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Active Stabilization of the Secondary Structure of Blood Proteins.
A New Approach Towards Biocompatibility.

Thesis submitted in accordance with the requirements of the University of Porto, Faculty of Engineering, for the degree of Doctor of Philosophy (Ph.D).

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I. Introdução

As proteínas são macromoléculas de elevada complexidade necessárias ao suporte da vida. Todos os sistemas biológicos estão dependentes da funcionalidade das proteínas pois elas controlam a actividade de "milhares" de tarefas diferentes como o transporte de substâncias orgânicas e inorgânicas, a catálise de reações, a defesa contra doenças, têm funções estruturais, etc. É interessante imaginar que a funcionalidade de cada proteína é regulada pela combinação de apenas 20 amino-ácidos diferentes (apêndice A). Este pequeno número de aminoácidos consegue dar origem a mais de 100,000 proteínas diferentes. Eles ligam-se entre si formando cadeias de diversos tamanhos que definem a base estrutural de cada proteína. Estas cadeias vão então enovelar-se (folding) dando origem a estruturas com formas distintas que definem a conformação, e como tal a função, de cada proteína. Vários campos de investigação surgiram para tirar partido das diferentes funções oferecidas pelas proteínas: libertação de drogas, biomateriais, biosensores, testes imunológicos [1-4].

Com este trabalho procurou-se desenvolver uma nova estratégia para preparar biomateriais. A síntese de superfícies biocompatíveis exige a estabilização da conformação das proteínas adsorvidas o que evita reações antigénicas. Por este motivo foi necessário encontrar grupos químicos capazes de satisfazer esta exigência e desenvolver um modelo que explique as interacções observadas. Mais tarde modificaram-se polímeros utilizando estes grupos químicos e estudou-se a conformação das proteínas adsorvidas. Estas superfícies tornaram-se também potenciais modelos para curar doenças relacionadas com a mudança de conformação das proteínas.

Para atingir os objectivos acima propostos dividiu-se o trabalho em diversas secções. A primeira parte vai dedicar-se principalmente ao estudo da estrutura das proteínas em presença de álcoois. Álcoois têm sido utilizados em estudos envolvendo enovelamento (folding) das proteínas – compreendendo o modo como enovelam é possível compreender o modo como funcionam. Particularmente Trifluoretilanol (TFE) [5-7] é um dos álcoois que produz a maior curiosidade devido à particularidade de introduzir estruturas α-hélicais na maioria das proteínas. Este carácter singular de TFE será aplicado para reconverter a estrutura secundária das proteínas após denaturação por calor. Apesar do elevado número de estudos desenvolvidos com este álcool o mecanismo da interacção entre álcool e proteína não está esclarecido [8,9]. Neste trabalho vai ser proposto um mecanismo para a formação de α-hélice por álcoois fluorinados baseado nas propriedades da mistura água/TFE. Mais tarde será estudado o impacto de TFE na funcionalidade das proteínas adsorvidas.

A segunda parte desta tese vai estudar a conformação das proteínas adsorvidas pois invariavelmente quando adsorvem numa superfície solidá as proteínas alteram a sua estrutura [10-12]. Uma técnica antiga, Dicroismo Circular [13], será utilizada para resolver um problema antigo: alteração de conformação, como medir? Esta técnica provará ser muito sensível a alterações estruturais de proteínas adsorvidas mostrando que estas alterações estão directamente relacionadas com a função da proteína e dependentes da química e estrutura das superfícies.

Na terceira parte vai-se utilizar o impacto peculiar de TFE em proteínas para o "design" de superfícies com os grupos químicos Fluor (-CF₃) e Hidróxi (-OH). As superfícies propostas mostraram ser uma nova aposta para a estabilização estrutural de proteínas adsorvidas. Numa primeira aproximação ao problema utilizou-se uma mistura de alcano-tiois tendo os grupos terminais -CF₃ e -OH. Tiois são excelentes modelos para a obtenção de monocamadas
bem definidas visto estas só dependerem do controlo da química em solução [14-18]. A mistura de diferentes percentagens destes dois compostos químicos em solução originaram superfícies de variadas energias sendo então possível controlar os processos de adsorpção. A própria natureza usa este conceito no controlo das suas actividades: por exemplo o endotélio vascular, conhecido pelas suas propriedades antitrombogénicas, é constituído por microestruturas com domínios hidrofóbicos e hidrofílicos, respectivamente de baixa e alta energia [19].
Numa segunda aproximação utilizaram-se polímeros modificados com unidades TFE ou então completamente fluorinados. Os polímeros oferecem fortes vantagens em relação aos dois visto que é possível modular as suas propriedades mecânicas, absolutamente indispensáveis para aplicações biológicas (por exemplo imobilização de enzimas e adsorpção de proteínas).
Através de um material artificial – polímero – a química encontra a biologia [10, 20-22]. É nesta interface onde realizamos a maior parte das experiências tendo em vista provar a possibilidade de adaptação dos polímeros fluorinados como materiais biocompatíveis.

Alterações estruturais são tão importantes e com tão grandes consequências que no corpo humano existem doenças baseadas em alterações estruturais: Alzheimer, BSE (com o nome vulgar de doença das vacas loucas), a doença de Creutzfeldt-Jakobs [23-26]. Em comum têm todas uma transição estrutural de α?β de pequenos peptídeos, chamados prions, que agregam sob a forma β e precipitam no cérebro. Nos últimos anos foi descoberto que os β-peptídeos podem induzir β-estruturas em um peptídeo α [25]. De onde vem o primeiro peptídeo e qual o mecanismo que o faz transitar numa estrutura β não está ainda clarificado. Para curar a doença é então necessário bloquear a agregação das estruturas β ou então reconverter o peptídeo.
O peptídeo responsável pela doença de Alzheimer, amilóide, será utilizado na última parte desta tese em estudos efectuados com TFE e superfícies fluorinadas com vista a reconverter a estrutura β em estáveis α-hélices.
I. Introduction

Les protéines sont de grandes macromolécules complexes nécessaires pour le développement de la vie. Tous les systèmes biologiques dépendent de la fonctionnalité de celles-ci, elles peuvent être des transporteurs, catalyser des réactions, défendre l’organisme, être des structures de support, en fait, elles contrôlent l’activité de "milliers" de tâches différentes. Il est intéressant d’imaginer que la fonction normale d’une protéine est produite seulement par la combinaison de 20 acides aminés différents (annexe A) ayant pour résultat plus de 100.000 protéines différentes. Les acides aminés forment des chaînes qui définissent la structure de la protéine, et c’est cette structure qui va définir sa fonctionnalité. Plusieurs champs d’application ont été développés pour tirer profit des différentes fonctions offertes par les protéines: transport de principes actifs, matérielles biologiques, monitourisation biologique (biosensing), immunodosages (immunoassays) [1-4]. Toutes ces applications exigent des structures stables.

L’objectif de ce travail est de développer une nouvelle stratégie pour préparer une surface biocompatible. Afin d’éviter des réactions antigéniques, celle-ci doit être modifiée par des petites molécules qui vont stabiliser activement la structure adsorbante des protéines. Pour cela des groupes appropriés ont dû être recherchés. Leur mode d’action a d’abord dû être clarifié, ils ont alors été reliés aux surfaces et la conformation et la fonction liante des protéines sur ces surfaces ont été étudiées. Une autre application envisageable est l’emploi de ces surfaces pour le traitement des maladies liées aux changements de conformation et une tentative dans ce sens est décrite à la fin de cette thèse.

Ce travail est principalement consacré à l’étude de la structure des protéines. Une attention particulière a été prêtée à la manipulation de la structure des protéines par des alcools. Des alcools ont été utilisés dans les études impliquant le pliage des protéines – le terme pliage étant utilisé dans le sens de fonctionnement. En particulier, le TriFluorEthanol (TFE) [5-7] est un des alcools qui produit le plus de curiosité, due à la singularité d’induire les structures α-helicoïdales dans la majorité de protéines. Ce caractère singulier de TFE a été appliqué pour reconverter la structure secondaire des protéines, détruite après dénaturation par la chaleur. Mais, en dépit du grand nombre d’études réalisées, le mécanisme de l’induction de α-hélice par TFE n’a pas encore été établi [8,9]. Ce travail propose un mécanisme de formation de α-hélice par les alcools fluorés basés sur les propriétés du mélange de TFE et d’eau. L’impact de TFE sur la fonctionnalité des protéines adsorbées a aussi été étudié.

Les protéines adsorbées changent invariablement leur conformation [10-12]. Une ancienne technique a été employée, le dichroïsme circulaire, pour approcher un problème déjà ancien qui est de savoir comment mesurer les changements structuraux des protéines. Le dichroïsme circulaire [13] s’avère être une technique très sensible pour suivre les changements structuraux des protéines adsorbées, ces derniers étant directement lié au fonctionnement / conformation des protéines et dépendant de la chimie et de la structure de la surface.

L’impact particulier de TFE sur les protéines nous a conduit à la conception de surfaces basées sur les radicaux fluor (-CF₃) et hydroxyles (-OH). Les surfaces proposées montrent une nouvelle approche au problème structural de stabilisation des protéines adsorbées.
Dans une première approche un mélange de thiols d'alcane avec les radicaux -CF3 et -OH est utilisé. Les thiols sont de bons modèles car il est facile d'obtenir des monocouches bien définies juste en contrôlant la chimie de la solution [14-18]. Quand des composés possédant les radicaux -CF3 (hydrophobe) et -OH sont mélangés (hydrophile), il est possible de jouer avec la mouillabilité des surfaces et en conséquence de contrôler les processus d'adsorption des protéines. La Nature elle-même se sert du caractère hydrophobique / hydrophilique dans les systèmes complexes. Les endothélium vasculaires, connus pour leurs propriétés anti-thrombogénique, sont des structures de micro-phases séparées par des micro-domaines hydrophobes et hydrophiles [19]

Une deuxième approche, utilisant des polymères modifiés avec des unités de TFE ou complètement fluorés est aussi étudié. Les polymères offrent de nombreux avantages par rapport aux thiols car il est possible de moduler leurs propriétés mécaniques, ce qui est absolument nécessaires pour l'utilisation sur des bio applications différentes; ceux-ci incluent l'immobilisation des enzymes et des couches adsorbées de protéines. Par un matériel artificiel - un polymère - la chimie et la biologie se rejoignent. C'est dans cette rencontre interdisciplinaire que des études étendues sont actuellement réalisées [10,20-22]. Pour déterminer la réponse d'un corps à un matériau étranger, ou à l'activité des enzymes immobilisées, il est nécessaire d'étudier la fonctionnalité de la couche adsorbée de la protéine.

Les changements structurels sont si importants et avec les conséquences tellement importantes que dans le corps humain, il existe deux groupes de maladies basées sur des changements de structure secondaire: Alzheimer et la BSE (généralement connu sous le nom de maladie de la vache folle) et la maladie de Creutzfeldt-Jakobs [23-26]. Elles ont en commun la transition de α en β de petits peptides, appelés prions, qui s'agrègent et précipitent dans le cerveau. Ces dernières années, il a été découvert que les β-peptides peuvent induire une β-structure dans un peptide en hélice [25]. D'où les premiers β-peptides proviennent et à partir de quel mécanisme ils transitent dans cette β-structure reste inconnu. Pour traiter la maladie, il est alors nécessaire de bloquer la β-agrégation ou de reconvertis le peptide. Le peptide responsable de la maladie d'Alzheimer, l'amyloïde, est utilisé dans le dernier chapitre de cette thèse en combinaison avec des alcools fluorés et des surfaces fluorées. La "puissance" des alcools fluorés est utilisée comme moyen de reconvertis le peptide en α-structure stable.
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I. Introduction

Proteins are large complex macromolecules necessary for the development of life. All biological systems are dependent on the functionality of proteins: they are carriers, they catalyze reactions, they defend, they support structures, they control the activity of "thousand" different tasks. It is interesting to imagine that the normal function of a protein is produced only by the combination of 20 different amino-acids (appendix A) resulting in more than 100,000 different proteins. The amino-acids will form chains that define the protein structure, and the structure will define the functionality. Several fields of application emerged to take advantage of the different functions offered by proteins: drug delivery, biomaterials, biosensing, immunoassays [1-4]. Any of these applications requires stable structures.

This work was motivated by the aim to develop a new strategy to prepare a biocompatible surface. The surface should be modified by small molecules that actively stabilize the structure of proteins adsorbing and hence avoid antigenic reactions. Hence suitable groups had to be found. Their mode of action was to be clarified, they were then to be connected to surfaces and the conformation and function of proteins binding to these surfaces was to be studied. As an offspring one could also envisage to use these surfaces to cure diseases related to conformational changes and an attempt into this direction is made at the end.

This work is mainly dedicated to the study of the structure of proteins. Particular attention will be paid to the manipulation of protein structure by alcohols. Alcohols have been used in studies involving the folding of proteins – by understanding the way they fold it is understood the way they function. Particularly TriFluorEthanol (TFE) [5-7] is one of the alcohols that produces the most curiosity due to the singularity of inducing α-helical structures in the majority of proteins. This singular character of TFE will be applied to reconvert the secondary structure of proteins, destroyed after heat-denaturation. Despite the large number of studies performed the mechanism of α-helix induction by TFE remains still obscure [8,9]. This work will propose a mechanism for the α-helix formation by fluorinated alcohols based on the mixing properties of TFE and water. Further on it will study the impact of TFE on the functionality of adsorbed proteins.

Adsorbed proteins invariably change conformation [10-12]. An old technique will be used, Circular Dichroism, to approach an old problem, structural changes of proteins – how to measure them? Circular Dichroism [13] will prove to be a very sensitive technique to follow structural changes of adsorbed proteins and these will be directly related to the function/conformation of proteins and dependent on the surface chemistry and structure.

The peculiar impact of TFE in proteins became then appealing for the design of surfaces Fluor (-CF₃) and Hidroxi (-OH) based. The proposed surfaces show a new approach to the structural stabilization problem of adsorbed proteins. In a first approach a mixture of alkane thiols, -CF₃ and -OH terminated is used. Thiols are great models as it is easy to obtain well defined monolayers just by controlling the chemistry in solution [14-18]. When mixing -CF₃ (hydrophobic) and -OH (hydrophilic) terminated compounds it is possible to play with the wettability of surfaces and as a result control the protein adsorption processes. Nature itself makes use of the hydrophobic/hydrophilic character on their complex systems. Vascular endothelium, known for their antithrombogenic
properties, are microphase separated structures of hydrophobic and hydrophilic microdomains [19].
As a second approach polymers modified with TFE units or completely fluorinated are used. Polymers offer strong advantages to thiols as it is possible to modulate their mechanical properties, absolutely necessary for different bioapplications; these include immobilization of enzymes and adsorbed layers of proteins. Through an artificial material – polymer chemistry and biology come together. It is at this point that extensive studies are being performed [10,20-22]. To determine the body response to a foreign material, or the activity of immobilized enzymes, it is necessary to study the functionality of the adsorbed protein layer.

