in presence of \textit{l}_o phase, \textit{m}-cresol does not interact with the bilayer and the small values obtained may be due to a self-quenching and energy transfer phenomena between \textit{m}-cresol molecules. These possibilities can occur when \textit{m}-cresol molecules are located at the membrane surface between phospholipid heads and they are close enough to allow the occurrence of these phenomena. This explanation is corroborated by the results obtained with the NBD-labeled lipids (Figure 4), since the observed intensity-weighted average fluorescence lifetime decrease can be caused by dynamic self-quenching or by the hydration increase at the bilayer core. The results obtained with \textit{t}-PnA (Figure 6) suggest then that \textit{m}-cresol should act preferentially on membrane surface and not at the
hydrophobic core level, and the decrease of the intensity-weighted average fluorescence lifetime is only explained by dynamic self-quenching phenomena.

The observation of \textit{m}-cresol effects on SLB composed by raft-forming mixtures through AFM showed notoriously a preferential interaction with \textit{l}_d/\textit{l}_o domains boundaries (Figure 7) in such a way that leads to a progressive reduction of the lipid rafts size over time and for higher concentrations, to the elimination of the ordered phase. Once again, this observation is confirmed by the results from fluorescence spectroscopy with NBD probes (Figure 4) and di-4-ANEPPS (Figure 5), indicating that \textit{m}-cresol effects are related essentially with an alteration on lipid phase proportions and the size of \textit{l}_o domains. Additionally, results obtained from FRET experiments (Figure S2) are in agreement with this behavior, since FRET efficiency between the donor, localized in \textit{l}_o phase, and the acceptor, with preference for \textit{l}_d phase, decreases in presence of \textit{m}-cresol. This result is in agreement with the expected, since an average increase of the \textit{l}_o domains size results in phase separation and therefore in an increase in donor-acceptor distance, as reported [18, 34].

Globally, \textit{m}-cresol interaction with lipid bilayers should involve an initial interaction with the bilayer surface of both \textit{l}_o and \textit{l}_d phases through hydrogen bounds between OH group from \textit{m}-cresol and phospholipid heads. This effect should be restricted to the bilayer surface, without perturbation of the phospholipid acyl chains packing. Since membrane dipole potential is higher at \textit{l}_o phase, at this phase, \textit{m}-cresol dipolar moment aligns with membrane dipole. Thus, \textit{m}-cresol insertion into the bilayer is energetically compensated by the dipolar moments interaction. This interaction is the explanation for the \textit{m}-cresol preference for \textit{l}_o phase. The packing of this phase is not perturbed during the interaction since \textit{m}-cresol should locates preferentially at the surface, aligned with the planar structure of the sterol rings. However, since new hydrogen bounds with \textit{m}-cresol are formed, the hydrogen bounds sterol-phospholipid are replaced, contributing to the further \textit{l}_o phase destabilization. This superficial interaction should happen preferentially on the interfaces between \textit{l}_o and \textit{l}_d domains probably because kinetically is more easy, leading to the disappearance of the small \textit{l}_d domains and to a progressive reduction of the \textit{l}_o domains size. This change on bilayer organization can be described as a displacement of the ternary mixture phase diagram in the direction of the disordered phase or a shift to higher temperature. At the final stage, bilayer should correspond to a disordered phase, but with distinct properties from the initial \textit{l}_d, since it contains large amounts of Chol. At \textit{l}_d phase, hydrophobic interactions should avoid \textit{m}-cresol contact with water molecules and it will have an orientation with more freedom. Globally, at this phase, the interactions will not be so strong as those at the \textit{l}_o phase, since there are no dipolar interactions between \textit{m}-cresol and the bilayer. The behavior based on dipolar moment interactions is similar to the one described to explain the different solubility of two fluorescent
lipid probes containing comparable apolar structures and different polar head groups, the NBD-hexadecylamine and the RG-tetradecylamine [44].

Knowing that in Humulin formulation, m-cresol concentration is c.a. 38-fold higher that of insulin, it was also convenient to study the effects of this formulation on membrane mimetic models in similar conditions as those used for pure m-cresol. The results obtained are in agreement with m-cresol behavior, suggesting that Humulin effects are largely triggered by m-cresol. The m-cresol toxicity level was recently tested and compared to insulin solutions containing m-cresol as excipient [16]. The results revealed that m-cresol displays the same level of cytotoxicity as the commercial insulin formulations, whereas insulin solutions without excipients did not cause cell death. Besides that, since m-cresol is also a preservative agent, the fact that the trend in intrinsic fluorescence is similar for pure m-cresol (Figure 3 – A, B) and Humulin (Figure 3 – C, D) is already quite surprising. This conclusion is based on the fact that, in formulation, m-cresol may interact with insulin itself and, possibly, with other components such as glycerol and hence, a more complex trend in the data should be expected. Actually, the system has a large number of components and, for example, a simple membrane/water partition of m-cresol is not sufficient to quantitatively describe the data. This is a problem regarding the study of this formulation using cells, since it is not possible to predict whether effects detected are due to m-cresol or insulin. This occurs because cells have innumerable receptors for insulin and thus, the application of Humulin will lead to activation of regulation pathways that will not be activated in presence of m-cresol alone. Therefore, this kind of studies has to be performed using model systems, like what was done during the study here presented.

Results here revealed may be of some medical importance since m-cresol is present in pharmaceutical formulations already in market and, particularly, Humulin is a widely used drug formulation prescribed as an adjunct to diet and exercise for patients with type 1 and type 2 diabetes mellitus. Furthermore, Humulin administration may be done subcutaneously or through intravenous injection. Thus, a fast release of formulation components leads to a fast spreading of m-cresol over the body, including blood circulation and the contact with cells, especially with cell membrane. Once in contact with the outer leaflet of plasma membrane, it may act on membrane constituents, namely with lipid rafts via mechanisms here described with possible implications on cellular viability. As a matter of fact, some of the skin irritation cases reported [4] using insulin-pump therapy containing m-cresol may be due to the m-cresol effects here described. Hence, this study will certainly contribute to unravel the mechanisms of pharmaceutical formulations containing m-cresol toxicity, at the membrane level, like insulin preparations.
5. References


Supplementary Material

**Figure S1.** AFM topographic images of an SLB composed of DOPC/PSM/Chol (2:2:1 mol:mol:mol) deposited on mica in the beginning of the scanning (A), 1h (B) and 2h (C) after scanning the surface. The images were obtained in a liquid cell at room temperature. The image corresponds to an area of 20x20µm². Z = 7nm.