Structural changes are so important and with so strong consequences that in the human body there are a couple of diseases based on secondary structure alterations: Alzheimer, BSE (commonly known as the mad cow disease), Creutzfeldt-Jakobs disease [23-26]. In common they have an $\alpha \rightarrow \beta$ transition of small peptides, called prions, which aggregate and precipitate in the brain. In the last years it was discovered that a $\beta$-peptides can induce a $\beta$-structure in a helix peptide [25]. Where the first $\beta$-peptides comes from and by which mechanism they transitaire into this $\beta$-structure remains unknown. To cure the disease it is necessary then to block the $\beta$-aggregation or to reconvert the peptide. The peptide responsible for Alzheimer, amyloid, will be used in the last chapter of this thesis in combination with fluorinated alcohols and fluorinated surfaces. The "power" of fluorinated alcohols will be used to reconvert the peptide into a stable $\alpha$-structure.
II. Basic principles

This chapter will give an overview about the protein structure and the substrates. It will include a brief description of the techniques used in the experimental part as well as the theoretical approaches that fit experimental data.

II.1. Materials: proteins and substrates

II.1.1. What defines a protein

Proteins are macromolecules constituted by long chains of amino-acids (appendix A). Despite the reduced number of amino-acids, 20, existing in nature their combination results in an incredible number of complex structures with various functionalities [27-29]. A human being produces 50,000-100,000 different proteins! The functionality of the proteins depends on the sequence of the amino-acids and on their spatial distribution which defines the structure of proteins. For a better characterisation of a protein is divided its structure in four parts: a primary structure defined by the amino-acid sequence; a secondary structure formed by intra- or intermolecular bonds between amino-acids relatively close to each other, forming the so called α-helices, β-structures and random coils; a tertiary and quaternary structure which builds the 3D picture of the secondary structure by approaching amino-acids far apart in a linear sequence and relates it to the function of the protein (figure II.1). Quaternary and tertiary structures differ in the number of polypeptide chains: the first has more than one while the second has only one polypeptide chain called subunit.

![Protein structures](image)

Figure II.1 – Representation of the three different structures existent in one polypeptide chain of a protein. A - primary structure, the different colours denote the different amino acids. B - secondary structure, pink represents the α-helix, yellow the β-sheet, blue the random coil, and grey the β-turn; C- tertiary structure. This last structure shows that proteins are very compact structures - their atoms occupy a fraction of space of 0.72-0.76, comparable to the compactness of a solid (0.75).
Knowledge of the protein structure is fundamental to the understanding of biological functions of the protein. In this work it will be referred a lot to conformational changes or denaturation of proteins which is nothing but loss or gain in functionality. If proteins change in conformation their internal structure will be broken. This means that the original interactions that stabilise the protein function are changed.

The forces responsible for the stabilisation of protein structures can mainly be described by four reversible non-covalent interactions: H-bonds, electrostatic, van der Waals and hydrophobic interaction. The first interactions, H-bonds, in biological systems can have the O or the N atom both as a proton donor or as the acceptor. These bonds involve energy on the order of 10 to 30 kJ/mol and a distance between the atoms of around 3 Å. The electrostatic interactions are stabilizing via the attraction of two oppositely charged amino-acids being optimal at a distance of ~2.8 Å. This attraction can be described by Coulomb’s law, with the energy being roughly the same as in case of the H-bonds. Van der Waals are nonspecific forces that describe attractive interactions between molecules. Finally the hydrophobic forces result from the interaction between hydrophobic amino-acids which cannot form H-bonds with water. The high energetic cost of solvation of such amino-acids is responsible for the “collapse” of these molecules. These amino-acids are found in the interior core of the proteins, away from the water.

Despite the high number of interactions that stabilize the protein’s structure only a relatively small amount of energy is necessary to change their native conformation. The differences in energy between folded and unfolded states are only of the order of 20-65 kJ/mol. This relatively low amount of energy needed for conformational changes of proteins is necessary for the easy adaptation to their tasks: binding of molecules to proteins, insertion in membranes, energy production, all these functions require flexible structures. Usually all these processes are reversible.

The question remaining is - being the differences in energy between folded and unfolded state so small - why proteins prefer the folded conformation. Unfolded proteins are highly H-bonded to the water. When folding they break these bonds and form internal ones with the same energy, so that the net difference between folded and unfolded state is nearly zero. Why then proteins adopt the folded conformation is caused by the hydrophobic amino-acids of proteins which are unfavourably solvated by water and by the large solvation layer formed around the unfolded state - causing a decrease in entropy. If structured water is released, then internal bonds between amino-acids are formed, the protein folds and the entropy increases.

II.1.2. The secondary structure of proteins.

As this thesis will refer a lot to the secondary structures of proteins it is presented a brief description of the different secondary structures: α-helix, β-structures and random coil (figure II.2).

The α-helix is a rod-like structure stabilized by H-bonds which line up across the helix and with the R-groups (appendix A) protruding outward from the helical backbone. The internal H-bonds are formed between the NH and CO groups of the n to n+4 amino-acid in the linear chain. Each amino-acid is related to the next one by a rise of 1.5 Å along the helix axis and a rotation of 100°, what gives 3.6 amino-acids per turn of helix. This results in a distance per turn of 5.4 Å.

In the β-sheets the backbone is extended in a zig-zag conformation. These zigzag backbones orient themselves parallel or antiparallel to each other forming intermolecular H-bonds with
the R groups of adjacent amino-acids protruding in opposite directions, almost perpendicularly to the plane of the backbone. The axial distance between adjacent amino-acids is 3.5 Å.

The β-turns are structures responsible for the change in the direction of a polypeptide chain. These structures can form a tight turn (~180°), and they involve only 3-4 amino-acids, bonded again by H-bonds.

The random coil - as the name says - hasn’t any defined structure, it is randomly oriented. It is stabilized by the high entropy allowed to the backbone and by H-bonds with the water. It is usually found at the ends of proteins.

![Diagram of α-helix and β-sheets](image)

**Figure II.2 – Representation of the main structures present in nature.**

* A - the α-helix shown is a right handed helix as, with rare exceptions, is the structure found in nature; one can also see the alignment from the helix dipole. The helix dipole is formed by adding up the small electrical dipole from each amino-acid which adds up across the hydrogen bonds in the helix. This dipole is stabilized by negatively charged amino-acids found at the end of the peptide chain, near the N-terminus, which have a stabilizing interaction with the positive charge of the helix dipole, being the opposite also true for the C-terminus.

* B - the β-sheet can be parallel or antiparallel, in which the orientation of the amino-terminal to carboxy-terminal is inverse;

* C - β-turn where the three aminoacids form a tight turn.

The secondary structures of proteins can be manipulated by external parameters, i.e. changes in pH, ionic strength, temperature, or addition of organic compounds like Sodium dodecylsulfate (SDS), Urea and alcohols [5-7,30,31]. The particular interest shown in urea and alcohols is caused by their ability to change the folded and unfolded conformation of backbones - they have opposite effects. The real mechanism of the folding and unfolding transitions induced by these compounds has not yet been understood [8,9,30]. Nevertheless they provide nice simulations of the folding and unfolding processes of peptides and – in the case of alcohols - even of the hydrophobic environment of phospholipids.
II.1.3. Proteins and adsorption to solid surfaces.

All the protein measurements in this thesis have been carried out with three human globular proteins, Immunoglobulin (IgG), Human Serum Albumin (HSA) and Fibrinogen (Fin), and one polypeptide – Amyloid (chapter VI). The structures of the globular proteins can be found in appendix B and some of their properties are listed in table II.1.

HSA, IgG and Fin are the three major constituents of blood plasma. Albumin represents 50-60 % of all blood proteins, IgG has a share of 15-25 %, while Fin makes only around 5 %. It has the smallest diffusion constant, but biggest mass, while HSA has the highest diffusion constant and lowest mass. These properties are important for biomaterials. If an implantable material (biomaterial) is put in contact with blood, then HSA will be the first one to adsorb in high quantities due to its highest content in plasma and highest diffusion coefficient. After some time IgG will displace HSA, and later on IgG will be replaced by Fin, due to higher mass of one relatively to the other. This became known as the Vroman effect [32].

This process reveals some of the reasons for the incompatibility of synthetic materials with the human body. Fin is the protein responsible for activating the biologic systems. When adsorbed, in the majority of the surfaces, it changes conformation and polymerizes. Its polymerization on the surface results in clotting formation and in cell adhesion.

HSA, however, is a protein that has no cell receptor known to its recognition. If it remains on the surface than this surface will be passivated against clotting [33]. So many people have tried to find surfaces which do not obey the Vroman effect by blocking the further adsorption of IgG and Fin. Thereby the adsorbed HSA has to maintain conformation when adsorbed, otherwise it will form a layer as foreign to the body as the implant itself.

This work follows an alternative strategy to produce a biocompatible surface. Rather than avoiding protein adsorption, that would lead to antigenic response due to the usual degradation on adsorption, it is prepared surfaces that actively maintain or even regenerate the native structure of adsorbed proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molec. weight</th>
<th>Diffusion constant (cm²/s)</th>
<th>Isoelectric point</th>
<th>% in Blood</th>
<th>Shape</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA (globular)</td>
<td>69000</td>
<td>6-8*10⁻⁷</td>
<td>5.4</td>
<td>50-60</td>
<td>Heart shaped</td>
<td>70% α-helix no β-sheet</td>
</tr>
<tr>
<td>IgG (globular)</td>
<td>160000</td>
<td>4*10⁻⁷</td>
<td>7.4</td>
<td>15-25</td>
<td>Y shaped</td>
<td>50% β-sheet no α-helix</td>
</tr>
<tr>
<td>Fin (fibrous)</td>
<td>340000</td>
<td>3*10⁻⁷</td>
<td>--</td>
<td>5</td>
<td>Long fibrous</td>
<td>30% α-helix and β-sheet</td>
</tr>
</tbody>
</table>

Table II.1 – Properties of the main globular proteins.

II.1.4. Substrates.

The substrates used for performing protein adsorption were quartz, octadecyltrichlorosilane (OTS), thiols (figure V.1, chapter V), polystyrene (PS), polyvinylmethylicetone (PVMK), a modified version of PVMK with TFE units, and several fluorinated polymers (FP) synthesised at the MPI by Andreas Thünemann (see appendix C for chemical structures).
The thiols were dissolved in ethanol with a concentration \(10^{-3}\)M and further Self Assembled (SAM) onto a gold surface for 12h. This gives a monolayer well ordered and well defined for protein adsorption. PS, PVMK and PVMK-TFE polymers were spin coated and dip coated onto a clean quartz slide using as solvents ethanol and acetone. The FP were solubilized in 1,1,1-3,3,3 - isofluoropropanol (IsoF) or 2,2,2 - trifluorethanol (TFE). These polymers were bound to the surface by dip coating (~2min) or simply deposited by evaporation of the solvent. The cross linking of this last polymers could be increased with temperature.

In order to perform Circular Dichroism measurements all quartz slides coated with polymers had to form stable thin films in buffer solution, to be optically transparent in the range 180-300nm and to be optically inactive.

II.2. Methods and theory.

II.2.1. Enzyme Linked Immuno Sorbent Assay (ELISA)

Enzyme Linked Immuno Sorbent Assay (ELISA) was used to detect conformational changes of proteins. ELISA uses a class of proteins, the immunoglobulins (IgG), also called antibodies, that recognize (binds) active parts of other proteins (called antigens). These active parts are named epitopes. Each antibody is specific for the recognition of only one epitope. Changes on the structure of this epitope leads to the nonbinding of antibodies.

Using this principle to detect conformational changes of adsorbed proteins it is necessary to place the surface coated with the proteins in a solution containing the antibody that recognizes the active epitopes of the protein (figure II.3). The detection of the first antibody (primary antibody) is made by adding a second antibody to the solution (secondary antibody). The last one is marked with an enzyme or by a fluorescence dye. The enzyme can be detected by a specific chemical reaction being then possible to measure the optical density of the reaction products.

![Diagram of ELISA](image)

Figure II.3 – Schematic representation of ELISA applied to solid surfaces
II.2.2. Physico-chemical methods

II.2.2.1. Surface tension ($\gamma$) of water/TFE mixtures.

The determination of the surface tension $\gamma$ was done by the ring-method (tensiometer TE 1C from Lauda). In this method the force acting on a platin ring of radius $r$ is registered while it is pulled out of a liquid. Just before the liquid film tears off, the force reaches its maximum, $F_m$, which is related to the surface tension by the formula:

$$\gamma = \frac{F_m}{4\pi r} f$$  \hspace{1cm} (1)

where $f$ represents a correction factor in order to obtain corrected values for the surface tension [34, 35]. This $f$ is a function of the mean ring radius $R r$, the force $F_m$, the ring radius $r$ and the difference in density of liquid and air $\Delta \rho$;

$$f = f\Bigl(\frac{R r}{r} ; \frac{R r^3 \Delta \rho}{F_m}\Bigr)$$  \hspace{1cm} (2)

In general the density of the solvent can be approximated to the density of the solution, but for TFE/water mixtures there is a strong deviation from both. Densities were therefore determined independently for each solution with a densiometer (TD1 from Lauda) (graph III.9).

From the surface tension, the adsorption at the interface $\Gamma$ can be derived by the Gibbs equation:

$$\Gamma = -\frac{1}{RT} \frac{\partial \gamma}{\partial \log a}$$  \hspace{1cm} (3)

where is $T$ the temperature, $R$ the ideal gas constant and $a$ the activity, which for diluted solutions can be approximated to the concentration $C$.

II.2.2.2. Measuring the viscosity ($\mu$).

The viscosity, $\mu$, of the TFE/water solutions was determined by the falling ball method at 25°C (BH2 from Höppler). The dynamic viscosity $\mu$ was calculated with the formula:

$$\mu = t (\rho_1 - \rho_2) K$$  \hspace{1cm} (4)

where $t$ is the time in seconds of the falling ball, $\rho_1$ is the density of the ball, $\rho_2$ is the density of the solvent and $K$ is the ball constant (parameters $K$ and $\rho_1$ dependent on the size of the ball - given in the Höppler manual).
II. Basic Principles

II.2.3. Spectroscopic techniques.

II.2.3.1. UV-vis spectroscopy.

The number density of adsorbed proteins was obtained by absorption measurements in the visible and ultraviolet with a grating spectrometer. According to the Lambert-Beer-law, the extinction coefficient $\varepsilon$ of a layer of material with the thickness $l$ and the number density $C$ is, for small number densities, related to the quotient of the light intensity before ($I_0$) and after ($I$) passing the layer. This quotient is called absorbance $A$, and is given by:

$$A = \log_{10} \left( \frac{I_0}{I} \right) = \varepsilon l C$$  \hspace{1cm} (5)

By comparison of the absorbance $A_{ss}$ of an adsorbed layer of proteins with that of a solution ($A_{sol}$) of the same protein with known number density at the wavelength of an absorption maximum, the number density of adsorbed proteins on a glass slide can then be determined. Here it is assumed that adsorbed proteins and proteins in solution have the same extinction coefficient.

II.2.3.2. Fourier Transform Infrared Spectroscopy (FTIR).

Vibrational bands in the infrared have been determined by Fourier-transformed infrared spectroscopy (spectrometer from Bruker, Equinox 55s, and from Nicolet, Protege 450): In Fourier spectrometers \([36]\), the light beam from a thermic light source is divided into two coherent partial waves. On the detector the superposition of both waves is measured in dependence of a phase difference $\gamma$, which is imposed on one of the waves by a phase shifting component (i.e. a moving mirror in a Michelson interferometer type). Mathematically, this means the formation of the autocorrelation function:

$$I(\gamma) = \int_{-\infty}^{\infty} E_1(\gamma) E_2(\gamma + dt) \, dt$$  \hspace{1cm} (6)

From this one derives with a Fourier transformation, the intensity over the frequency $\nu$:

$$I(\nu) = \int_{-\infty}^{\infty} I(\gamma) e^{-i2\pi\nu \gamma} \, d\gamma$$  \hspace{1cm} (7)

As all frequencies are contributing to the signal at the detector simultaneously, the signal to noise ratio of the fourier spectrometer is better than with a grating spectrometer, as the ones that are used for the measurements in the ultraviolet and visible. This is especially helpful in the far infrared due to the limited intensity of the available light sources. In addition, for the fourier spectrometer the energy throughput is not dependent on the chosen resolution, as it is with the grating spectrometer due to the chosen slit width.
The vibrational bands in the infrared determined with the Fourier spectrometer can be described as the frequencies $\nu$ of classical harmonic oscillators. Depending on its symmetry, each molecule can perform a number of vibrations at characteristic frequencies $\nu_i$. Their position is, in the classical model for diatomic molecules, determined by the force constant $F$ and the reduced mass $\mu$ of the molecular bond between atoms with the masses $m_1$ and $m_2$.

$$\nu = \frac{1}{2\pi} \sqrt{\frac{F}{\mu}} \quad \text{with} \quad \mu = \sum \frac{1}{m_i^2} + \frac{1}{m_1}$$  \hspace{1cm} (8)

These equations are no longer valid for anharmonicities as the force is no longer proportional to the mass displacement.

An infrared (IR) spectrum measures the absorption of radiated energy at a certain frequency $\nu$ which match the natural vibrational frequency of the molecule. A shift of the position of a band or a variation of its width is an indication for a change of the internal bond structure of a molecule, while a change of the intensity alone hints to a change in the number of molecules or change in the orientation of the dipole moment, that occurs as a result of molecular vibration.

For solutions, transmission spectra reveal the vibrational bands, if the volume and concentration are carefully chosen. Yet for the investigation of adsorbed monolayers with a small number of molecules in the beam path, special setups have to be chosen.

In the Grazing Incidence Reflection (GIR) method, p-polarized light is sent at grazing incidence (in this case at an angle of $84^\circ$ to the surface normal) on a thin film on a metallic substrate (figure II.4). The electric field parallel to the interface vanishes at the metal surface thus allowing detection of vibrations with the transmission dipole moment in the plane. The field perpendicular to the surface however is enhanced thus, vibrations perpendicular to the surface are detected.

This makes GIR sensitive enough to study monolayers. It is widely used to study thiol monolayers on gold surfaces and will be applied in this work with the same purposes.

For the investigation of adsorbed proteins at the interface between a solid substrate and the liquid protein solution, it was chosen Atemuated Total Reflection (ATR). With ATR the light has not to pass the liquid and thereby avoids any disturbing light absorption from the liquid. In ATR, light is entering a prism (in this work out of ZnSe) perpendicular to its surface, but is then guided in the prism by total reflection (figure II.4). The beam hits the surfaces of the crystal at an angle $\theta$ greater than the critical angle $\theta_{critical}$ with

$$\theta_{critical} = \sin^{-1}\left(\frac{\eta_1}{\eta_2}\right)$$  \hspace{1cm} (9)

with $\eta_1$ representing the refractive index of the ATR crystal and $\eta_2$ the refractive index of the outer medium. After $N$ reflections the light beam leaves the prism again through a surface with nearly zero incoming angle. $N$ can be easily calculated from the length of the ATR crystal $L$ and its thickness $d$ with the relation
\[ N = \left( \frac{L}{d} \right) \cot \alpha \theta \] (10)

Although the light beam is totally reflected at the interface between the prism and the outer medium, the light intensity in the outer medium is not zero. Due to the continuity conditions for the components of the electrical field an evanescent field \( E \) is present in the outer medium. It penetrates a small distance into the outer medium and propagates parallel to the surface in the plane of incidence. It can be shown that the evanescent wave has an exponentially decreasing distribution with distance, \( z \), from the waveguide/fluid interface [37]. In the absence of an adsorbed film the field of the evanescent wave can be described by:

\[ E = E_0 e^{-z/dp} \] (11)

As this evanescent field will interact with materials at or near the crystal surface, proteins and other material adsorbing from a liquid flowing on the outer medium, will show characteristic bands in the infrared spectrum within a defined distance of the surface, depth of penetration \( dp \). At \( dp \) the electric field falls \( e^{-1} \) of its value at the surface. The depth of penetration \( dp \) can be then defined by:

\[ dp = \frac{\lambda}{2\pi} \left( \sin^2 \theta - \eta_{21}^2 \right)^{1/2} \] (12)

where \( \lambda = \frac{\lambda}{n_i} \) and represents the wavelength of the light in the denser medium and \( \eta_{21} = \frac{n_2}{n_1} \) is the ratio of the refractive index of the less denser medium divided by that of the denser one. This \( dp \) gives an idea of how deep the experiment probes into the medium.

---

**Figure II.4 – Scheme of grazing incidence and of ATR optics with representation of the evanescent wave field. \( \theta_c \) is below 8° and \( \theta \) in ATR is equal to 45° (a ZnSe crystal was used for the ATR experiments in chapter III).**
II.2.3.3. Fluorescence Recovery after Photobleaching with Total Internal Reflection Fluorescence (FRAP - TIRF).

II.2.3.3.1. Method

Total Internal Reflection Fluorescence (TIRF) in combination with Fluorescence Recovery After Photobleaching (FRAP) was used to follow the absorption and diffusion of molecules (proteins and TFE) into/on a surface.

In the FRAP-TIRF experiments proteins were labeled with a fluorescence dye (FITC) so that they could be excited by the beam of an Ar\(^+\)-laser at \( \lambda = 488\) nm as light source. Protein and buffer solutions were flowing in the system in the laminar regime.

In TIRF the laser beam was coupled via a prism into a glass slide. As prism and glass slide are separated by an air gap, the light is guided in the glass slide by total reflection – similar to the ATR prism described above. In this case, however, the reflected light was not analyzed as in ATR, but the evanescent wave alone was used for the excitation of labeled proteins eventually adsorbed at the glass slide. The fluorescence signal was detected via a CCD camera (details of this setup can be found at [38]) or with a photomultiplier tube (details can be found at [39-41]). From its variation one could follow variations on the protein concentration at the surface. The TFE approach to the surface was detected by the strong increase in fluorescence signal due to dequenching of the FITC dye.

In FRAP, two beams were coupled via a prism into a glass slide with the same angle of incidence, chosen in such a way that they travel by total reflection inside the glass slide. Due to their slightly different azimuthal angles, the two beams formed an interference pattern of stripes perpendicular to their direction. By the evanescent waves of this interference pattern, a bleaching pattern was formed in the adsorbed protein layer. This bleaching pattern could be imaged by TIRF via a lens on the CCD-camera due to a further laser beam coupled in along the same path as one of the two bleaching beams, which excited the not bleached FITC to fluorescence. Diffusion can be followed by observing changes in the fourier transformation of the fluorescence intensity.

For performing the TIRF measurements a flow cell was designed with such dimensions (64\(\times\)12\(\times\)2 mm (figure II.5) that a laminar flow could be obtained in the cell [42]. Thereby well defined conditions could be achieved to control the protein and TFE diffusion onto the surface.

A laminar flow is provided for a Reynolds number inferior to 2100, which reynolds is calculated by:

\[
Re_y = \frac{\rho v d}{\mu}
\]  \hspace{1cm} (13)

being \( \rho \) the density of the liquid, \( v \) the velocity in the direction of the flow, \( d \) the thickness of the rectangular system and \( \mu \) the viscosity of the liquid. For the pure buffer system it was obtained a \( Re_y = 11.4 v \) and for the TFE buffer system it was obtained \( Re_y = 6.6 v \). The flow rate of the solvent varied between 1-2ml/min. The wall shear rates were then calculated with:
\[ \gamma = \frac{6Q}{d^2W} \] (14)

\(Q\) represents the volumetric flow, \(d\) the thickness and \(W\) the width of the flow chamber.

Figure II.5 – Side and front view of the TIRF flow cell apparatus. Two light beams are coupled and totally internally reflected. The CCD camera measures the fluorescence intensity from the probe. The TIRF optical set-up can be found in Mark Auch’s thesis (1999).

II.2.3.3.2. Estimation of the diffusion coefficients of proteins and of TFE.

To calculate diffusion coefficients from the FRAP-TIRF measurements it was used Fick’s second law for mass transfer in a transient medium. The diffusion perpendicular to the interface as well as along the stripes is not considered in the experiment, so the redistribution of adsorbed proteins after photobleaching was treated as diffusion in one dimension with

\[ \frac{\partial C(x,t)}{\partial t} = D \Delta C(x,t) \] (15)

Fourier transformation of this equation leads to:

\[ \frac{\partial \tilde{c}(\vec{k},t)}{\partial t} = -Dk^2\tilde{c}(\vec{k},t) \quad -\infty < |k| < +\infty \] (16)

with the solution:

\[ c(\vec{k}_x,t) = \tilde{c}_0(\vec{k}_x) + \tilde{c}_1(\vec{k}_x)e^{(-k_x^2D)t} \] (17)

where \(k_x = 2\pi/\lambda\) and \(D\) the diffusion coefficient of adsorbed molecules.
The determination of the diffusion coefficient of TFE into the surface was done assuming that there are no energetic barriers to the adsorption of TFE during the initial adsorption times. Then all molecules that adsorb will stick to the vicinity of the proteins. Fick’s law becomes for the initial adsorption times:

$$\frac{\partial \Gamma}{\partial t} = C_0 \left( \frac{D_{TFE}}{\pi t} \right)^{1/2}$$

with the integrated result for estimation of the total number of molecules adsorbed, $\Gamma$:

$$\Gamma(t) = 2C_0 \left( \frac{D_{TFE}}{\pi} \right)^{1/2}$$

$C_0$ represents the bulk concentration of TFE and $D_{TFE}$ the diffusion coefficient of TFE to the surface.

The relation between the $F$ and $\Gamma$ was obtained assuming a proportionality between both quantities [43]. The fluorescence, $F$, observed at a constant exciting light intensity $I_o$, is equal to the light absorbed, $I_a$, multiplied by the quantum yield $\phi$ and an instrumental factor $r$, $(p = \phi r)$, which is defined by the relation:

$$F = p \frac{(I_o - I_a)}{I_o}$$

The Beer-Lambert law (eq. 5) can be applied here as $I_a = I_o - I$, and eq 20 becomes:

$$F = p(1 - e^{-2.36CI})$$

For very small values of $\varepsilon CI$ the emitted fluorescence becomes directly proportional to the concentration of the fluorophore and it is obtained the relation:

$$F = p(2.36\varepsilon \Gamma) \quad \text{with} \quad \Gamma = CI$$

To estimate the rate of diffusion of TFE molecules in water it was applied the Stokes-Einstein equation. This equation derives from the Nerst-Einstein equation which states that the diffusivity $D_{AB}$ of a single particle or solute molecule of A through a stationary medium B is:

$$D_{AB} = kT \frac{\mu_A}{F_A}$$
where $\frac{\mu_A}{F_A}$ represents the steady-state velocity attained by the particle under the action of a unit force. Here molecule A has to be bigger than B. This formula depends on the validity of the Stoke’s equation:

$$F_A = 6\pi \mu_B \mu_A R_A$$  \hspace{1cm} (24)

with $\mu_A$ and $\mu_B$ being the viscosities for pure solvents and $R_A$ the radius of the diffusion particle. The substitution of equation 24 in 23 gives the Stokes-Einstein relation:

$$D_{AB} = \frac{kT}{6\pi \mu_B R_A}$$ \hspace{1cm} (25)

II.2.3.4. Surface Plasmon Spectroscopy.

Surface Plasmon Spectroscopy (SPS) \cite{44} was used to determine the thickness of thiols and adsorbed amount of proteins onto thiols.

In the SPS setup a laser beam enters a glass prism and internally reflects at its lower side. After leaving the prism, light hits a detector. The prism is attached against a sample with a liquid of the same refractive index as glass slide and prism. The sample is covered with a thin gold film of roughly 50 nm.

With increasing angle of incidence, the reflected light intensity grows, until after the critical angle when total reflection sets in. At a certain angle of incidence, a sharp dip in the reflection curve can be seen. This dip results from the excitation of oscillations of the electrons at the metal/air boundary by the evanescent light wave from the glass/metal interface. These oscillations are called surface plasmons.

Position, width and strength of the dip in the angle scan depend of the thickness and dielectric function of the gold film. With the help of Fresnel theory, which calculates the reflected intensity in dependence of the angle of incidence for such a system, the parameters of the gold film can be determined by comparison of simulation and experiment.

If a gold film of known thickness and dielectric function is covered with a further thin film (thiols), its thickness and dielectric function can be determined in the same way from the changes in the surface plasmon parameters. This was used to determine in situ the thickness of the thiol layer and the amount of proteins $A$ adsorbed from solution on the thiol film. Assuming a linear dependence of the refractive index $n_P$ of the protein film with the protein concentration $c_P$, one can use the relation

$$A = \frac{\eta_P - \eta_S}{d\eta_P} \cdot dP$$ \hspace{1cm} (26)

where $\eta_S$ is the solvent refractive index and $dP$ the thickness of the film. The refractive index
of the solvent as well as the increment \( \frac{d\eta_p}{dc_p} \) were taken from literature [45].

II.2.3.5. Circular Dichroism.

Chiroptical spectroscopy refers to spectroscopy using circularly polarized light. A right-circularly polarized beam has the electric vector rotating about the direction of propagation. It defines a right-handed helical pattern. The left-circularly polarized light has the opposite characteristics. (figure II.6). Mathematically the electric vector of circularly polarized light can be described by:

\[
E_\pm = E_0 (i \pm j) e^{i(kz - \omega t)}
\]  

(27)

with the plus sign referring to right, the minus sign to left circularly polarized light; \( E_0 \) is the amplitude of the light wave, \( i \) and \( j \) are unit vectors along the \( x \) and \( y \) coordinates, respectively, in a right-handed Cartesian coordinate system with the \( z \) coordinate in the direction of propagation; \( k = \tilde{n} k_0 \) the wavevector in a medium with refractive index \( \tilde{n} \).

Figure II.6 – Schematic representation of left (L) and right (R) polarized light. The photon is a particle with spin 1 (that is, it carries one unit of spin angular momentum), and in a state of circular polarization it has a definite helicity, or component of angular momentum along its directions of propagation. Left circularly polarized light consists of photons of one helicity and right circularly polarized light consists of photons of the opposite helicity.
Chiral molecules - molecules, which cannot be superimposed by their mirror image - are optically active, which means that they can rotate the plane of polarized light. These molecules then display Circular Dichroism (CD) \([19,29]\). That is, the molecules have different absorption coefficients of right and left circularly polarized light at any given wavelength. Applying eq. 5 it is possible to write:

\[
\Delta A = A_l - A_r = \varepsilon_l C l - \varepsilon_r C l = \Delta \varepsilon C l
\]  

(28)

where \(\Delta \varepsilon\) represents the decadic molar extinction coefficient of the solute for right and left polarized light. For achiral molecules and racemic mixtures then \(\varepsilon_l = \varepsilon_r\) holds for each electronic transition in an isotropic medium, so CD is not observed.

The original method of measuring CD \([46]\) took advantage of the fact that when plane polarized light passes through a circular dichroic medium, differential absorption of the two circular components converts the plane polarized light into elliptically polarized light, in which the tip of the vector traces out an ellipse rather than oscillating in a plane of forming a circle. Modern commercial instruments, like the one used here, uses a modulation technique to measure the generally very small \(\Delta A\).