**Figure S2.** Efficiency of FRET between the donor/acceptor pair NBD-DPPE/Rhod-DOPE in the absence and in presence of pure m-cresol 30 µM and m-cresol 1 mM from Humulin. Measurements were performed in presence of LUVs containing POPC/SM/Chol ternary mixtures with molar proportions 48.5/29.3/22.2 at room temperature.
Part II

Construction of Novel Sensitive Biosensing Interfaces for Tumor Markers Detection
Construction of Novel Sensitive Biosensing Interfaces for Tumor Markers and BOD Detection

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1. Introduction

Biosensors are produced combining a material of biological origin with a physical transducer (Figure 1). A biological recognition element, the functional component, is incorporated onto a transducer that is responsible for the conversion of chemical information, the interaction between the analyte and the biorecognition element, to a quantifiable signal. Transduction may be done by electrochemical or optical ways or by measurement of mass or heat and the resulting signal is then converted into a readout or display [45-48]. Enzymes, cell receptors, nucleic acids, microorganisms and antibodies (or antibody fragments) can be used as biological recognition elements. In this case, the biosensor is called immunosensor and makes use of the natural high affinity and specificity of the interaction antibody/antigen to detect the presence of a target analyte. Either an antigen or antibody is immobilized on a solid substrate, forming the sensing surface, and participates in the biospecific recognition of the analyte, allowing its detection and quantification [45, 48, 49]. The binding event can be directly detected through the antibody/antigen interaction (direct or label-free immunosensors) or a further label on one of the biomolecules can be used, such as an enzyme or a fluorescent dye (labeled or indirect immunosensors) [45, 50].

Regarding to the building of the sensing surface, through the immobilization of the biological receptor, it can be done using different procedures: entrapment behind a membrane, within a polymeric matrix, covalent bonding to self-assembled monolayers (SAMs), lipid bilayers, membranes or surfaces activated by means of functional groups or even by modification of the entire electrode material [47]. Among these immobilization strategies, lipid-based platforms are a highly promising approach since they provide a biomimetic environment for the support of biological sensing elements, like membrane-associated proteins, can reduce potential interferences and undesirable reactions with the sensing surface and avoid denaturation of the transducing element through contact with the metal surface [51-55]. At the same time, biomembrane models by themselves, namely supported lipid bilayers (SLBs), can be used to study membrane-related processes [56].

![Figure 1. General scheme of a biosensor. Adapted from [57].](image-url)
SAMs are a useful way to develop simple, effective and practical biosensing interfaces. They are usually prepared with a wide variety of alkanethiols that, over gold substrates or other electrode surfaces like silver and mercury, spontaneously self-assemble into monolayers. The choice between different alkanethiols (with shorter or longer alkyl chains and distinct terminal groups, for example) depends on the desired physical and electrochemical properties of the SAMs. The reproducibility, the easy preparation and the high stability and organization are the main advantages of this kind of surface modification [58-61]. Moreover SAMs may have amine or carboxylic acids as terminal groups, which can be easily used to covalently immobilize biomolecules. For instance, COOH-terminated SAMs can react with amine groups after a common activation step with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [62] (Figure 2).

Figure 2. Activation mechanism with EDC and NHS. EDC reacts with a carboxylic group on molecule 1 (a protein or a peptide), forming an intermediate that can react with other amine group from molecule 2 (a protein or a peptide). NHS ester intermediate (formed by reaction with NHS) as sufficient stability to permit two-step crosslinking procedures, which allows the carboxyl groups on one protein to remain unaltered. Adapted from [63].

An alternative system to thiolated SAMs on gold in biosensing is the in situ formation of dithiocarbamates (DTCs). DTCs are spontaneously formed with carbon disulfide (CS₂) and amine groups on a gold surface at room temperature and thus, they can be used to immobilize compounds with amine groups, such as proteins [64-69] (Figure 3). Using this simple, effective and practical method, antibodies can be immobilized to form an immunosensing interface with good sensitivity and strong capability to inhibit nonspecific adsorption [69]. Yet, the performance of this interface was only tested with IgG and Anti-IgG, not with cancer biomarkers, by SPR and conventional ellipsometry.
This report shows and discusses the main results obtained at Centro de Química e Bioquímica, at Lisbon University and at Institute of Mechanics from Chinese Academy of Sciences, in Beijing. It is divided in two different parts: 1) Covalent Immobilization of Carbon Disulfide and Protein A (Figure 3) for the detection of prostate-specific antigen (PSA), the most commonly used serum marker for prostate cancer and to monitor responses to therapy [70]; 2) Development of an interface containing a modified lipid covalently attached to gold surface for immunosensing (Figure 4). During this part of the work, commercial compounds were used with the intent of building a highly organized and stable platform to covalently bind IgG. As shown in Figure 4, different layers of molecules are deposited sequentially covalently linked between them through functional groups localized at their end, except for 2-hydroxioleic acid molecules, that are deposited between 1-myristoyl-2-(14-carboxymyristoyl)-sn-glycero-3-phosphocholine (DMPC-COOH) molecules. The carboxylic group of the 2-hydroxioleic acid molecules is used to covalently bind IgG after activation with EDC and NHS.

**Figure 3.** Schematic diagram of IgG immobilization mechanism on gold modified with DTC (formed by reaction between CS$_2$ and protein A). A gold slide is immersed into a solution containing CS$_2$ and protein A, immobilizing the DTC on the surface. Finally, IgG can be covalently immobilized to protein A and then specifically recognize Anti-IgG [69].

**Figure 4.** Schematic diagram of the immunosensing interface developed. First, gold surface was modified with 11-Amino-1-undecanethiol SAM. Before incubation with 1-myristoyl-2-(14-carboxymyristoyl)-sn-glycero-3-phosphocholine (DMPC-COOH), its carboxylic group was activated with carbonyldiimidazole (CDI) to covalently bind to amine group of SAM. Finally, between DMPC-COOH, 2-hydroxioleic acid molecules were deposited. The final step was their activation with EDC/NHS to covalently link IgG. (For more details see Methods).
When preparing a biosensor interface using a bottom-up approach, it is crucial to ensure that each modification step is successfully achieved. For this purpose, several characterization techniques were employed during the project execution. Conventional ellipsometry was used to estimate gold substrates thickness used in interfaces as well as to the screening of each step of the biosensor development. Atomic force microscopy (AFM) was also an important surface characterization tool, which enables a direct visualization of the modified surfaces during the process. Total internal reflection imaging ellipsometry (TIRIE), as well as surface plasmon resonance (SPR), were selected to perform the biosensing assays with selected antibodies in order to evaluate the performance of the biosensor platforms developed.

The basic principles and main biological applications of the advanced techniques used throughout this work will be briefly described.

2. Characterization Techniques

2.1. Atomic Force Microscopy (AFM)

AFM is a scanning probe microscopy, developed to provide information about atomic scale structure and processes. This surface characterization technique, based on the detection of repulsive and attractive surface forces [71], can resolve surface topography on the nanometer scale laterally and the angstrom scale vertically [72]. Nowadays, AFM is an essential tool in investigating the structure and function of biomembranes, either on living cells or on model systems, mainly due to the capability of high resolution imaging in aqueous environment in the order of one minute [73]. Furthermore, unlike other microscopy techniques, only microgram quantities of the sample are required, neither stains and contrast agents nor conductive coatings are required. Sample preparation is quite simple, quick and cheap and a large variety of materials with very different properties can be imaged. Additionally, this method is nondestructive, allowing the sample reutilization for additional analyses over time [72]. Time-dependent processes, like the interaction of lipid membranes with proteins, peptides and drugs, and the growth of single lipid domains can be imaged using AFM as a surface analytical tool [74].

2.1.1. Instrumentation

In scanning probe microscopies, sample surface is scanned by a probe, following a parallel raster path or zig-zag path, measuring a local interaction, and registering its value for each position. In AFM (Figure 5), the probe is an atomically sharp tip integrated on the end of a flexible cantilever, responsible for the signal transduction. The cantilever bends up or down as the tip contacts and is displaced by features on the surface, according to the forces between the atoms of the sample and those of the tip (Figure 6). These small movements are usually measured by a laser beam focused on the extreme of the cantilever, which is reflected onto a position sensitive
detector (photodiode). The variation of the point of incidence of the reflected beam on the photodiode allows the measurement of any minimal bending or twisting of the cantilever, by the induction of different voltage levels out of the detector. These voltages are sent to a computer for processing and display of the topographic image. Measurements with AFM can be performed by the movement of the probe or of the sample itself, which is assembled on a piezoelectric support [71, 72, 75].

Scanners used in AFM are made of piezoelectric materials, i.e. materials whose dimensions deform in response to an applied electric field. The utilization of this type of scanner allows the application of precise movements by the application of precisely controlled voltages. There are two ways of operate: the sample can be mounted directly onto the scanner or the cantilever can be mounted to a scanner tube. A small deflection of the cantilever changes its angle and changes the position of beam on the detector, producing a change in the voltage out of the detector. Since the distance between cantilever and detector is three orders of magnitude greater than the cantilever length, motions of the tip are amplified with greater sensitivity, generating a detailed topographic image of the surface [72, 75, 76].

Figure 5. Schematic diagram of the essential components of an AFM [71].
2.1.2. Modes of operation

AFM experiments can be operated in three different modes – contact, non-contact and intermittent. Each one of them is special indicated to different types of samples, according to their different surface properties, in order to optimize the topographical image obtained. Forces generated upon interaction of the tip with the sample surface are determined by the applied mode in each situation (Figure 6).

![Diagram showing modes of operation](image)

Figure 6. Plot showing the forces between tip and sample according their distance. The different modes of operation are shown. Adapted from [76].

In this work, tapping mode was used to characterize the modified surfaces. This mode was developed to overtake the problems inherent to other methods. It is known as tapping mode (or intermittent contact mode), since the cantilever oscillates at is resonance frequency, which implies that the tip is in contact with the sample surface only at the lower part of the oscillation. Depending on the distance between the tip and the sample, this mode is governed by both repulsive and attractive forces (Figure 6). Bringing the flexible cantilever close to the sample, causes a dampening of its oscillation amplitude, due to the forces that are established between the tip and the sample, that is adjusted by a feedback loop. In addition, as the tip oscillates up and down, a phase difference can be recorded in a phase image (simultaneously to the topographic image) that is the result of the mechanical properties of the sample as well as the force interactions between the tip and the sample. This mode of operation is one of the most popular, since it has several advantages. Firstly, both vertical and lateral resolution are very good, lateral forces are greatly reduced comparing with contact mode and thus, the damages in soft samples can be minimized. Adhesive interactions between the tip and the sample are reduced, enabling analysis of molecules that are not strongly attached to the surface. This operation mode can be used in liquid environment and is the most generally used for imaging biological samples [71, 72, 75-77].
2.1.3. Sample Preparation

The procedures traditionally applied in preparation of electron microscopy samples are complex, making difficult the analysis of biological samples. Even more, sample preparation methods commonly leads to sample shrinkage and changes in its morphology. Since AFM allows the operation in different environments, sample preparation procedures are not so difficult, biological samples can be studied in their native environment without special need for processing and live cells can also be monitored. Usually, the biological sample has to be deposited on a solid substrate that is attached to the base of the microscope or, like in this work, substrates can be covalently modified and the resulting surface imaged. The substrate has to be smooth at the atomic level and it should have a high affinity for the studied molecules. The most common substrates used are glass and mica that present negative surface charges in neutral aqueous solution and thus, allows the adsorption of samples positively charged. Samples negatively charged can also be imaged on these substrates through the utilization of divalent cations that act as intermediates in the interaction between the sample and the substrate. Highly ordered pyrolytic graphite (HOPG) can also be used as substrate and, like mica, are the smoothest substrates and their surface can be easily cleaned by cleavage with a scotch tape. In contrary, glass and silicon, another possible substrate, can be cleaned by acid treatment. Different conductive surfaces are too possible substrates for AFM measurements. Among all the possibilities, gold has been one of the most used, since presents the electrical, biocompatible and optical properties desired for this application. A wide range of materials can be imaged in AFM many times only limited by their physical dimensions. Nevertheless, the method used for sample preparation has to be suitable for properties of each sample and importantly, the sample binding to the substrate has to be strong enough to avoid the dragging by the tip and at the same time cannot cause structural changes in the system [71, 75, 78].

2.1.4. Biological applications

In the field of biosciences, AFM has become an essential tool to provide structural, mechanical and functional information at high resolution level. The study of nucleic acids was the first attempt of AFM application in biology. Since then, the improvement of the equipment and methodologies allowed the realization of more elaborated studies. The possibility of observation of samples in nearly physiological conditions was an important improvement regarding the application field of AFM. Within biological molecules, proteins are also an important subject of experiments conducted with AFM, by direct adsorption to the substrate or by chemical bonds between proteins and functionalized substrates. The crystallization of the sample on the desired substrate allowed the study of many membrane proteins or, more recently, through their deposition in planar membranes, more dynamic studies were performed. This kind of studies are usually performed with model membrane systems deposited on solid supports,
including solid-supported lipid bilayers and monolayers, polymer-cushioned lipid bilayers, hybrid bilayers, tethered bilayers, suspended lipid bilayers or supported vesicular layers. From these type of experiments, information about phospholipids organization can be extracted. Furthermore, AFM imaging is crucial to study lipid domain formation, protein adsorption to membranes and membrane lipid-small molecules interactions [73, 74, 79, 80].

2.2. Surface Plasmon Resonance (SPR)

SPR technique is one of the most important analytical tool to study biomolecular interactions. Mass changes caused by biomolecular interactions are detected by SPR using a thin metal surface (usually gold or silver) through changes in the refractive index of the sensed medium. This results in an alteration of the resonance condition between the metal surface plasmons and the interacting optical wave.

2.2.1. Principles

SPR technique is based on an optical phenomenon that involves excitation of free oscillating metal electrons of a thin metal film containing the sample (Figure 7). When polarized visible light impinging on the prism contacts with the metal surface, the energy carried by its photons can be transferred to valence electrons of the metal surface. This transference only occurs when the energy of the light photons is exactly equal to the metal electrons quantum energy level, i.e. under conditions of attenuated total reflection. It is the incident angle (θ) that defines if the conditions are suitable to excite the electrons. When it occurs, a plasmon resonant wave is generated at the interface between the metal and the dielectric material and connects with an evanescent electromagnetic field. A change in the refractive index of the dielectric material results in a change of the incident angle and thus in an alteration of the propagation constant of the plasmon resonant waves. When the incident angle decreases (in consequence of an alteration in the refractive index near the metal surface), the intensity of the reflected light increases, meaning that the electrons went back to the fundamental state and the plasmon resonant wave disappeared [81-86].
Figure 7. Schematic representation of the working principle of SPR. A ligand is immobilized over the sensor surface while the analyte flows across it. The changes in the refractive index of the solution at the interface are monitored in real-time. The sensorgram depicts changes in SPR angle in real-time, with responses measured in resonance units (R.U.) vs time. Adapted from [82].