However, circular dichroism is still expressed in terms of the molecular ellipticity \(\theta\), which has its origin in the original method. The molecular ellipticity \(\theta\) is then defined by the ratio of the semiminor and semimajor axes of the traced ellipse, which is the tangent of the angle \(\theta\). Since the angle \(\theta\) is generally very small, \(\tan \theta\) can be approximated by \(\theta\) in radians:

\[
\theta \approx \tan \theta = \frac{E_l}{E_l + E_r} = \frac{|E_l - E_r|}{E_l + E_r} = \frac{\ell^{-A_l/2} - \ell^{-A_r/2}}{\ell^{-A_l/2} + \ell^{-A_r/2}}
\]  

(29)

Expanding the exponentials, neglecting the terms of the order of \(\Delta A\) in comparison with unity, and converting to degrees gives the relation:

\[
\theta (\text{deg}) = 180 \ln 10 \frac{\Delta A}{4\pi} = 32.98 \Delta A
\]  

(30)

Thus the ellipticity is directly proportional to the CD. For comparison it is necessary to remove the linear dependence on path length and solute concentration by defining a molar ellipticity:

\[
[\theta] = 100 \frac{\theta}{C_l}
\]  

(31)

Combining equation 28 with 31 it is obtained the relation between \([\theta]\) and \(\Delta \varepsilon\):

\[
[\theta] = 3298 \Delta \varepsilon
\]  

(32)

which can be rewritten as a function of \(I_l\) and \(I_r\) (the intensities for left and right handed polarized light respectively) using Beer-Lambert law:
\[
[\theta] = \frac{3298}{lC} \log_{10} \left( \frac{I_R}{I_L} \right)
\]  

(33)

When the difference between \(I_L\) and \(I_R\) becomes rather small, it becomes desirable to measure the difference between both like it is done in the instrument used. Introducing the parameters \(I_A\) and \(S\),

\[
I_A = \frac{1}{2} (I_R + I_L)
\]  

(34)

\[
S = I_R - I_L
\]  

(35)

one can write,

\[
[\theta] = \frac{3298}{lC} \log_{10} \left( 1 + \frac{S}{2I_A} \right)
\]  

(36)

As \(\frac{S}{2I_A} \ll 1\), equation 36 becomes

\[
[\theta] = \frac{3298}{lC} \frac{S}{I_A} \log_{10} e
\]  

(37)

The CD spectrometer used here (J-720 from Jasco) consists of a Xe lamp as light source, two monochromators, a photoelastic modulator, the sample cell, and a photomultiplier tube (figure II.7).

The double monochromator composed of slits and two prisms separates the wavelength, reduces the stray light and creates linear polarised light oscillating in horizontal direction, by using suitable oriented crystals for the prisms.

The modulator consists of a fused quartz oriented with its principal axis at 45° to the linear polarised light, which is put under periodic stress by a piezoelectric transducer. Thereby periodic circular polarised light is created, which causes a DC signal proportional to \(I_A\) at the photomultiplier and an AC signal proportional to \(S\) – in case there is a dichroic sample.
Figure II.7 – Optical set-up of the used CD spectrometers. Mo ~ M5 Mirrors; LS Light Source (Xe lamp 150W); S1 ~ S3 Slits; P1, P2 Prisms; L Lens; F Filter; CDM Modulator; SH Shutter; S sample holder; PM Photomultiplier Tube

For measuring CD of proteins in solution and adsorbed on a substrate it is necessary to use quartz cuvettes or slides which are optically transparent until at least 190nm. To measure CD of adsorbed proteins the sample holder represented in figure II.8 was designed.

Figure II.8 – Sample holder for transmission measurements of an adsorbed film of proteins. The samples are placed perpendicularly to the beam and are separated by hollow Teflon pieces where it is possible to put the solvent.

Analysing the CD spectra of adsorbed films one makes the assumption that there are no effects on $\Delta \varepsilon$ due to orientation of proteins. This assumption is often reasonable as the protein adsorption process is random, and - being globular - the proteins have secondary structures
oriented in several directions. This was checked by rotation of the measured sample, which induced no changes in the CD spectrum.

Optical artifacts contributing to the CD spectra of adsorbed films could also be neglected due to the small thickness range of adsorbed films (5-8nm). Another argument against optical artifacts is that the film ellipticity ratio at $\theta_{222}/\theta_{208}/\theta_{190}$ of adsorbed films and solution are approximately maintained.

II.2.3.5.1. Applying CD to proteins.

Chiral units are necessary to observe a CD signal in proteins. This chiral units are provided by each amino-acid.

The amide group of the amino-acids has three $\pi$ centers and so provides three $\pi$ orbitals with two main $\pi\pi^*$ transitions. One of the transitions is observed at $\approx$190nm (the other transition hasn't been attributed yet [19]). The transition at 190nm is electrically allowed and is directed approximately along the N→O direction (figure II.9).

In addition to the $\pi$ orbitals there are two lone pairs on the carbonyl oxygen ($\sigma$ orbital). This orbital provides a $\pi\sigma^*$ transition (figure II.9). This transition is electrically forbidden but charge displacement will induce a magnetic dipole transition moment orientend along the carbonyl bond at a wavelength between 215-220nm, dependent on solvent.

![Dipole Transition Moment](image)

Figure II.9 – The electric dipole transition moment of the amide $\pi\pi^*$ transition ($\mu_{\pi\pi^*}$) and the magnetic dipole transition moment of the amide $\pi\sigma^*$ transition ($m_{\pi\sigma^*}$).

A CD spectrum plots the difference of the molar absorption coefficients for right and left circularly polarized light, $[\theta]$, against wavelength, $\lambda$.

Proteins have a characteristic CD spectrum dependent on their secondary structure. Each secondary structure is represented by characteristic absorption bands at certain wavelengths seen in figure II.10.
Based on the transitions observed for the amide group (figure II.9) it is possible to attribute to the different secondary structures a $\pi\pi^*$ and $n\pi^*$ transition for each absorption band observed in figure II.10.

To the $\alpha$-helix is attributed a $n\pi^*$ transition at 222nm with negative $\Delta\varepsilon$ [48,49] and a $\pi\pi^*$ transition at 208 and 190nm with negative and positive $\Delta\varepsilon$, respectively. The existence of these two last bands results from exciton splitting of the $\pi\pi^*$ absorption band due to the helix symmetry, with the 208nm component polarized along the helix axes and the 190nm polarized perpendicular to the helix axes [50-52].

The $\beta$-sheet has a negative band near 215nm assigned to the $n\pi^*$ transition and the $\pi\pi^*$ absorb at 198nm with a negative $\Delta\varepsilon$. The magnitude of these two bands is dependent on the twisting of the $\beta$-sheets; low degree of twisting implies bands with the same magnitude while highly twisted sheets leads to a 200nm band much stronger than the one at 215nm.

There are two types of CD spectra refering to $\beta$-turns. Figure II.10 shows type II which has absorption bands similar to the $\beta$-sheet but red-shifted for around 5-10nm. Type I $\beta$-turns are also similar to $\alpha$-helices but the band at 208nm is smaller and the band at 190nm has half of the absorption intensity.

The random coils have also two bands, a negative band at 197nm corresponding to the $\pi\pi^*$ transition and a small positive band at 217nm corresponding to the $n\pi^*$ transition.

**II.2.3.5.2. Calculating secondary structures.**

The previous section showed that it is possible to identify the different structures existent in nature by looking at the CD spectra. The problem is that proteins in nature are combinations of the different structures, rather being constituted by one single structure. The resulting CD spectra of measuring globular proteins is then a linear combination of all the structures present. The CD spectrum, $cd(\lambda)$, of a globular protein can be expressed by:
\[ cd(\lambda) = \sum_{k=1}^{N} f_k b_k(\lambda) \]  

(38)

\( f_k \) represents each structural fraction of conformation \( k \), \( N \) is the number of secondary structures present and \( b_k(\lambda) \) is a set of reference CD spectra of known structures based on X-ray analyses. The constrains for \( f_k \) are:

\[ \sum_{k=1}^{N} f_k = 1 \text{ and } f_k \geq 0 \]  

(39)

Based on this linear dependence between structure fractions and spectra several mathematical methods have been proposed for the calculation of the secondary structure of proteins. The methods used in this work are CONTIN, [53] and self consistent method, SELCOM, [54]. These methods were chosen as they better represented the variations in secondary structure represented by the CD spectra measured along this work. Description of the mathematical procedures used to compute the fractions of each structure are described in appendix D.

Much simpler than these mathematical approaches is the estimation of the \( \alpha \)-helical content by looking at a single wavelength by the following relation:

\[ f_H = \frac{[\theta]_{obs} - [\theta]_C}{[\theta]_H - [\theta]_C} \]  

(40)

\([\theta]_{obs}\) is the mean residue ellipticity measured at 222nm, \([\theta]_C\) is the mean residue ellipticity of a complete random coil (+640) and \([\theta]_H\) is the mean residue ellipticity of a complete helix [55]. The last is obtained by:

\[ [\theta]_H = -\frac{42500}{1 - x/n} \]  

(41)

\( x \) is the number of non H-bonded peptide CO groups and \( n \) is the number of residues in a peptide chain.

This method can predict rather good \( \alpha \)-helix contents of an \( \alpha \)-helix->r.coil transition on polypeptides. The disadvantage is that there is no information concerning \( \beta \)-sheets and \( \beta \)-turns.
III Overlooking un/folding properties of major human proteins.

Manipulation of the conformation by changing external parameters (pH, temperature, specific binding, ionic strength, solvent) helps to understand the folding and unfolding process of each protein and later on to relate this knowledge to unfolding when in contact with a solid surface. The exchange of information between a protein and a surface determines the rate of unfolding suffered by this same protein. So it is important to have a clear picture of the different un/folding possibilities adopted in solution prior adsorbing any protein to a surface.

The conformation of the three main blood proteins (Fibrinogen (Fin), Immunoglobulin (IgG) and human Serum Albumin (HSA) - table II.1 and appendix B shows some of the properties of these proteins) was manipulated by changing the solvent composition with the intuition learning about the refolding properties of globular proteins.
Fin, IgG and HSA are the proteins that first adsorb as soon as a foreign material is put in contact with blood (chapter II) so it is rather significant to know their response when subjected to different media, in this case alcohols. Understanding the different conformations adopted in presence of different solvents could help the design of surfaces that maintain the conformation of adsorbed proteins.

III.1 CD to measure the folding of homopeptides

The first section shows the effect of charges and alcohol on the un/folding process of homopeptides.
Polyglutamic acid (PGA) is used as a model. The pK of this homopeptide is 4.25 and the number of amino-acids is 405 to which corresponds a molecular weight of 61200.

III.1.1 The r.coil→α-helix transition.

PGA is known [56] to fold as an α-helix for low pH values and as a random coil for a pH above 10 (see graph III.1).
At a pH above 10 there is high repulsion between the negatively charged side groups -COO\(^{-}\) from the peptide and -OH\(^{-}\) in solution and consequently the peptide forms a r.coil. The peptide is stabilized by the high entropic effects resulting from repulsion and H-bondings with water (figure III.1). For low pH values the -COO\(^{-}\) side group becomes protonated overcoming the repulsion between the positively charged amino groups from the peptide. Internal H-bondings are favored to increase stability. Peptide folds as a helix with the -COOH groups linked to water by H-bondings.
Graph III.1 – CD spectra of polyglutamic acid for pH 2 (—) and pH 11.5 (—) with a solution concentration of $1.6 \times 10^{-5}$. The structures represent an α-helix and a r.coil, respectively.

\[
\text{NH}_3^+ - R - N - R - N - COO^- \\
\]

**pH 2:** helix stabilized by H-bonds in the peptide backbone and H-bondings between water and COOH.

\[
\text{H}_2\text{N} - R - N - R - N - COOH \\
\]

**pH 11.2:** random coil stabilized by high degree of conformational entropy and H-bondings with water.

Figure III.1 – Difference between chains after pH change. At pH 2 there is excess of [H⁺] so \( R = -\text{CH2-CH2-COOH} \) and at pH 11.2 there is a lack of [H⁺] so \( R^- = \text{CH2-CH2-COO}^- \).
The same results were obtained with polylysine which is a positively charged polypeptide at low pH values with the following R group: -CH2-CH2-CH2-CH2-NH3+. This peptide folds as a helix for pH 11 and is a random coil for pH 2. The charged environment is responsible again for the transition α↔r.coil.

This set of experiments show examples of two structures, usually found in nature, where manipulation by electrostatic forces decided the adopted conformation. By itself a homopeptide does not favor the formation of a helix dipole which leads to the stabilization of the α-helix (figure II.1). This requires negatively and positively charged amino-acids at opposite ends of the peptide chain.

III.1.2 Folding of peptides by alcohols.

We have seen in the previous section the impact of charges on folding of peptides into α-helices. Alcohols are also known to be capable of inducing such changes by a mechanism not fully understood. They can be strong proton donors and as a result they work as acid compounds. It is important to know then to which extent the acid effect of these alcohols can influence the folding of the previous peptides.

We chose three different alcohols to work with different acid properties, ethanol<n-propanol<TFE (TriFluorEthanol). Titration curves of PGA solution at pH 11.2 with different amounts of TFE are shown in graph III.2. The pH was maintained constant for the different set of experiments at 11.2 where PGA assumes the conformation of a r.coil.

Graph III.2 - TFE dependent conformational transition of PGA measured by CD at pH 11.2. The percentages are given by volume.

The graph shows a two-state transition, r.coil→α-helix, as the CD spectra has an isodichroic point at 204nm. There is a critical concentration corresponding to the conformational transition at 70→75% v/v sol TFE, for a high content of this compound. At this percentage we have a change on the environment that surrounds the polypeptide, from water we change to an alcohol phase. The overall pH of the solution was maintained constant but if on one side TFE
molecules have preferential attraction to the peptide the local pH changes and the peptide would fold by a local acid-folding effect. On the other hand we have the desolvation effect of the backbone by the increased amount of TFE molecules in solution. Water molecules are released and the polypeptide forms internal H-bonds of similar strength to water-peptide. PGA then folds into an α-helix stabilized by the high degree of weak interactions and water increases in entropy by the release of structured water from the polypeptide. Weaker proton donors than TFE are ethanol and n-propanol. So it was proceeded with the titration of the solution with different amounts of these alcohols (graph III.3) to check if the folding into α-helix was due to the local acid effect. If so larger amounts of both alcohols should be needed to introduce the α-helical structure.

Graph III.3 – Graph a) and b) plots the titration of PGA solution with different percentages of ethanol and n-propanol, respectively. Graph c) plots the ellipticity at 222nm as a function of volume of TFE, n-propanol and ethanol.
Again it was observed the same isodichroic point at 204nm due to the two-state transition. But this time the critical concentrations at which occurred the r.coil→α transition for each alcohol was different from TFE. Ethanol became the most effective alcohol as at 60% v/v$_{soi}$ we had already a helix while n-propanol needed 85% for the transition. This point is easier seen by graph III.3C which shows clearly at which alcohol percentage occurs the phase transition.

Acidity is not then the main factor for the observed transition as we obtained an order of folding as ethanol > TFE > n-propanol. For higher concentrations (>85%) of any alcohol the difference between TFE and these two alcohols is regarding stability, as the α-helix is maintained for TFE and unfolds and precipitates for any of the others.

Observing the chemical formulas of the different alcohols and -R groups of the peptides (figure III.2) than we can advance an explanation for the faster folding of ethanol when compared to TFE. Ethanol and TFE have approximately the same chain length of PGA -R groups what facilitates alcohol-peptide side chain interactions. The molecular difference then between these two alcohols is on the end group, -CH$_3$ and -CF$_3$, which per se causes already higher affinity from ethanol to the R group of the peptide than TFE. As -CF$_3$ is bulkier it can also be sterically repelled by the side chains. After neutralizing the -COO$^-$ group repulsion won't play any more an important role and the peptide stabilizes by forming internal H-bonds. We are assuming that the alcohol molecule is oriented with the hydrophobic tail towards the peptide backbone.

The same experiments were performed on polylysine at pH 5.2. At this pH the peptide has a r.coil structure. Titration of polylysine solution showed that TFE was the most effective alcohol for α-helix inducing. It was needed 80% TFE to induce the transition against 90% ethanol and n-propanol. As was discussed in the previous section an acid environment makes this peptide to fold with a r.coil rather than α-helix. Acidity from the alcohol molecules then cannot play a role in the r.coil→α transition of poly-L-lysine otherwise it wouldn’t be possible to obtain an α-helix.

![Chemical formulas of the different alcohols used and -R group of polylysine and polyglutamic acid.](image)

One rather interesting experiment done by Arankumar et al. [57] was the conversion of poly-L-lysine from an α-helix into a β-sheet by heating the solution to 50°C. Then they titrated the solution with TFE and ethanol. 5% TFE immediately reconverted the peptide back into an α-helix while ethanol wasn’t able to reconvert the peptide at any concentration. They didn’t present any explanation for the observation.
This set of experiments showed that folding by alcohols is independent from the acidity scale of the alcohols as the acidity series was not respected and acid peptides are also folded into \( \alpha \)-helices. On the other hand folding wasn’t specific, as high concentration from any of the alcohols was needed to be able to perform a transition into a non-native conformation. At this high alcohol concentration the peptides are solubilized in alcohol rather than water. The desolvation of the backbone will require stabilization by internal H-bonds. This is entropically unfavorable as the random coil structure is also stabilized by the high entropy of the peptide but it is entropically favorable for water.

TFE was more effective than any of the two alcohols used, except for poly-L-glutamic acid where steric hindrance was important showing that alcohol-peptide interaction can play a role. TFE was also the strongest hydrophobic alcohol. Arankummar’s results showed exactly the strong hydrophobic power of TFE compared to ethanol as a \( \beta \)-sheet offers a strong hydrophobic site for the alcohol to bind.

### III.2 HSA and Fin in TFE

In the previous experiments it was shown that alcohols are molecules with the ability to induce helicity non-specifically in peptides, however one needs high alcohol concentrations, \(~80\%\), to observe a change. To test the capabilities of renaturation of human proteins by TFE we used HSA, Fin and IgG. They are globular proteins with 70\%, 30\% and 0\% helical structure, respectively.

To perform the next experiments unfolded forms of HSA and Fin are needed. IgG has already no \( \alpha \)-helix in it structure so no need for heat-denaturation. By heating up the solution of these proteins to 80\(^\circ\)C it was then obtained the denaturated forms of these two proteins. The formation of helices is an enthalpy-driven process, so unfolding would occur with temperature increase. The denaturated and native solutions were then treated with TFE (graph III.4b and III.5).

The unfolding path of HSA was followed by looking to changes in ellipticity at 222nm (\( \theta_{222} \)) (graph 4a)). After heating the solution to 80\(^\circ\)C the \( \theta_{222} \) value observed for the native state decreased due to unfolding. If we cool down the solution slowly we observe the final state is not native but the protein has refolded 85\% of it’s structure. If the cooling down was done fast than we obtain an unfolded state near the one at 80\(^\circ\)C with 40\% of native structure lost. It was this unfolded state that we treated with TFE. In parallel we also added TFE to native HSA. The helicity increased in both solutions over the all range TFE added but differently. If we look at the unfolded protein we are able to recover the ellipticity value that matched the native for ~30\% of TFE. On the other hand 30\% TFE induced only ~8\% helix on the native protein. When comparing the \( \theta_{222} \) curves of native and denatured state we see that the denatured state folds faster and above the maximum in ellipticity obtained by the native structure. This indicates that the native structure is more stable and “resistant” to the “TFE effect” than the denatured state, due to a different tertiary structure. We have to have in mind that heating weakens not only the local interactions (due to nearby amino acids) but also the non-local interactions (due to distant amino acids) that stabilize the globular structure of proteins. So a resistance to TFE should be expected from the native state as the denatured state is represented by a more “open” solvated structure and is thus more sensitive to TFE.
Graph III.4 - a) CD intensity versus time representing denaturation of HSA by heating the solution from 23°C to 80°C and slowly cooling down back to 23°C. The blue curve (—) is HSA in phosphate buffer and the red curve (—) is HSA in 30% TFE.

b) CD signal (T=23°C) versus TFE concentration representing native HSA folding properties by addition of TFE (—●—) and the refolding of HSA upon addition of TFE to its denatured conformation obtained after quick cooling of a heat denatured sample (—□—).

It was seen that a TFE environment recovers the α-helical state of HSA. So the next question was concerning the blocking of HSA against denaturation. To check the resistance of unfolding of HSA with TFE we heated up a solution of native HSA with 30% TFE to 80°C and cooled it down to 23°C (graph III.4a).

The percentage of unfolding with TFE at 80°C was ~20% smaller than without TFE. At 23°C the refolded molecule had a $\theta_{222}$ only 4% smaller than in the beginning. We had a capping from the TFE molecules to unfolding of the protein. The unfolding paths followed by both proteins were nevertheless different. The small peak, due to folding, appearing at 7 min heating was felt stronger by HSA with TFE. This folding peak is due to hydrophobic interactions which are enlarged by the presence of TFE. When cooling down HSA treated with TFE refolded gradually into almost native state.

From these results we can say that TFE blocks denaturation of the proteins.

The same tests were performed with Fin: addition of TFE to Fibrinogen's native state showed an increased $\theta_{222}$ (graph III.5a); the denatured state didn't recover in structure into the native conformation (graph III.5b). It is important to have in mind that heat-denaturation is a harsh process applied to the structure of proteins and that irreversible forms can be derived from this process.

If the amount of secondary structure given by the spectra at 60% in v/vsol is calculated one obtains ~25% α-helix. This shows that TFE can only stabilize the α-helical structure in Fin. Fin has only ~30% content α in the native structure (table II.1).
Graph III.5 – $[\theta]$ versus TFE concentration and wavelength for Fib upon addition of TFE to its native secondary structure - graph a) - and denaturated secondary structure - graph b).

III.3 IgG in TFE

Titration of the IgG protein solution allowed radical changes in its native conformation (Graph III.6a). From a $\beta$-sheet it has changed to an $\alpha$-helix. The helix was stabilized for values above 45% in TFE. Nevertheless the $\alpha$-helix induction was specific. Contrarily to the polypeptides this protein has an intrinsic tendency to fold as a helix, dictated by the amino-acid sequence which is typical for $\alpha$-helices. Adding 50% TFE to an IgG solution can then stabilize intermediate $\alpha$-helical structures observed on the early stages of folding. The folding of a protein is thought to begin by clustering of the hydrophobic amino-acids and by a statistical preferential formation of turns and helices [31, 58-60]. These structures are formed in different regions inside the protein and are rather unstable, exchanging permanently with the unfolded state. They are stabilized by short-range interactions performed by nearby amino-acids. After the formation of these structured local microdomains they approach each other by diffusion and collision. This approach is going to influence the final secondary structure remaining and $\alpha$-helices can refold into $\beta$-sheets. The global secondary structure of the protein is formed, which is already globular but non-compact. Long range interactions start to play a role. The secondary structure formation has been found to be rapid and the rate-limiting step is the coalescence of the secondary structure into the native compact form of the IgG protein.

The stability of IgG intermediates was checked against dilution and interestingly the structure refolded back to almost native secondary structure (graph III.6b). TFE was then not able to induce an energetically stable helix in this protein. Calculation of the secondary structure percentages are tabulated in chapter VI, pag.82. Possibly this refolding path is a simulation of the folding process undertaken by IgG when building up its native structure, it starts by forming the $\alpha$-helices which coalesce into $\beta$-sheets!
III Overlooking un/folding properties of major Human Proteins

Graph III.6 – Graph a) represents TFE dependent conformational transition of IgG measured by CD at pH 7.3 while on graph b) we have plotted the effect of diluting a solution containing 50% TFE on the secondary structure.

From these results we can conclude that TFE only induces stable α-helices in proteins where it is energetically favorable, considering the interactions within the native protein.

III.4 The effect of other alcohols.

The comparison between TFE helix induction effect and other alcohols is now required to try to find out the influence of fluoro atoms on the folding properties of HSA and IgG. Previously was shown that ethanol can perform the r.coil→α-helix transition in peptides for high alcohol concentrations but is not able to induce β-sheet→α-helix transition. Contrarily TFE can perform both transitions.

In graph III.7 is plotted the ellipticity changes against maximal %v/v of alcohol added to an IgG solution without precipitation of the protein and secondary structures calculated from each curve is given as a table. IgG solution was titrated with ethanol, n-propanol and 1-fluoroethanol. None of these alcohols was able to quantitatively transform the β-sheet conformation into a complete α-helix, the maximal amount of helix introduced was 16% by 1-fluoroethanol. Direct analyses of the graphs show that the sheets changed conformation as the original peak for an IgG solution in buffer at 217nm shifted. This is special for twisted β-sheets and α/β proteins where we can find present on the same chain for example areas with β-α-β structure. From the results it is deduced the necessity of at least three Fluorine atoms to induce a complete β→α transition.
Graph III.7 - Alcohol dependent conformational transition of IgG measured by CD at pH 7.3. The percentages are given by volume. The table represents the secondary structure percentages of IgG after titration the solution with different solvents.

<table>
<thead>
<tr>
<th>% secondary structure</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>r.coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG in Buffer</td>
<td>0</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>IgG 60%v/vsol Ethanol</td>
<td>9</td>
<td>37</td>
<td>53</td>
</tr>
<tr>
<td>IgG 50%v/vsol n-propanol</td>
<td>1</td>
<td>52</td>
<td>38</td>
</tr>
<tr>
<td>IgG 75%v/vsol 1-Fluoroethanol</td>
<td>16</td>
<td>42</td>
<td>41</td>
</tr>
</tbody>
</table>

Increased stability for lower concentrations of alcohol is achieved whenever fluorine is introduced into the alcohol molecular structure. It would be then important to look to other fluorinated alcohols.

To compare effects from fluorinated alcohols HSA solutions, native and denatured, were titrated with 1-fluoroethanol and hexafluoroisopropanol (IsoF).

The first alcohol was not able to induce a recovery from the denatured state into a native state in terms of secondary structure. Again we need –CF$_3$ groups for the transition. The second alcohol in fact induced an α-helix but introduced new changes in the secondary structure of the protein. If we qualitatively analyze graph III.8a and III.8b then we see that a β-sheet structure appeared on the global structure of the protein, as an intermediate step to the recovery of HSA. This is seen by the reduction of the peak at 209nm. Maybe this β-structure is one intermediate on the folding process of globular HSA.

The “power” of fluorine atoms was too strong and the protein refolded with a structure that is stabilized by hydrophobic forces. Until now only one example came in literature where there was the possibility of stabilization of a β-sheet structure by fluoroalcohols. In general alcohols help to induce the α-helix [62].
Graph III.8 – Variation in secondary structure of native (nn) and denatured (dd) HSA solution after titration with IsoF given by CD spectra, curves a) and b), and calculated by SELCOM algorithm, curve c). The denaturation was performed at 80°C.

These results are rather interesting as there is the stabilization of a new structure, the β-sheet, for low alcohol concentrations, 10% v/v sol IsoF, (10%v/v sol IsoF represents 50 molecules of water per each molecule of IsoF). In general the stabilization of β-sheet structures is done through hydrophobic interactions and intermolecular H-bonds. So 10-30 % IsoF has to provide the hydrophobic environment necessary that two backbone chains can diffuse and interact. This could be done by direct interaction of IsoF molecules with the amino-acids from HSA or by creating the “right” hydration shell around the peptide which orients backbones parallel to each other. 43%v/v sol refolds the structure back to α-helix. In this situation we have one alcohol molecule per each 10 water molecules in solution so dehydration from the protein is very probable. Each water molecule binds to other 4 [63] to form a cage around a hydrophobe solute so as there are two apolar groups (-CF₃) then all water molecules would be involved in the solubilization of IsoF. This is not possible or the protein would precipitate. It is necessary then to take in account clustering of IsoF molecules and/or direct interaction of IsoF molecules with the protein to observe folding of HSA into an α-helix without precipitation of the protein.

The motivation for all these tests was the search for agents capable of recover the secondary structure of globular proteins. It was seen that there is a match between the structure of denatured and native HSA by the use of ~30% TFE. 1-Fluoroethanol was not able to recover HSA to any percentage of this alcohol and on the other side IsoF, with two –CF₃ groups, introduced new structures on globular HSA by stabilizing an intermediate β-sheet for 10-30% v/v sol of this compound. This could mean that there is in fact an optimum number of –CF₃ groups for stabilization of secondary structures. Fib at 30% alcohol was not able to recover its complete secondary structure but it presented already 30% helix which is the same
III. Overlooking un/folding properties of major Human Proteins

value as native Fib. IgG changed its β-structure into an α-helix by TFE. The changes were reversible and the protein recovered almost its initial secondary structure.

The next problem that comes from these results is an interpretation of the mechanism by which proteins change conformation.

III.5 The mechanism of α-helix induction by TFE.

It was seen that TFE is a special molecule capable of inducing α-helices in globular proteins. A complete structural recover from denaturated HSA, a typical helical protein, to native HSA was obtained for ~30% TFE. IgG, a β-sheet protein, showed a total conformational transition into α-helix for ~45% TFE. It is now required to understand the possible mechanism behind these transitions.

III.5.1 Physical-chemical behavior of TFE-water mixtures.

The physical-chemical properties of alcohol-water mixtures are rather important as to obtain information on the structural properties of these two compounds when mixed at different ratios. These characteristics will influence solubility, diffusion constants, size, energy and structure of proteins. It will be tried to use the macroscopic behavior of the mixture to infer the microscopic interaction between protein and alcohol.

III.5.1.1 Viscosity, density and Surface Tension.

In graph III.9 we have plotted the variation in viscosity and density against %v/v_{sol} of alcohol. By observation of the graphs we see that viscosity increases until a maximum of 1.89mPa.s is reached at 50% TFE and then remains approximately constant at the value of pure TFE (1.8mPa.s). Ethanol reaches its maximum of 2.3 for 40% ethanol and then decreases fast to 1.06mPa.s.

The maximum viscosity for ethanol has no impact in the observed folding properties of any of the proteins used. Rather opposite, the region of the TFE maximum viscosity is the region where IgG folds into helices and where Fin/HSA have a steep increase in helicity (graph III.4, III.5 and III.7). The viscosity value observed at 80% ethanol and the one observed at 80% TFE correlates well with the folding of peptides observed for high amounts of these alcohols in solution (graph III.2 and III.3). At this point the number of water molecules is rather reduced (1 H2O : 1.23 Ethanol and 1 H2O : 1 TFE molecules) so there are many free uncompensated backbone –NH and -C=O groups which finally bind as the stabilizing energy given by the α-helix at these concentrations is large enough to overcome repulsion.
Graph III.9 — Physical-chemical properties of water–alcohol mixtures at $T=23^\circ C$. In graph a) we have plotted the variation in viscosity for ethanol/water [64] and TFE/water, TFE/buffer mixtures and density for TFE/buffer solutions. In graph b) it is represented the surface tension of different alcohol/water solutions against concentration (M). For TFE/water mixtures it is shown the percentage in volume at which the alcohol starts to aggregate.