2.2.2. Biological applications

SPR allows to follow biomolecular interactions in real-time and in a non-invasive way. Furthermore, it provides highly accurate kinetic and equilibrium results from label-free measurements, where only one of the interacting partners has to be immobilized on a sensor surface. SPR has been used as a tool to biomolecular affinity assays, to monitor adsorption kinetics, to perform thermodynamic analysis, to study interaction mechanisms and drug screening, among others. Thus, comparing with traditional laboratory analytical techniques, SPR is a central tool for molecular interactions in a fast, non-labeled, specific and sensitive way [81, 83-86].

2.3. Ellipsometry

Ellipsometry is an optical characterization technique that measures changes in polarization of light reflected on samples in order to estimate their thickness (Figure 8). In the usual mode of operation, a beam of polarized light encounters a planar surface and is reflected in a different polarization state, generally an elliptically polarized state. When the surface is covered by a thin film, the light beam is reflected in a polarization state different from the first. This change in the polarization is measured to obtain the thickness and optical properties of the film. Firstly, the measurement must be performed at oblique incidence and, secondly, surface roughness of the samples analyzed by ellipsometry has to be smaller, since the roughness increases the light scattering. This effect reduces the intensity of the reflected light and thus increases measurement errors [87-90].
Figure 8. Schematic representation of a reflection at a bare substrate (A) for a two phase model (ambient – substrate) and at a film covered substrate (B) for a three-component system (ambient – film – substrate). Adapted from [88].

2.3.1. Principles

When light passes from one medium (air or solution) into another medium, some of the light is reflected back and does not enter the second medium and other part enters the second medium and is refracted (Figure 8). The complex index of refraction (defined by Eq. 1) describes the interaction between the light that enters the second medium and the material [87-89].

\[ \tilde{N} = n - ik \quad \text{Eq. 1} \]

Eq. 1 is a combination of a real part and an imaginary part, where \( n \) is the index of refraction and \( k \) is the extinction coefficient. The extinction coefficient is defined as:

\[ k = \frac{\lambda}{4\pi\alpha} \quad \text{Eq. 2} \]

where \( \lambda \) is the wavelength of the incident light beam and \( \alpha \) denotes the absorption coefficient. For a dielectric material, none of the light is absorbed (the medium is transparent), \( k = 0 \) and thus \( \tilde{N} = n \).

The refracted light, \textit{i.e.} the part of the light beam that enters the material at an angle \( \theta_0 \), does not continue in the same direction and is refracted to a different angle \( \theta_1 \). This behavior is described by the Snell’s law:

\[ \tilde{N}_0 \sin \theta_0 = \tilde{N}_1 \sin \theta_1 \quad \text{Eq. 3} \]

which is written as:

\[ n_0 \sin \theta_0 = n_1 \sin \theta_1 \quad \text{Eq. 4} \]
for dielectric materials.

Regarding the part of the light that is reflected, before it hits the surface, the light is linearly polarized and after the reflection there is a shift of its phase and amplitude. This shift is different for both parallel, p, and perpendicular, s, (to the plane of incidence) components and thus the resultant light is elliptically polarized. The phase change, \( \Delta \), and the light amplitude change, \( \Psi \), upon reflection are the two values measured by ellipsometry. These values can be from zero to 360° and 90°, respectively and are related by the fundamental equation of ellipsometry:

\[
\tan \Psi e^{i\Delta} = \frac{R^p}{R^s} \quad \text{Eq. 5}
\]

where \( R^p \) and \( R^s \) are the total reflection coefficients for parallel and perpendicular polarizations, respectively, which are given by:

\[
R^p = \frac{r_{01}^p + r_{12}^p \exp (-i 2\beta)}{1 + r_{01}^p + r_{12}^p \exp (-i 2\beta)} \quad R^s = \frac{r_{01}^s + r_{12}^s \exp (-i 2\beta)}{1 + r_{01}^s + r_{12}^s \exp (-i 2\beta)} \quad \text{Eq. 6}
\]

where \( r_{ij} \) is the Fresnel reflection coefficient for the interface between medium i and medium j. Eq. 6 is used for film covered surfaces (Figure 8 - B), once additional reflections and refractions that occur at the film-film and film-substrate interfaces are taken into account. \( \beta \) is the film phase thickness given by the:

\[
\beta = 2\pi \frac{d}{\lambda} \bar{N}_1 \cos \theta_1 \quad \text{Eq. 7}
\]

where \( d \) is the film thickness.

The Fresnel reflection coefficient, \( r \), is the ratio of the amplitude of the reflected wave to the amplitude of the incident wave for a single interface (Figure 7 – A) and is given by:

\[
r_{01}^p = \frac{\bar{N}_1 \cos \theta_0 - \bar{N}_0 \cos \theta_1}{\bar{N}_1 \cos \theta_0 + \bar{N}_0 \cos \theta_1} \quad n_{01}^s = \frac{\bar{N}_0 \cos \theta_0 - \bar{N}_1 \cos \theta_1}{\bar{N}_0 \cos \theta_0 + \bar{N}_1 \cos \theta_1} \quad \text{Eq. 8}
\]

After the measurement of \( \Delta \) and \( \Psi \) values by the ellipsometer, these quantities are used along with an assumed optical model to calculate material properties, such as the thickness of the film deposited on top of the surface [87-89].

### 2.3.2. Instrumentation

To perform an ellipsometric experiment a monochromatic light source is required, typically a laser (Figure 9). The light beam produced by the light source passes through the polarizer that changes the polarization state of the incident light. The resulting linearly polarized light output is converted into elliptically polarized light in the compensator and reaches the
sample. The light undergoes an amplitude and phase shift during the reflection and the resultant light beam goes directly to the analyzer that determines its state of polarization. Finally, the light intensity is measured by the detector and results are interpreted in terms of an assumed sample [87-89, 91].

![Schematic representation of an ellipsometer](image)

**Figure 9.** Schematic representation of an ellipsometer [88].

### 2.3.3. Total internal reflection imaging ellipsometry (TIRIE)

The detection of antibody-antigen interactions have been one important application of ellipsometry [91]. Since the detection can be performed on solid supports, the high sensitivity for detection of thin organic layers with a resolution of less than 0.01nm, makes the ellipsometry one powerful tool in this field. Furthermore, the possibility of follow adsorption kinetics on those substrates without destruction of the sample, even for less than monolayers, allied with the high lateral resolution allows its application as an analytical device for the detection of biomolecular interactions in real-time [92]. More recently, imaging ellipsometry was developed with large applications in the biosensing field [93-95]. This technique is an improvement of standard single beam ellipsometry that allies the power of ellipsometry as an analytical tool with microscopy as an imaging technique. The application of imaging ellipsometry allows the achievement of high spatial resolution, on the order of micrometers laterally and on the order of sub-nanometers vertically, of a large viewable area [50]. Thus, imaging ellipsometry has been used as an automatic analysis technique for protein detection, multi-protein analysis and detection of protein interaction processes. More recently, combining a micro-fluidic reactor array with imaging ellipsometry, a total internal reflection imaging ellipsometry (TIRIE) was developed. TIRIE was specially developed for *in situ* real-time detection of biomolecular interaction and is based on the ellipsometric principle of detection of phase changes applied to a large view field typical of imaging ellipsometry. The micro-fluidic reactor array system, coupled with the optical sampling system by a prism, is used for biomolecule interaction and is controlled by an operation system, providing high sensitivity and high throughput. Moreover, this technique provides the possibility of mimic the solution environment *in vivo*, observe, measure and analyze the biological reaction process in real-time.
2.3.3.1. Principles and Instrumentation

The TIRIE biosensor is based on the principle that each ligand, immobilized to a surface, has a receptor in solution, (e.g. antibody and antigen) to which it assembles to form a complex due to their affinity. The immobilization of the ligand to the gold surface is made in a way that forms a monolayer that, when exposed to the solution containing the corresponding receptor, they assemble into a complex due to their specific affinity. The TIRIE equipment is composed by two essential elements: the optical system and the micro-fluidic array system. In the optical system (Figure 10 – A), a coupling prism on the back side of the substrate is used for the internal reflection phenomenon and the incident angle is fixed at a value larger than the total internal reflection angle. The micro-fluidic reactor array system is used for sampling and receptor-ligand interaction, which generally occurs in PBS. This interaction between ligand and receptor changes the polarization state of the evanescent wave that appears at the interface of the substrate and the receptor layer. Then, the polarization state of the reflection is changed by the successive depositions of biomolecules. This process is accompanied through an ellipsometric image and the reflection intensity is expressed in grayscale, showing the variation of the surface concentration. As shown in Figure 10 - B, the surface concentration distribution can be easily identified by different grayscales, i.e. higher surface concentration corresponds to a higher grayscale value. An optical image in grayscale is captured at each moment and finally, a series of images in grayscales with the time reflects the whole kinetic process of the biomolecules interaction [50, 94, 96, 97].

![Figure 10](image.png)

**Figure 10.** Principle of the TIRIE biosensor (A) to detect a ligand-target interaction that is visualized through a series of images in grayscale with the time and (B) then converted to a curve signal vs time. Adapted from [96].

Generally, a Xe lamp and a monochromator are used as the light source (Figure 11). The light beam formed is guided through optical fibers to the polarizer, the compensator and then penetrates to the coupling prism perpendicularly and finally onto the sensing surface do detect biomolecules interaction. The light beam is totally reflected at the interface of the substrate, passes through an analyzer and penetrates to the imaging system, composed by imaging lens and the
imaging device of CCD (charge-coupled-device). The micro-array reactor is attached to a gold layer surface on glass to form 24 individual cells, containing each one an inlet and an outlet for the solution. The micro-fluidic system is responsible for delivering the solution containing the reactants to each cell, where they will interact with their ligands [94, 96-98].

![Figure 11. Schematic diagram of the TIRIE biosensor [96].](image)

2.3.4. Biological applications

Regarding the features related with thin films characterization described above, ellipsometry can be a powerful tool to investigate macromolecular adsorption on solid surfaces. Furthermore, the adsorption on solid supports prepared to mimic a natural interface allows the formation of monolayers of macromolecules and through ellipsometry, their dielectric properties can be investigated. Thus, ellipsometry can provide an interface between biology and electronics. The visualization and quantification of the surface mass of a specific substrate is another important biological application of ellipsometry, in particular of imaging ellipsometry [91]. These properties allowed the creation of ellipsometrically based biosensor systems that can be used, for example, as gas sensor systems or, more commonly, as affinity biosensors providing information on antigen-antibody interactions, as described above [50, 91]. In this field, the combination with AFM can give some insights on the study of interactions between a variety of biomolecules, like what was performed recently to detect interactions of antibodies with bacterial endotoxins [99]. Furthermore, this techniques combination allows the monitoring of the SLB formation [100]. Regarding model membrane systems, ellipsometry by itself is a versatile tool to characterize their structural and functional properties [101] and the interaction with different biomolecules [102, 103]. SLB can also be a good platform for ellipsometry monitored formation of three-dimensional nanostructures [104].
3. Methods

3.1. Preparation of the gold surfaces

Two different kinds of gold surfaces were used. Gold coated glass slides with 50nm were used for TIRIE experiments. They were cleaned with ethanol and water several times, dried with pure nitrogen flow and, after that, placed in a UV cleaner for 40min. For ellipsometry, gold film deposited on borosilicate glass (prelayer of chromium, 2-4nm) with 200nm was first cleaned in piranha solution for a few minutes (H₂SO₄/H₂O₂ = 3:1, v/v), and then also placed in a UV cleaner for 40 minutes.

3.2. Covalent Immobilization of Carbon Disulfide and Protein A for PSA detection

Gold slides were immersed into a mixed solution (1:1, v/v) of 0.1M CS₂ in water and 20 µg/mL protein A in PBS solution for 2h and then washed with water and dried by nitrogen, according to the reported procedure [64]. During TIRIE and SPR experiments, IgG, casein blocking buffer (BB), fetal bovine serum (FBS), Anti-IgG, bovine serum albumin (BSA), PSA and Anti-PSA were injected.

3.3. Development of an interface containing modified lipids for immunosensing

Gold surfaces were modified with a solution containing 1mM 11-Amino-1-undecanethiol hydrochloride in ethanol overnight at 4°C, washed with ethanol and then dried by nitrogen. The formation of the resulting SAM was confirmed by cyclic voltammetry and ellipsometry. 17.5mg (= 5.0 mM) of 1-myristoyl-2-(14-carboxymyristoyl)-sn-glycero-3-phosphocholine (DMPC-COOH) and 3.55mg (= 4.4 mM) of carbonyldimidazole (CDI) were mixed in 5mL of anhydrous chloroform during 2h at room temperature, in a glove box under N₂. After that, previously modified gold slides were immersed into the above solution for 4h at room temperature under N₂, washed with chloroform and ethanol and dried carefully with flow of nitrogen. Samples were then soaked in a 1mM 2-hydroxyoleic acid solution in ethanol for 6h at room temperature and rinsed with PBS pH 7.4. The activation of carboxylic group of 2-hydroxyoleic acid with 0.2M EDC and 0.05M NHS was done in situ, during TIRIE experiment. For SPR and ellipsometry measurements, activation was done ex situ, during 1h with 0.2M EDC and 0.05M NHS in PBS pH 4.8. The samples were incubated with IgG and Anti-IgG during 30min and washed with water. After each step, the thickness was measured by ellipsometry and imaged by AFM. For SPR and TIRIE, IgG, BB and Anti-IgG were injected during the assays.
4. Results and Discussion