The surface tension, $\gamma$, (graph III.9b) variation with alcohol concentration shows that both alcohols again have a distinct behavior when mixed with water. $\gamma$ for TFE/water solutions showed typical micelle formation after 30% v/vol as $\gamma$ is maintained constant. Micelles are not possible to be formed by TFE due to the small chain length of this molecule, so rather than micelles TFE could form alcohol clusters in solution. The appearance of these clusters occurs for values higher than 30% v/vol, again on the region of high impact from this alcohol in the tested proteins.

In the low alcohol region for TFE and for the whole range of alcohol concentrations for ethanol, the curves show a preferential exclusion of both alcohols from the water environment: accordingly with the Gibbs equation (chapter II) the surface excess for TFE and ethanol increases with concentration. TFE has nevertheless a greater impact on the surface tension than ethanol, small amounts decrease $\gamma$ faster.

III.5.2 FTIR on water/TFE solutions.

The previous results show that TFE/water mixtures have a rather interesting behavior leading to the formation of alcohol clusters. To further analyze the solution IR was used to follow
variations on the vibrational bands of water and TFE molecules. The spectra of pure TFE was analyzed having as reference published IR data on this molecule [65-67].

H-O-H has it’s bending vibrational band (v2) at 1636 cm$^{-1}$. Shifts on the peak position of this band are a measure on the variation of the H-bond strength. This band doesn’t overlap with any band from the alcohol so it is a good reference to interpret the water structure. Graph III.10 shows that this peak doesn’t change position for any of the alcohol/water concentrations used. In other words water molecules are basically bond to each other independently of how much alcohol is in solution. Mizuno et al [68] performed the same experiments with other halogenoalcohol-water mixtures (X-C$_2$H$_4$OH, X = BR, Cl, I) and found a red shift of the water bands for the whole range of concentrations. This shift is the result of the breakdown of water structure with the order Cl< Br< I. According to this result it is very probable that TFE causes no shift on the v2 of water as it is first on the periodic table, and thus there is no breakdown of the water structure.

One point that should also be discussed from this spectra is related to the width of the bands at half-maximum. With the increase in alcohol concentration the band becomes less symmetrical which means that H-bonds strength distribution is changing. This is due to the fact that water molecules around –CF$_3$ and –OH groups form a different H-bond network: the higher is the alcohol content the lower is the number of water molecules in solution and the more prominent becomes the water structuring effects around alcohol clusters.

Analysing then the –CH$_2$– vibrations (graph III.10c), symmetric and asymmetric, one observes a blue shift towards the –CH$_3$– vibrations from pure alcohol. A blue shift represents an increase on the electron density of the –C-H bond which is the result of the increase of the number of molecules in the alcohol clusters.

In graph III.10b it is plotted the -OH stretching region. Pure TFE is known to exist in a gauche conformation [66]. This conformation allows the formation of an intramolecular dipole interaction between the O-H--F- which is determined by the stretching band at 3633 cm$^{-1}$. After TFE is mixed with water a new band appears at 3664 cm$^{-1}$, shifting to higher frequencies with alcohol concentration and increasing in intensity. This new band is attributed to non-bonded –OH groups from the alcohol rather than from the water. This fact is because, as was said before, the structure of water is maintained for all range of alcohol concentrations. If the number of non-bonded water molecules was increasing in the solution than a red shift due to the weakening of the H-bonds in water should have been observed.

From FTIR results it is shown that alcohol molecules tend to aggregate in the whole range of alcohol concentrations used. On the other hand the water H-bonds strength remain as in bulk water assuming that when alcohol and water coexist in solution they form small aggregates of both compounds.
Graph III.10 – FTIR spectra of TFE/water solutions. Graph a shows the H-O-H bending vibrational bands; graph b the typical stretching vibrational region for non bonded –OH groups, graph c the –CH2- assymetric and symetric stretching region of TFE.

III.5.3 X-Ray diffraction studies

To check for clustering of alcohol molecules X-ray studies in volume phase were performed for TFE/water mixtures. In the low-angle region no diffraction was observed. The results for the wide-angle region are plotted in graph III.11. One observes a broad peak existing only for TFE concentrations above 25% and the maximum shifts slightly to lower s for higher concentrations.

From this results it is clearly seen that TFE molecules aggregate in solution. The measured repeat distance d (s = 1/d) calculated at the maximum-half width was of ~18, 20, 23Å for 50, 75 and 100% TFE. The van der Waals radius of the –CF3 group is ~5.6Å so the number of molecules involved in clustering is 3-4, in a one dimension system. 25% of alcohol forms no
aggregates. These results agree well with the surface tension measurements which show a kind of micellar formation only for v/v_{sol} > 30%.

Graph III.11 – X-ray diffraction in volume phase for TFE/water solutions.

III.5.4 What can be the possible reason for the observed aggregation?

From the physical-chemical behavior of the solutions we can see that there are distinct regions for the alcohol/water solutions: a region of low alcohol concentration where the mixture is homogeneous and a region of higher alcohol concentration, for TFE > 30% v/v_{sol}, where the TFE molecules start to aggregate giving origin to heterogeneities.

In figure III.1 gives a schematic representation of the water/TFE mixing properties accordingly to the results obtained above. In the low alcohol concentration region the structure of the water will be disrupted in the vicinity of the alcohol molecules. It is known that water forms "cages" around the solutes when dissolving them [63]. This coordination layer is rather oriented in order to maximize the H-bonds of water around the solutes and to orient charges towards the solution to form further H-bonds with the bulk water. Nevertheless the entropy of water remains approximately the same; the decrease in entropy due to the orientation of water molecules around the solutes is compensated by the increase in entropy of free water molecules. The same happens with the enthalpy, so the overall free energy of the system remains approximately constant. This phenomenon is called the hydrophobic effect [63,69-71]. In the high alcohol concentration region the coordination layers start to overlap and the molecules of alcohol interact with each other via strong dipolar interactions between fluorine groups. On the other hand the aggregation of the alcohol allows the formation of areas rich in water molecules by the release of structured water, a situation thermodynamically favorable. This phenomenon is called the hydrophobic interaction. It was shown in graphs III.9-11 a deviation from the linearity on the behavior of the properties of the mixtures and an increase in scattering by the solution when increasing the alcohol concentration due to alcohol clusters in solution. This aggregation is a result of the hydrophobic interaction.
III.5.5 The mechanism of α-helix stabilization.

Proteins are surrounded by three different types of layers of water; a first layer where molecules are strongly bound and oriented, a second layer where the water molecules are more loosely bound and a third layer in permanent exchange with water which resembles bulk water [69,72,73]. Now the TFE solvent is added to the protein-buffer solution. Comparing figure III.3 with the behavior of proteins in buffer/TFE solutions it is possible to establish a relation:

Until 30% the change in helicity of native HSA (graph III.4b) is rather small - region A; beyond 30% the change becomes more pronounced driving the folding of the protein to values above that of the native state - region B and C. With denatured HSA there is also region A until ~30% TFE and a steeper increase in the helix values beyond 30% TFE - region B and C. Graph III.5 shows a similar effect for Fin. Looking at the total disruption of the IgG structure this is completed at ~45% vol/vsol (graph III.6a) - region C. Until 25% - region A - there is in fact no disturbance of the secondary structure. The breakdown of the IgG structure begins with 40% TFE, from region B to C.

There are several works published [74-76] where the authors state a β→α-helix transition by TFE at ~40% alcohol. Looking to this results it is when actually the hydrophobic interaction region in solution approximately has begun that the major transitions are observed on the proteins. Ethanol was not able to perform this transition as its hydrophobic interaction isn’t strong enough (no clustering has been observed for ethanol [77,78]).

The relation between water/TFE properties and protein folding may be explained by the following mechanism (figure III.4): in region A when adding TFE to water-protein solutions there will be competition between alcohol and protein for water molecules. If protein releases
water from the coordination layer then the –NH and -C=O groups of the backbone are free to interact with each other. The protein will fold by forming internal H-bonds. The released water will be then involved in the solubility of the alcohol. When 30% of alcohol is reached only \( \text{X} \) molecules of water are present per each molecule of alcohol and protein. This is **region B**. This is the transition region between free and clustered alcohol, where the folding rate of the protein is also changing. When 40% is reached the alcohol concentration is rather high – **region C**. To maintain the bulk water structure, seen by FTIR, the refolding of protein should proceed at a higher rate. The release of water from the protein is this time contributing to the entropy of water. Proteins like the alcohol can aggregate. In **region D** the alcohol concentration is too high. Nevertheless until 95% TFE it is possible to solubilize the protein. It is clear that at this high alcohol concentrations the proteins have to aggregate due to the incapability of forming enough intermolecular H-bonds, as the amount of water is too reduced to hydrate the protein.

It is not possible to exclude binding of alcohol molecules to the protein: proteins offer lots of binding sites for alcohol molecules as their structure possesses lots of hydrophobic amino-acids in their interior core. Denaturation will expose these amino-acids to water/TFE mixtures (graph III.4) which can offer a binding site to an alcohol molecule excluded from the water environment. Native proteins have the hydrophobic core buried inside the protein so the interaction between TFE-protein wont play a role. It will be shown further on that TFE in fact likes to be in the vicinity of the protein for adsorbed denaturated proteins (chapter IV). In the case of the homopeptides figure III.3 doesn’t hold. The introduction of helicity in these large macromolecules is a non-specific effect; they do not possess a native helical structure dictated by the amino-acid sequence like HSA, IgG and Fin. As a result there are two contributions to the energetic barrier necessary to overcome in order to observe folding: the entropy of the side-chains and the high expulsion between charged amino-acids.

**Figure III.4** – **Schematic representation of TFE/water mixtures interacting with proteins.**
III.6 Conclusions

It was shown that homopeptides fold from r coil $\rightarrow$ $\alpha$-helices by non-specific alcohol induction. This transition isn't due to an acid effect but to a change in the environment surrounding the peptide as it is observed for high alcohol concentrations (75-85%). When using the same alcohols in globular proteins the effects become specific as is induced an intrinsic structure from the proteins, the $\alpha$-helix. Denaturated and native protein structures are changed by IsoF, TFE and 1-Flouroethanol.

Fin didn't recover the complete secondary structure by the use of any alcohol. This protein has 30% $\beta$-sheet structure which is usually destroyed by the presence of fluorinated alcohols. IgG underwent a $\beta$$\rightarrow$$\alpha$-helix transition only with TFE which none of the other alcohols was able to perform. The new formed $\alpha$-helix was reversible against dilution. 

~30% TFE and 50% IsoF induced a secondary structure in denaturated HSA that matched the native structure. The secondary structure formed by 30% TFE in HSA has proved to be stable against dilution. It was expected that IsoF would be a strong $\alpha$-helix inducer but in fact small amounts of IsoF stabilized an intermediate $\beta$-sheet structure. These results suggested an optimum hydrophobic environment to induce $\beta$-sheets in HSA. On the other side it will be discussed in chapter VII peptides which have strong hydrophobic regions known to underwent $\alpha$$\rightarrow$$\beta$ transformations in the blood by unknown processes.

The physico-chemical behavior of the water-TFE mixtures showed a strong correlation with the observed folding transitions (figure III.1) so it was possible to divide the TFE effect in four regions. In the region where clustering from alcohol molecules was observed IgG folded as an $\alpha$-helix, HSA and Fin increased the rate of refolding.

Correlation of physical-chemical properties and folding suggested a mechanism of $\alpha$-helix induction by TFE: changes in the protein solvation layer, indirectly induced by restructuring of water around $-\text{CF}_3$ groups from TFE. Direct binding of alcohol molecules can not at this point be excluded from the mechanism as there are lots of hydrophobic amino-acids to which proteins can bind specially in heat denaturated proteins.

These results brings up the question how would blood proteins react in contact with a surface covered with TFE? Denaturation of adsorbed proteins has been for years an obstacle to the design of biocompatible materials. By the observed impact of TFE in native structures we would assume the existence of a certain surface coverage with TFE molecules at which no conformational changes should be observed or at which $\alpha$-helical structures of adsorbed proteins are conserved.
IV Surface-protein interactions

Understanding and controlling protein adsorption has been a subject of great interest for many years in different fields. The adsorption of proteins is rather important for biocompatible materials, for biosensing, for drug delivery, as all require that proteins maintain their function. Nevertheless it is known that adsorbed proteins denaturate [79, 81]: denaturation means changes in the structure and as a result loss in functionality. This denaturation is caused by the marginal stability of proteins (see chapter II) which results in a rather small energetic barrier towards the unfolded state. Researchers search for the optimal surface conditions to adsorb proteins without inducing structural changes [82]. If the need is to avoid denaturation then it is necessary to understand surface-protein interactions. With this chapter it will be tried to explain some of the parameters influencing denaturation induced by surfaces on globular proteins. Further on TFE will be used to recover the structure of adsorbed proteins with new consequences for the mechanism of α-helix induction by this alcohol.

IV.1 CD spectroscopy applied to homopeptides.

To study conformational changes of adsorbed proteins is needed a suitable technique that allows to measure in situ conformation of adsorbed proteins. CD has been used mainly to study conformational transitions of proteins in solution [83-85]. To test the sensitivity of CD towards one monolayer of adsorbed molecules PGA was used as a model.

PGA was spread at the air-water interface in a Langmuir-Blodgett Trough, with the bulk phase at pH 2. It was shown in chapter III that at this pH the peptide adopts the conformation of an α-helix. The film was compressed until a pressure of 14.7mN/m was reached (isotherm is shown as in graph IV.1a). At this pressure the film was deposited in a quartz slide. After drying, the slide was placed in a sample holder specially designed to measure CD (chapter II). The results are plotted in graph IV.1.

Graph IV.1 – Graph a) shows the LB isotherm of PGA. In graph b) it is plotted the CD signal of the PGA monolayer deposited on a quartz slide at 14.7mN/m and PGA dissolved in 87% TFE.
By analysis of the CD spectra it is clear that the major structure represented is a helix with the two minima at 222 and 206nm and the maximum at 195nm. Calculating the fraction of α-helix in deposited PGA by equation 40 we obtained a value of 66% against 86% from solution, so we lose helicity in the film. This is clearly seen by the reduction in intensity from the band at 222nm. This reduction can be the result of introduction of some β-sheet structure in the system. Schladitz made IRRAS studies with PGA monolayers and showed that the major secondary structure component in the monolayer is the α-helix but some β structure is also present.

The area/molecule of the deposited monolayer was around 4860 [Å²], 40% smaller than the area if PGA forms an ideal α-helix in the extended position with all the amino-acids contributing to helix formation. So it is clear that not all amino-acids are contributing and some other structures are present; the β-sheet which connects side chains and the random coil. The β-sheet formation can be the result of compression which squeezes water molecules out of the monolayer and forces the approach of peptide backbones. Finally deposition can also induce conformational changes by substrate-protein interaction.

IV.2 Conformational transitions of adsorbed globular proteins onto hydrophilic and hydrophobic surfaces.