4.1. Covalent Immobilization of Carbon Disulfide and Protein A for PSA detection

In situ dithiocarbamate formation (Figure 3) was used to prepare an immunosensor interface (Figure 12). After injection of IgG solution the signal increased (Table 1), suggesting that IgG was immobilized on the surface and, after washing with casein blocking buffer solution (BB), the signal did not decrease meaning that Fc region of IgG should be interacting specifically with Protein A. This interaction should happen through the several IgG binding domains that Protein A contains [105]. Furthermore, the slightly increase observed after BB addition (Table 1) means that there are still few sites not occupied by IgG molecules available for BB molecules adsorption. However, previous studies indicate that the addition of a small percentage of Tween 20 in the buffer solution can drastically reduce the nonspecific adsorption of the modified surface [69]. After Anti-IgG solution injection, grayscale values also increased as a consequence of specific interaction with IgG molecules (Figure 12 – A), through the IgG Fab portion. When bovine serum albumin (BSA) was injected instead of Anti-IgG (negative control), the signal maintained the same level (Figure 12 – B) showing that the surface was protected from non-specific interactions. The decrease in the grayscale during BSA pumping is due to the different refractive index of the BSA solution regarding that of PBS. It is worth noting the very high ratio between Anti-IgG and IgG, which taking into consideration that non-specific interactions have been eliminated proves the excellent performance of this simple interface.

Figure 12. TIRIE data showing the interaction of 0.1mg/mL IgG, BB and A) 0.1mg/mL Anti-IgG and B) 0.1mg/mL BSA with protein A immobilized on gold surface modified with CS₂. After injection of BSA (B), the signal decreased because this solution was prepared in water instead PBS.
This biosensor interface was then used to test PSA. For that purpose, a solution of Anti-PSA was injected, interacting specifically with the surface and making possible specific interaction with PSA (Figure 13 – A, B). When PSA concentration was increased (Figure 13– B), the signal also increased (Table 2), showing that this surface is sensitive to the amount of PSA that is injected. Nevertheless, the grayscale value increase after injection of both Anti-PSA and PSA (Table 2) is not comparable with the one observed after injection of IgG and Anti-IgG (Figure 12), mainly because the molecular weight of this antibody (28.4 kDa) is much smaller than IgG molecular weight (ca. 150 kDa). When BSA was used as a negative control, the grayscale value almost did not change (Figure 13 – C, table 2), since BSA does not interact with Anti-PSA. When PSA was injected after IgG, it did not interact with IgG (Figure 13 – D) and thus, grayscale value did not increase (Table 2).

**Table 1 – Grayscale variation of TIRIE data shown in figure 12.**

<table>
<thead>
<tr>
<th></th>
<th>Grayscale Variation</th>
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<tbody>
<tr>
<td></td>
<td>Panel A</td>
</tr>
<tr>
<td>IgG</td>
<td>946</td>
</tr>
<tr>
<td>BB</td>
<td>594</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>4132</td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
</tr>
<tr>
<td>Anti-IgG/IgG</td>
<td>4.4</td>
</tr>
</tbody>
</table>
Figure 13. TIRIE data showing the interaction of A) 0.1mg/mL Anti-PSA, BB and 0.075mg/mL PSA; B) 0.1mg/mL Anti-PSA, BB and 0.15mg/mL PSA; C) 0.1mg/mL Anti-PSA, BB and 0.1mg/mL BSA; D) 0.1mg/mL IgG, BB and 0.1mg/mL PSA with protein A immobilized on gold surface modified with CS$_2$.

Table 2 – Grayscale variation of TIRIE data shown in figure 13.

<table>
<thead>
<tr>
<th></th>
<th>Panel A</th>
<th>Panel B</th>
<th>Panel C</th>
<th>Panel D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PSA</td>
<td>599</td>
<td>527</td>
<td>515</td>
<td>-</td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>527</td>
</tr>
<tr>
<td>PSA</td>
<td>103</td>
<td>142</td>
<td>-</td>
<td>-47</td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
<td>-</td>
<td>89</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 14. TIRIE data showing the interaction of A) 0.1mg/mL Anti-PSA, BB and 0.15mg/mL PSA; B) 0.1mg/mL Anti-PSA, BB and 0.15mg/mL BSA; C) 0.1mg/mL IgG, BB and 0.15mg/mL PSA with protein A immobilized on gold surface modified with CS$_2$. 
Since Anti-PSA interacts with the surface through binding to protein A, in order to improve PSA recognition by the biosensor, protein A concentration was increased from 20 to 50 µg/mL (Figure 14). With this increase, the amount of Anti-PSA and PSA specifically bound to the surface also increased (Figure 14 – A, Table 3), suggesting that the biosensor performance was improved. This conclusion is corroborated by the result with the negative control (Figure 14 – B), since the signal almost did not change after BSA injection (Table 3) and the variation in grayscale value after PSA injection (Table 3) when there was no Anti-PSA bound to the surface (Figure 14 – C) is small. In spite of this, it is clear that when the surface is washed with PBS after PSA injection, the signal decreases (Figure 14 – A, C). This result seems to indicate that the Anti-PSA/PSA specificity may be altered, possibly in consequence of their denaturation.

**Table 3** – Grayscale variation of TIRIE data shown in figure 14.

<table>
<thead>
<tr>
<th></th>
<th>Panel A</th>
<th>Panel B</th>
<th>Panel C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PSA</td>
<td>623</td>
<td>708</td>
<td>-</td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
<td>-</td>
<td>1123</td>
</tr>
<tr>
<td>PSA</td>
<td>173</td>
<td>-</td>
<td>108</td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
<td>47</td>
<td>-</td>
</tr>
</tbody>
</table>

Globally, the results here presented are very promising, since they show that this immunosensor platform is sensitive to the detection of PSA and then, it can be used in clinical experiments to detect this cancer biomarker. Yet, further experiments must be performed in order to increase the immunosensor sensitivity regarding PSA detection. Additionally, different proteins, namely different cancer biomarkers, can be used to evaluate the immunosensor performance.

**4.2. Development of an interface containing modified lipids for immunosensing**

The first step to build this lipid-based immunosensing interface was the choice of an alkanethiol containing a terminal amine group (for the covalent attachment of the lipid molecule) for SAM formation. Two different molecules were tested: 2-aminoethanethiol (cysteamine) and 11-Amino-1-undecanethiol (C₁₁NH₂). After the modification of the gold surface with both compounds separately, a SPR experiment was performed with both IgG and Anti-IgG to test their non-specific adsorption to the modified surfaces (Figure 15). The amount of IgG that was adsorbed to the surface modified with Amino-1-undecanethiol was smaller than the amount that was adsorbed on bare gold and on gold modified with cysteamine (Table 4). On bare gold, a high amount of IgG and Anti-IgG was adsorbed through non-covalent bonds. Thus, bare gold cannot
used as immunosensor by itself, since all the proteins adsorb to its surface without any kind of specificity. Comparing the IgG amount adsorbed to gold surfaces modified with cysteamine and C_{11}NH_{2} (with longer alkyl chain), in presence of the shorter SAM more IgG adsorbed non-covalently, despite the smaller amount of Anti-IgG adsorbed.

**Figure 15.** SPR data showing the interaction of 0.1mg/mL IgG, and 0.1mg/mL Anti-IgG with bare gold and gold modified with 2-aminoethanothiol (cysteamine) and with 11-Amino-1-undecanethiol SAM (C_{11}NH_{2}).

**Table 4.** R.U. variation of the SPR data shown in figure 15.

<table>
<thead>
<tr>
<th></th>
<th>ΔR.U.</th>
<th>Anti-IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>501</td>
<td>609</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>266</td>
<td>222</td>
</tr>
<tr>
<td>C_{11}NH_{2}SAM</td>
<td>86</td>
<td>300</td>
</tr>
</tbody>
</table>

At the same time, in order to prove that the alkanethiol-based SAM with longer alkyl chain was compact, to prevent direct exposure of gold surface, a cyclic voltammetry was performed (Figure 16). The reduction peak at ca. -1.1 V in the cyclic voltammogram corresponds to the desorption of sulfur rom gold, indicating the presence of a compacted SAM with a surface coverage of 5.1 x 10^{-10} mol cm^{-2}.

These first results show that the non-specific adsorption to the surface can be reduced using a well packed alkanethiol-based SAM with longer alkyl chain. Thus, the development of the biosensor was performed using a SAM with Amino-1-undecanethiol.
Figure 16. Cyclic voltammogram of bare gold modified with 11-Amino-1-undecanethiol SAM (C\textsubscript{11}NH\textsubscript{2}).

The thickness of an 11-Amino-1-undecanethiol SAM on bare gold was estimated as ~1.6 nm by conventional ellipsometry and, after reaction with a DMPC modified with a carboxylic group previously activated with CDI (Figure 18) during 4h, the thickness increased ~1 nm. The amount of IgG that adsorbed to the surface after these modification steps (Figure 19) is lower than the amount that adsorbs only in presence of SAM (Table 4). This amount of IgG that was adsorbed was physically bond to the surface, since there was not any functional group on the surface available to covalently link IgG. Thus, the presence of phospholipids reduces non-specific adsorption of IgG, as it was shown through this SPR experiment (Figure 19). The IgG that adsorbs to the surface is not removed even after addition of BB and fetal bovine serum (FBS) that contains different molecules with a wide range of molecular masses. This last step was performed to confirm that after injection of BB and FBS the non-covalently bonded molecules were removed more efficiently. Since that the amount of IgG adsorbed to the surface was small, the amount of Anti-IgG adsorbed was also small (Figure 19), confirming an Anti-IgG/IgG ratio close to the expected assuming that each IgG molecule can specifically bind two Anti-IgG molecules.

Figure 18. Acid carboxylic general activation mechanism with CDI. Adapted from [106].
Figure 19. SPR data showing the interaction of 0.1mg/mL IgG, BB and 0.1mg/mL Anti-IgG biosensor containing SAM/DMPC-COOH deposited on gold. The inset shows the R.U. variation of the SPR data.

Samples were then incubated with 2-hydroxyoleic acid during 6h (thickness increased ~0.3nm), introducing a carboxylic group into the system that, after activation with EDC and NHS, can be used to bind proteins covalently. By ellipsometry, after incubation with IgG, the thickness increased 2.5nm and 2.7nm after Anti-IgG. IgG was covalently adsorbed on the surface when carboxylic acid groups of the 2-hydroxyoleic acid molecules were activated (Figure 20 – A), since the grayscale value obtained by TIRIE did not decrease after washing with BB (Table 5). In agreement with the ellipsometric data, the signal also increased after injection of Anti-IgG solution (Table 5) as a consequence of the specific interaction with IgG molecules adsorbed on the surface. When carboxylic acid groups were not activated (Figure 20 – B), signal decreased after washing with BB (Table 5), meaning that IgG was non-covalently adsorbed on the surface and it was washed away or partially replaced by casein molecules from BB. This explains why the increase after Anti-IgG solution injection is smaller than the one observed when activation (Figure 20 – A) was done. These results clearly show that this interface is very sensitive to recognize Anti-IgG and it has good capability to inhibit non-specific interactions.

<table>
<thead>
<tr>
<th></th>
<th>ΔR.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>51</td>
</tr>
<tr>
<td>BB</td>
<td>153</td>
</tr>
<tr>
<td>FBS</td>
<td>-40</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>82</td>
</tr>
<tr>
<td>Anti-IgG/IgG</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Figure 20. TIRIE data showing the interaction of 0.1mg/mL IgG, BB and 0.1mg/mL Anti-IgG with the biosensor containing SAM/DMPC-COOH/2-hydroxyoleic acid deposited on gold. A, C) The injection of 0.2M EDC and 0.05M NHS corresponds to the activation of carboxylic groups of 2-hydroxyoleic acid. A, B) The time of incubation with DMPC-COOH was 4h and 6h to the 2-hydroxyoleic acid. C, D) Instead of 4h, the incubation time with DMPC-COOH was 2h and 4h to the 2-hydroxyoleic acid.

Table 5 – Grayscale variation of TIRIE data shown in figure 20.

<table>
<thead>
<tr>
<th>Grayscale Variation</th>
<th>Panel A</th>
<th>Panel B</th>
<th>Panel C</th>
<th>Panel D</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>2818</td>
<td>3833</td>
<td>906</td>
<td>4322</td>
</tr>
<tr>
<td>BB</td>
<td>965</td>
<td>-855</td>
<td>536</td>
<td>-1259</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>7651</td>
<td>2547</td>
<td>709</td>
<td>2771</td>
</tr>
<tr>
<td>Anti-IgG/IgG</td>
<td>2.7</td>
<td>0.4</td>
<td>0.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

One of the requirements for a good biosensor performance is its easy preparation. In spite of the good results obtained with the immunosensor tested interface, the whole procedure for its preparation is quite long and requires specific equipment for some steps; for instance CDI is sensitive to water and its manipulation has to be done inside of a glove box, under N2. In order to decrease the procedure duration, the time of incubation with DMPC-COOH was reduced to 2h (instead of 4h) and to 4h for 2-hydroxyoleic acid (instead of 6h). The incubation time with DMPC-COOH was enough, since after this step the thickness increased 1.2nm. However, after incubation with 2-hydroxyoleic acid, the thickness decreased 0.3nm, probably meaning that some DMPC-
COOH molecules were replaced by a few molecules of 2-hydroxyoleic acid. This conclusion is corroborated by TIRIE results (Figure 20 - A, D) because after activation, the amount of IgG that adsorbed to the surface was about three-fold less than when the incubation time with 2-hydroxyoleic acid was 6h (Table 5). This suggests that there were not enough 2-hydroxyoleic acid molecules on the surface with carboxylic groups available to covalently link IgG. Once again, when carboxylic groups were not activated (Figure 20 – D), grayscale value increase was larger (Table 5), but after washing with BB the signal decreased. This result is quite intriguing because in absence of activation of carboxylic groups, IgG does not link covalently to the 2-hydroxyoleic acid molecules on the surface and then, the grayscale value only increase a few units. Thus, without activation, the grayscale value should just increase a few units. The explanation for this phenomenon has still to be found. Nevertheless, the results in Table 5 clearly proved that covalently linked IgG can specifically detect some Anti-IgG.