In section IV.1 it was shown that it is possible to measure the conformation of a deposited monolayer of homopeptides. So the next step is to use CD as the technique to measure conformational changes of adsorbed globular proteins. The biggest advantage of this technique related to FTIR is the exclusion of effects from water which disturb greatly FTIR measurements as what we measure is momentum transitions of chiral molecules. The disadvantage is concerning information relating tertiary structural changes of adsorbed proteins.

HSA, IgG and Fin with initial concentrations of 1*10⁻⁶M were adsorbed onto clean quartz slides for 30 minutes.

The CD spectra of the adsorbed monolayer is registered in graph IV.2a and the CD spectra of proteins in solution is seen in graph IV.2b.

By comparison of both graphs it is shown that the major structural components of each protein are retained in accordance with what is said in general about hydrophilic surfaces, low conformational changes. This is seen by the small reduction in [θ] values from adsorbed proteins.

With this experiment it is shown that it is also possible to measure the CD spectrum of monolayers of adsorbed globular proteins and quantify changes in conformation.
Graph IV.2 – HSA, IgG and Fin adsorbed on a quartz slide for 30 min (a) and in solution (b). All spectra are normalized by concentration and adsorbed amounts.

IV.2.1 CD can detect multilayers

Proteins are described as to adsorb with a first layer irreversibly bond to the surface and with a second layer in exchange with solution [10,79]. The layer in contact with substrate is supposed to suffer the major conformational changes dependent on substrate type. CD was used to test the possibility of measuring different conformations in adsorbed layers. Quartz was used again as a substrate model.

HSA, IgG and Fin were adsorbed for two hours into clean quartz slides (B), after they were thoroughly rinsed (C) and inserted into buffer for 24h (D). In a new experiment the proteins were adsorbed for 24h (E) and then rinsed (F). After each step the CD spectra were taken. The variation in the secondary structure of the adsorbed proteins is shown in the bar graph below and adsorbed amount variations measured by UV are given in table IV.1.

<table>
<thead>
<tr>
<th>Adsorption/Desorption steps</th>
<th>HSA (mg/m²)</th>
<th>IgG (mg/m²)</th>
<th>Fin (mg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B - 2h adsorp.</td>
<td>2.2</td>
<td>2.1</td>
<td>11.5</td>
</tr>
<tr>
<td>C - Rinsing 1</td>
<td>1.2</td>
<td>2.1</td>
<td>6.0</td>
</tr>
<tr>
<td>D - 24h buffet.</td>
<td>1.2</td>
<td>2.1</td>
<td>5.5</td>
</tr>
<tr>
<td>E - 24h adsorp.</td>
<td>4.2</td>
<td>2.6</td>
<td>20.4</td>
</tr>
<tr>
<td>F - Rinsing 2</td>
<td>2.7</td>
<td>2.1</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table IV.1 – Variation in the adsorbed amount of HSA, IgG and Fin calculated by UV spectroscopy after each CD measurement (values for two monolayers, one each side of the quartz slide). Irreversibility in this set of experiments is defined as the layer remaining after the rinsing steps.
Graph IV.3 – Variation of the secondary structure of HSA, IgG and Fin adsorbed onto quartz. The CD spectra were taken after each cycle: adsorption of the proteins in steady-state for 2h without rinsing, rinsing, placing the slide against buffer for 24h, adsorption for the period of 24h, then rinsing. The first two bars represent the structure of protein in solution.

After adsorption IgG loses ~20% of the β-sheet structure in favor of the random coil. This decrease is maintained approximately constant during all steps except for 24h adsorption where the β-sheet structure increases. This increase is due to further adsorption of proteins. After both rinsing steps the adsorbed amount of proteins remaining on the surface is the same showing that after 2h adsorption the monolayer is saturated. The further increase in β-sheet for the 24h step is then due to the new proteins, from the loosely adsorbed layer, on the surface (table IV.1). These new proteins are weakly interacting with proteins already on the surface and thus can be removed by rinsing. Placing the adsorbed film against buffer for 24h introduced only small changes on the adsorbed layer showing that the IgG conformation is rather stable when interacting with quartz.

Fin after 2h adsorption and after performing the rinsing step 1 showed small changes in structure despite ~50% desorption (table IV.1). Structural changes become pronounced when samples are placed against buffer for 24h. The α-helix is reduced ~20% in favor of the β-sheet. This shows that Fin molecules are unfolding with time in the search for a stable conformation or strengthening lateral interactions as mobility on the surface will lead to collisions. During the 24h adsorption time Fin decreased also by 40% in α-helix content. More molecules are coming to the surface and protein-protein interaction plays an important role in denaturation of Fin. The rinsing step 2 recovered partially the Fin structure showing...
that the loosely bound layer is responsible for the high content of $\beta$-sheets present. In total it is possible to show that there is also more than one monolayer adsorbed shown by the 15% increase in the amount of irreversible bond proteins after the rinsing step 2 (24h) when compared to the rinsing step 1 (2h).

Step C, D and F are not shown for HSA as the very small signal of the adsorbed monolayer after rinsing didn’t allow a reliable measurement. In step B and E HSA showed always loss in helical structure in favor of the $\beta$-sheet for the two adsorption steps, like Fin and IgG. Again the amount of protein in the irreversibly adsorbed layer increased with adsorption time showing that lateral protein-protein interactions are important and help to determine the final conformation of the protein.

Comparing the performance of the three proteins IgG was the most stable on the quartz surface. It has been shown in chapter III that this protein has a rather good resistance to environment, only 45% TFE induce a conformational transition on the protein. IgG is rather important in the recognition process of other proteins and is widely applied in the biosensoring industry which requires stable adsorbed proteins.

The secondary structure of Fin and HSA is more dependent on the amount of adsorbed proteins than IgG’s. The formation of $\beta$-sheet is the preferred structure even in HSA which has no $\beta$-sheets in the native conformation. Nevertheless this protein can form non-native $\beta$-sheets when in contact with 10-30% IsoF (graph III.8). The phenomenon was explained by the reduction in the amount of water molecules from the coordination layer of the proteins to an extent that brings backbone chains together. It is shown in table IV.1 that the adsorbed amount increases from 2h to 24h, approximately to double values for Fin and HSA, so the number of proteins per unit area is increasing. A reduction in entropy from the water molecules of the coordination layer of the adsorbed protein should be expected due to overlapping layers and water molecules should be released to the bulk. The adsorbed layer should be less hydrated than the proteins in the bulk and so the $\beta$-sheet formation should stabilize the proteins. Compressing of a PGA monolayer (graph IV.1a) has shown to introduce the $\beta$-sheet structure in the protein due also to proximity of the backbone chains and squeezing of water out of the monolayer.

From the above observations it is possible to determine:

- Two reasons responsible for the formation of the $\beta$-sheets in adsorbed proteins (figure IV.1): the hydration state of proteins in the bulk and on the surface is different, the concentration/area is higher on the surface obliging backbone structures to interact.
- Two pictures on the origin of the $\beta$-sheets: proteins are desolvating and interact via formation of H-bonds between adjacent chains from two proteins and form $\beta$-sheets; proteins form internal $\beta$-sheets and interact via peripheral amino-acids.

From all the results a two step denaturation process was confirmed: the first occurring between proteins and surface and the second between adsorbed proteins and newly adsorbed ones, forming multilayers. The multilayer adsorption process observed for Fin, HSA and IgG is possible to describe as (figure IV.1): a first layer irreversibly adsorbed which changes with time by influence of the substrate and a second removable layer which is bound to the irreversible layer by $\beta$-sheets.
IV Surface-Protein Interactions

Figure IV.1 – Scheme of interaction between human proteins and a hydrophilic surface. Denaturation of the first adsorbed layer is due to surface–protein interactions. This layer is irreversibly bound. Increase in adsorption time will lead to protein-protein interactions via β-sheet formation and further denaturation.

IV.2.2 CD as a tool to detect variation in adsorbed structures with time.

The results above showed that proteins vary their conformation if adsorbed for 2h or adsorbed for 24h. It was then appealing to follow the denaturation of an irreversibly adsorbed layer with time (graph IV.4). Fin was the chosen protein as it has the highest adsorbed amount and so it is easier to detect changes with CD.

Graph IV.4a plots Fin adsorption isotherm obtained by incubating samples at different adsorption times. Graph IV.4b represents the variation in ellipticity of each sample against time.

By observation of the graph it is seen that the decrease in helical structure accompanies the increase in adsorbed amount. These results are rather interesting as they show that the structure of Fin for very small adsorption times results in α-helix induction. This could be explained by strong changes in the hydration layer of isolated adsorbed proteins. With the increase in adsorption time proteins adapt their structure to the surface and unfold decrease in helicity. When the amount of adsorbed protein increases the compactness on the surface increases and proteins interact also with each other as was shown in figure IV.1.
Graph IV.4 – Graph a) shows the adsorption isotherm of irreversible adsorbed Fin and in graph b) it is plotted the variation in secondary structure for each irreversible adsorbed layer.

IV.2.3 CD as a tool to detect the influence of solution concentration in adsorbed proteins.

Concentration has shown to be rather important in determining the final structure of proteins on the surface (graph IV.4). So at this point it became necessary to look at concentration dependent measurements. Quartz and OTS were used as substrates where Fin was let to adsorb for 30min with different initial concentrations. The variation in secondary structure and the adsorption isotherms are shown in graph IV.5a and IV.5b, respectively.

It is very interesting to notice the difference in behavior of Fin when adsorbed on quartz and when adsorbed on OTS. For high concentrations the α-helix is the preferred structure on OTS and the β-sheet on quartz; for low concentrations the β-sheet is the preferred concentration on OTS and the α-helix on quartz (as was seen in graph IV.4 for low adsorption times).

It is possible to correlate the behavior of Fin to differences in the surface energy and area/molecule. A hydrophobic surface interacts with the protein through hydrophobic forces. With low concentrations this requires higher unfolding of the protein as hydrophobic aminoacids are buried in the interior core of the protein. This is possible as the number of molecules/area is small and protein-protein interactions do not play a role. The result is loss in helicity and increase in β-sheet stabilized by hydrophobic forces. High concentrations means that molecules are interacting with the surface and neighboring proteins. There is not enough space for proteins to unfold and maximize hydrophobic interactions so the release of structured water from the surface to the bulk is entropically favorable for water. This loss in water from the surface implies α-helix stabilization in the protein. A hydrophilic surface is the opposite, low concentrations don’t require strong unfolding of the protein as the hydrophobic core wants to be away from water - α-helix stabilization. High concentrations imply that protein-protein interactions become stronger than surface-protein interactions - β-sheet formation.
Graph IV.5 – The bar graphs show a variation of the secondary structure as a function of the bulk concentration for Fin adsorbed in quartz coated with OTS and in hydrophilized quartz. It is also plotted the adsorption isotherms of the irreversible adsorbed layer (each value for the concentration \( c_1 \) was measured after rinsing the substrate).

This section shows the high sensitivity of CD towards changes of adsorbed proteins: a relation between adsorption times and conformation, and adsorbed amounts and conformation was found; structural changes induced by model hydrophobic and hydrophilic surfaces, with the same surface roughness, could be quantified.

The next chapter will study adsorption of HSA onto thiol and polymer surfaces and will be shown that variation of the chemical nature or roughness of the surface varies the adsorption and conformational behavior of this protein.

### IV.3 Renaturation of adsorbed proteins by TFE.

It was shown that proteins denaturate upon adsorption into model hydrophilic and hydrophobic surfaces. If denaturation doesn’t occur in the first contact times between surface and protein than unfolding will be observed with time. The question now concerns the possibility of recovering these adsorbed denatured proteins. In chapter III it was found that TFE has the possibility to induce helical structure in denatured proteins: 30% TFE recovers denaturated HSA and 60% recovers the \( \alpha \)-helix content in denatured Fin. It is possible to detect if the newly recovered protein has also recovered its function by taking advantage of
the specificity of antibodies towards antigens [86,87]. Antibodies (key) recognize active domains of a protein (lock) (see chapter II). If the structure of these domains is lost the binding should not be observed. So the combination of both informations defines the next experiments.

HSA and Fin were let to adsorb on a quartz slide coated with OTS for 30min. The adsorbed proteins were then denaturated by heating the slide up to 80ºC. The denaturated adsorbed film of proteins was treated for 5min in a solution containing 30% TFE for HSA and 60% for Fin. The antibody key-lock principle was used to detect conformational recovery in the renaturated adsorbed proteins to OTS. The slides were immersed in a solution containing a first antibody responsible for recognizing active domains of a protein. A second antibody marked with an enzyme is then added to the solution. This antibody is specific for the first. The results (graph IV.6) showed that antibody binding was enhanced after treatment of the denaturated films with TFE; ~8% in the case of albumin and in the case of Fin ~15%. By CD it was also seen that in fact there was only partial recovery of the adsorbed proteins. It is necessary to refer that heating the films of adsorbed Fin and HSA provokes denaturation at a level that never occurred with these proteins, in any surface used in the previous section. So as in solution denaturation has been performed too harshly.

It is then demonstrated that not only secondary structure is recovered but also function of the proteins by the use of TFE. These results also show that the refolding induced by TFE occurs in regions of the protein where native structures were observed before unfolding, otherwise binding of antibodies should not occur. Extrapolating these results to solution when adding 30% and 60% TFE in a solution containing denaturated HSA and Fin the newly observed helices were refolded where native helices have been stabilized before.

Graph IV.6 – Antibody binding to adsorbed, denaturated and recovered by TFE HSA and Fin. The ELISA signal is normalized to the observed signal of adsorbed proteins without denaturation.
IV.4 Understanding the effect of TFE in irreversible adsorbed proteins: FRAP.

It was seen in section II that an irreversible film of adsorbed proteins, when placed against buffer for 24h, changes conformation. Proteins unfold with time. They diffuse on the surface without desorption. This is possible if a protein is attached by several weak bonds to the surface. Each bond has the order of magnitude of kT but the sum of the adsorption free energy of all bonds is large [79]. So the probability that all detach at the same time is rather low: desorption does not occur. In this section FRAP (description in chapter II) is used to follow the lateral mobility of irreversibly adsorbed proteins under buffer and under 30% TFE.

It was shown in the previous section that TFE recovers the conformation of adsorbed proteins under rather harsh conditions (heat-denaturation). The motivation was then to learn about the TFE effects in proteins adsorbed on hydrophilic and hydrophobic surfaces. For the following experiments a cell was designed in order to obtain laminar flow with Rey = 12 and ρ(s-1) = 4.5 (see chapter II). In this way all experiments were performed in a diffusional regime.

IV.4.1 FRAP measures mobility.

HSA-FITC labeled is let to adsorb for 2h on a quartz surface. After adsorption buffer is flown through the system to remove the weakly bound proteins and so the irreversible adsorbed layer remains. At this point a pattern is bleached with strips of fluorescent and non-fluorescent labeled proteins (see figure II.5). After bleaching the total fluorescence intensity (I₀) of the system remains constant and then variations on the Fourier transform of the intensity (I₁) can be fitted to equation 17 to obtain the diffusion coefficient of the proteins, irreversibly adsorbed on the surface (graph IV.7).

Graph IV.7 shows the variations in I₀ and I₁ for HSA-FITC adsorbed on quartz. After injecting buffer a protein solution containing HSA-FITC is flown again. The fluorescence intensity increased in time due to the excitation by the evanescent wave of FTIC from proteins in the bulk, and not due to a great increase in the adsorbed amount. After buffer is flown again I₀ returns to approximately the initial values. The mobility of the proteins determined by fitting the curve from graph IV.7b to equation 17 gives a diffusion coefficient of 4.0*10⁻¹¹ cm²/s. The diffusion coefficient of HSA in solution is 6.0*10⁻⁸ cm²/s (table II.1), several orders of magnitude higher than for adsorbed proteins.