The whole procedure was followed by AFM experiments (Figure 21), confirming that for each step, there were visible changes on the gold surfaces indicating that a successfully surface modification was achieved. The deposition of C_{11}NH_{2} SAM follows the gold globular morphology, typical of the SPR indicating that a smooth layer is formed (Figure 21 – A). With the deposition of DMPC-COOH over the SAM, the topographic image is clearly different (Figure 21 – B), showing areas with different height, i.e. the DMPC-COOH deposition over the surface modified with the SAM was not uniform and there are some areas that are not occupied by DMPC-COOH molecules, as further demonstrated by Figure 21 – C, acquired on a modified flat gold surface (Au (111)). After the last step (Figure 21 – D), the topographic image shows that the surface becomes more uniform. This means that the deposition of 2-hydroxyoleic acid molecules was successfully achieved and, possibly, the 2-hydroxyoleic acid molecules fill in the DMPC-COOH patches, since the whole surface is more uniform and, thus, more suitable for recognition of IgG molecules after activation.
Figure 21. AFM images of gold modified with 11-Amino-1-undecanethiol SAM (A) and with SAM/DMPC-COOH (B). Detailed image of a more flat gold substrate (Au (111)) containing SAM/DMPC-COOH (C). Gold modified with SAM/DMPC-COOH/2-Hydroxy Oleic Acid (D). The images correspond to an area of 1.0 µm x 1.0 µm. Z = 15.

5. Conclusions and Future work

The results presented in this report show that it is possible to covalently immobilize carbon disulfide and protein A on gold substrates and that this interface can bind antibodies for immunosensing. The main features of this approach are the simplicity of the procedure, the ability to inhibit non-specific interactions and good sensitivity when IgG is used as antibody. Regarding PSA recognition by this surface (with anti-PSA), the results may still be improved. Despite the well-successfully detection of PSA, the interface sensitivity was not good enough to use this platform as an immunosensor to detect this biomarker. In order to improve it, a different detection system that allows a signal amplification can be used. Additionally, the procedure may be extended to a different antigens in order to prove that this interface has potential to develop an immunosensor for detection of biomolecule interactions.

The biosensor containing modified lipids was the other approach studied. This new interface is clearly more complex than others, such as the system studied in the first part of this work, but it has more potential regarding non-specific interactions inhibition. This feature can be important regarding its application in clinical diagnosis since, generally, the samples used are mixtures of proteins that can potentially be adsorbed to the immunosensor surface, preventing the recognition
of the target molecule. More experiments must be performed to evaluate the immunosensor performance as well as its sensitivity using different antigen concentrations, denatured IgG and Anti-IgG as control experiments, and also typical proteins like BSA. Globally, for the first time, the covalent immobilization of lipid molecules was successfully employed to build a stable monolayer that can be employed in the assembling of new biosensor platforms.

6. Acknowledgements

I would like to thank to Professor Jin, Dr. Ana Viana, Dr. Rodrigo Almeida and Dr. Niu for the opportunity to work in a foreign country, for the trust placed in me and for all the support. To Liu, I would like to thank for the help during my stay in Beijing and to Kang for the help to carry out the experiments.
Concluding Remarks
Concluding Remarks

Membrane model systems are well established as important platforms to study membranes structure, dynamics and function [107, 108]. In order to characterize the interaction between \textit{m}-cresol and biological membranes, liposomes (LUVs) and SLBs were used. Deposited lipid layers were also employed in the assembling and development of immunosensor interfaces. These studies were performed using a range of biophysical characterization techniques, including different fluorescence spectroscopy techniques, AFM and ellipsometry, all of them with great applicability in this field and with compatibility with the model membrane systems.

As described above, one of the main goals of the work developed during this thesis was the biophysical characterization of \textit{m}-cresol interaction with biological membranes. Fluorescence spectroscopy was a crucial technique to characterize this interaction, either using \textit{m}-cresol intrinsic fluorescence or membrane fluorescent probes in presence of ternary mixtures mimicking lipid rafts. \textit{m}-cresol effects were also evaluated through real-time experiments using tapping-mode AFM with a methodology well described. Raft-forming ternary mixtures displaying \textit{l}_o/\textit{l}_d phase separation were deposited on mica and the dynamic morphological changes of the SLB caused by \textit{m}-cresol were evaluated \textit{in situ}. Together, the results obtained showed an interaction mechanism based on the alteration of the bilayer components natural organization as consequence of the promotion of a more disordered phase formation, although with properties different from the typical disordered phase. Given the high \textit{m}-cresol utilization, as excipient, in pharmaceutical formulations [14, 16], the results presented throughout this work may rise some concerns about its potential effects during the usage of those formulations. In fact, some of the cytotoxicity situations detected [2, 4, 5] could be caused by \textit{m}-cresol, as reported in a recent cytotoxicity study [16]. This conclusion is corroborated by the results here presented, since it was shown that when integrated in Humulin, \textit{m}-cresol has effects on biological membranes. Thus, \textit{m}-cresol potential effects while excipient is now and more than never, a matter of deep concern and further studies focused on its effects must be performed. Otherwise, the utilization of high amounts of \textit{m}-cresol in formulations has to be replaced by other excipients whose cytotoxic effects are well characterized.

The development of purposed interfaces was the other goal of the work developed during this thesis. For that, the first step was the utilization of a well-characterized platform based on gold surface modification with DTCs [69]. The immunosensor
performance was evaluated with both IgG and Anti-IgG using TIRIE, an automatic method for real-time analysis of protein interaction processes with merits of label-free, quantitative and high throughput technique [50, 98]. Furthermore, using this interface, the tumor markers PSA and Anti-PSA were successfully immobilized. Thus, it was shown that the DTCs formation principle can be employed in the detection of tumor markers, an important application of the biosensors industry [46, 109, 110] that had been facing a huge expansion over the last years, and predictably will continue in the near future [111].

An immunosensor composed by 1-undecanethiol SAM on gold surface, DMPC modified with a COOH group in the end of the acyl chain and 2-hydroxioleic acid was built to create a biomimetic and robust interface with high sensitivity for immunosensing, also preventing non-specific adsorption. IgG and Anti-IgG immobilization was successfully achieved with this new approach, but further optimization is needed in order to accomplish the main goal. Moreover, it was shown that this lipid-based immunosensor interface can potentially avoid non-specific adsorption of proteins, an important requirement of the immunosensors [112], which makes it suitable to use optical methods to detect the antibody-antigen biorecognition event [113, 114].
References