Now a buffer solution containing 30% TFE is introduced in the system. I₀ increases by dequenching of the FITC dye in presence of TFE. This shows that molecules of TFE have approached the surface. The result is an increase of the diffusion constant of adsorbed proteins to 8.9*10⁻¹¹ cm²/s. Graph III.10 shows that the viscosity increases with the amount of TFE mixed. Viscosity is inversely proportional to diffusion by the Stokes-Einstein law (equation II.29) so if viscosity has an impact than TFE should decrease the diffusion rate on the surface contrarily to what was observed. In solution the diffusion constant in presence of 30% TFE decreases to 3.2*10⁻⁷ cm²/s, approximately half of the initial value. After rinsing again the total intensity decreased slowly to ~20%, showing that there was desorption of HSA molecules. The diffusion coefficient was the same as in the presence of 30% TFE. This desorption of proteins has an influence on the diffusion constant, so one of the reasons of it's increase is caused also, but not only, by this phenomenon.
Graph IV.7 - HSA-FITC adsorbed in quartz and OTS. Graph A shows the variation in fluorescence intensity ($I_0$) after adding labeled protein, buffer, 30% TFE and buffer again. Graph B shows the calculated values of the diffusion coefficients for each step.

It was shown in chapter III that TFE refolds proteins in solution by introducing the α-helix. In the previous sections it was seen also that adsorbed proteins recover their conformation with TFE. On the other side it was shown that protein-protein interactions occur also via the β-sheet formation. This structure is easily converted by TFE into α-helices (chapter III and VI). If the protein refolds when a buffer containing 30% TFE is flushed the number of interactions between surface and protein, protein and protein, is reduced and proteins are more easily desorbed. Another consequence of this weakening of interactions due to refolding is the faster diffusion of proteins on the surface.

The same experiments were performed in OTS (graph IV.7). The diffusion coefficients on this surface are smaller than on quartz. Two reasons are the origin of this reduction: on hydrophobic surfaces proteins interact via hydrophobic amino-acids usually found on the interior core of the protein, so, the mobility is reduced due to the stronger interaction between protein-surface; another reason is related to the fact that HSA adsorbs ~3.5 times more proteins in this surface than in quartz so there has to be a hindrance of the rate of diffusion by increase in the collision probabilities between proteins. After adding TFE the diffusion constant increased again to the double. So again refolding should be on the origin of this
change in diffusion constant together with the desorption of proteins from the surface. This can be qualitatively seen in figure IV.2 when comparing the bleached stripes from buffer→TFE (the last are more diffused). The addition of buffer immediately reduced the diffusion constant contrarily to what was observed with quartz. The decrease in fluorescence intensity was ~10%, half of the decay in quartz after TFE, showing that proteins are stronger bound on OTS than on quartz.

IV.4.2 Estimating TFE diffusion constants.

One interesting effect worth to analyze is related to the increase in fluorescence intensity when flowing 30% TFE (graph IV.8). In both cases this increase is linear with time for short times. Fitting the initial fluorescence increase to a line, a slope ~10 times larger for OTS than for quartz is obtained:

\[ I_{OTS} = 3.0 \times 10^4 t^{1/2} \quad \text{and} \quad I_{Q} = 2.9 \times 10^3 t^{1/2} \]

(42)

Applying the Lambert-Beer (eq. 5) to fluorescence together with the diffusion equation in transient state it is possible to obtain an estimation of the diffusion coefficient of TFE to the surface.

In diffusional regime it is obtained \( D_{OTS} = 6 \times 10^{-10} \text{ cm}^2/\text{s} \) and \( D_{Q} = 6 \times 10^{-10} \text{ cm}^2/\text{s} \). In solution by applying stokes-Einstein and considering the TFE molecule similar to a prolate a diffusion coefficient of TFE in water of \( 9 \times 10^{-6} \text{ cm}^2/\text{s} \) is obtained. So diffusion to the surface is several orders of magnitude slower than in solution.

The difference in diffusion coefficients could be explained by the difference in interactions between surface and protein. In quartz the interaction between protein and surface occurs via H-bonds and in OTS via hydrophobic interactions. So TFE diffusion should be slow into the first surface and fast into the second as TFE is an hydrophobic organic compound (graph IV.8). The same effect seen for OTS was shown for a surface coated with PS and on hydrophobic thiols (chapter V). On the other hand IgG adsorbed on quartz showed also a slow increase in fluorescence intensity, like HSA.

Looking back to the graph IV.8a after TFE has diffused to the surface (it takes around 2min for observing a first maximum increase in fluorescence intensity) then HSA-FITC desorbs. Contrarily in quartz after these initial ~3 min the fluorescence intensity increases slowly for 4 min and after a new maximum in intensity is reached.

On OTS proteins behave like unfolded in solution and fast refold repels them from the surface – desorption. On quartz proteins behave like folded so it takes time until TFE, or higher concentrations of TFE near the protein are able to further refold the protein. This refolding will imply mobility and rotation of the proteins and so higher exposure of FTIC dye molecules to TFE what can justify a second maximum in fluorescence intensity.

The surface hydrophobicity and as a consequence orientation of the hydrophobic amino-acids should be driving TFE's diffusion rates into the surface. Extrapolating this result to proteins hydrophobic amino-acids are the "driving" reason for approaching TFE molecules to proteins. Graph III.4 shows that the α-helix induction of HSA by TFE is faster when proteins are unfolded and so when the hydrophobic amino-acids are more exposed to solution.
From these results it is possible to imagine HSA lying on a hydrophobic surface with conformation E and on a hydrophilic surface with conformation F (see picture in appendix B).

Graph IV.8 – *Calculation of the diffusion constants of TFE into the surface for OTS (a) and quartz (b). It was fitted the linear part of the curve to the equation of diffusion in transient state (II.23 and II.26).*

IV.4.3 Measuring refolding of adsorbed HSA in OTS.

Graph IV.9 plots the time dependence of the CD signal in presence of 30% TFE. It represents the renaturation of HSA adsorbed on OTS.

The induction of the α-helix is not immediate and takes approximately 12 min until it is reached a steady conformation. After 2 min proteins start desorbing from the surface but renaturation has only occurred to 10% and 12 min corresponds already to great desorption when the renaturation has its maximum, seen in graph IV.8a and IV.9. Altogether this result shows that there are different populations of proteins adsorbed with different strengths, rather than one single adsorbed conformation.
In solution the process is faster than 5s (time limited by experimental conditions). So there are forces opposing renaturation of adsorbed proteins and these come of course from surface-protein, protein-protein interactions. Hence is observed on the surface, when adding TFE, a transition of the type:

\[ \text{Unfolded(U)} \rightarrow \text{Intermediate(I)} \rightarrow \text{Partially Folded(PF)} \]

This PF conformation is rather interesting as it leads to aggregation of proteins on the surface (figure IV.2).

After renaturating the proteins on the surface with TFE and after flowing buffer (graph IV.7 OTS) a new solution of FITC-HSA was flushed into the cell. Immediate aggregation of the proteins on the surface is seen (Figure IV.2C). These aggregates will grow with time (it is not shown in the picture).

This is the result of an incomplete I→PF transition on the surface due to the surface-interaction forces opposing to refolding and that is why in solution the folding process by TFE is faster, no surface-protein interactions are present! These results also imply that aggregation in solution by the presence of TFE is possible until the native secondary structure is reached and that several intermediates I are induced.

Figure IV.2 – HSA adsorbed in OTS. Left image shows the bleached pattern and right images show the bleached pattern after flowing TFE and later HSA-FITC. In the last picture one clearly observes the aggregation of HSA molecules and strong disappearance of the bleached pattern.
Graph IV.9 – Renaturation time of HSA adsorbed into OTS followed by CD at 222nm. The α-helical structure is introduced slowly in time. The final content in ellipticity is not the native one ($-2 \times 10^4 \text{ deg} \cdot \text{cm}^{-1} \cdot \text{dmol}^{-1}$).

IV.5 Understanding the effect of TFE in irreversible adsorbed proteins: FTIR-ATR.

FTIR-ATR is a good technique to identify organic groups adsorbed to a surface due to its sensitivity to selected functional groups. Checking conformation of adsorbed proteins is a rather tricky task due to the H–O–H bending vibration at 1640cm$^{-1}$ which overlaps with the amide I region of the protein [88]. Subtraction of water bands is possible but identifying variations in α-helices can be confused with random coils due to the proximity in absorption of both structures [86,89,90]. In this section FTIR-ATR was used to identify TFE on the surface rather than follow conformational changes of HSA. FRAP demonstrated that TFE diffuses to the surface and that the diffusion rate is limited by the substrate. After reaching the vicinity of proteins this molecule induces refolding and as a consequence desorption from the surface. The remaining proteins are in an unstable conformation maintained by surface-protein interactions.

HSA was adsorbed for two hours on hydrophilic ZnSe and hydrophobized ZnSe (coated with OTS). This protein denaturates when contacting with these surfaces for long periods of time. After adsorption the irreversibly bond proteins were removed by rinsing with buffer and a new buffer solution containing 30% TFE was flown in for 30 min. Washing again with buffer resulted in a new recovered film of irreversibly adsorbed proteins in a PF conformation. The results are plotted in graph IV.10.
Graph IV.10 – FTIR-ATR spectra of TFE absorption bands when proteins adsorbed into hydrophilic and hydrophobized ZnSe were treated with 30% TFE. The spectra were taken several times during 24h. For OTS new buffer was flown after 14h: the TFE molecules assumed a disordered conformation which reorganized in time.

The region between 1000-1400 cm⁻¹ was chosen to analyze the TFE bands as proteins do not absorb in this region.

Graph IV.10 shows that TFE molecules remain in the vicinity of the protein for long periods of time. The molecules rearrange with time on the surface and desorb (the intensity of the bands tends to decrease). Reorientation of the TFE molecules is the result of rearrangements of the proteins on the surface which will exposed different chemical groups to the solution. Analyzing closer, immediately after flowing buffer the -CF₃ symmetric and antisymmetric bands observed in solution appear with varied frequencies for adsorbed TFE as a result of molecules bound to different regions in the protein and/or clustered in the vicinity of the protein (different chemical environment). This leads to the assumption that the -CF₃ group is
pointing in the direction of the protein with -OH bound to water. The frequency of this group (1088 cm⁻¹) is maintained all over the time. Comparing surfaces it is seen that in OTS the reorientation of -CF₃ yields to the same frequencies as observed in solution, suggesting that TFE molecules are no longer bound to the protein groups but clustered in the vicinity of the hydrophobic regions in the protein. New buffer disturbs the TFE molecules but time makes the process reversible and reorganization is achieved (graph IV.10b).

The quartz surface only allows small reorganization on TFE as the hydrophobic groups from the protein are not exposed which doesn't allow stabilization of TFE clusters via hydrophobic interactions. Rather single molecules have to find the free hydrophobic groups that can stabilize the protein-TFE hydrophobic bonding or to desorb into the bulk.

In chapter III the mechanism of α-helix induction by TFE was discussed. Disturbance on the entropy of water was referred as a main reason for the transition. Now it is possible to add another piece to the puzzle, interaction between protein amino-acids and TFE is possible. This molecule diffuses into the vicinity of the protein, disturbs the water shell, the protein reacts by refolding and desorbing. The remaining proteins are PF with some amino-acids free to interact with TFE.
IV.6 Conclusions

In this chapter it was shown that it is possible to monitor by CD conformational changes of adsorbed globular proteins in optically inactive transparent films. This technique has proved to be rather sensitive to the different parameters influencing protein conformation:

1. Different adsorption layers,
   For quartz the process of adsorption is a multilayer process, with an irreversibly adsorbed layer in close contact with the surface and a weakly bound layer in contact with the adsorbed film. Each layer is conformationally different:
   - In the irreversibly adsorbed layer surface-protein interactions play a role in denaturation.
     In this layer the α-helix is the lost structure. Fin and HSA irreversible adsorbed layers unfold with time while IgG has maintained conformation.
   - For reversibly+irreversibly adsorbed layers a strong increase in the overall β-sheet content of the film was obtained with all proteins. The new proteins can bind to proteins already on the surface through β-structures.

2. Conformational changes of the irreversible layer with time,
   Fin was shown to change conformation dependent on the adsorption time. For early adsorption times the α-helix is the preferred structure with fractions above native ones while high adsorption times lead to unfolding of the protein.

3. Concentration dependent conformational changes.
   Conformation of irreversibly adsorbed Fin is dependent on the concentration in the bulk and on the surface energy. For high concentrations hydrophilic surfaces induce β-sheets while low bulk concentrations induce the α-helix. The opposite is also true for OTS.

Adsorbed proteins denaturate so TFE was used to recover the structure of adsorbed proteins. Fin and HSA have recovered their functionality seen by an increase in the antibody binding. These results imply that the newly formed helices are induced where native structures are formed when these proteins are active.

TIRF-FRAP has shown that irreversibly adsorbed proteins have different diffusion constants on the surface dependent on the surface chemistry, on hydrophobic surfaces proteins diffuse slower. This technique was used to monitor TFE influence on adsorbed proteins: as a result of the presence of 30% TFE proteins refolded and desorbed. Consequently the diffusion constant increased to approximately the double. Desorption was greater on quartz than on OTS proving that the strength of interaction between surface and protein is smaller on quartz.

It was possible to estimate the diffusion coefficient for TFE into the vicinity of the protein and the value proved to depend on the protein conformation, proteins adsorbed in OTS have their amino-acids exposed to the surface while in quartz they are hidden in the interior of the protein. By TFE the α-helix is increased leading to steric repulsion as the backbone is not bound any more with surface and proteins desorb. The remaining adsorbed proteins have an unstable, partially folded conformation.

FTIR-ATR showed a new facet of TFE, this molecule can bind to the protein showing that the mechanism of α-helix induction by TFE should include TFE binding to the amino-acids of the protein. The binding is irreversible for proteins adsorbed in quartz.
V Protein adsorption on fluorinated surfaces. Design of biosurfaces.

In the previous chapter it was shown that during the process of adsorption strong structural changes are induced in the proteins by surfaces. If these surfaces are designed to be implanted then the formed monolayer of proteins is as foreign to the body as the implant itself [1,82,91,92]. But with an ideal biosurface the proteins adsorb without further modification of their structure so that the implant is covered by a layer accepted by the body. Surfaces that do not change the structure of adsorbed proteins are hard to achieve so the present demand is to optimise surfaces in order to diminish the immunological responses.

In the last chapters it was tried to understand the effect of TFE on proteins. It was found out that TFE diffuses and remains in the vicinity of adsorbed proteins. As a result the proteins recover their α-helical structure. In solution high quantities of this molecule have adverse effects and the amount of helicity introduced goes beyond that of the native structure. The main reason to use TFE isn’t to denature the structure of proteins but to preserve it! With this chapter it is tried to search for the effects of TFE in proteins if this compound is covalently attached to the surface. The main objective is to preserve the structure of adsorbed proteins.

As a first approach mixtures of thiols -CF₃ and -OH terminated were used as models to study adsorption and conformational changes of proteins on surfaces with different properties. Further on were synthesised polymers with -CF₃/-OH groups and finally completely fluorinated polymers were used as surfaces.

HSA was used to perform the majority of experiments in this chapter. This protein is well known to have no cell receptors in the body so when preadsorbed on a surface it leads to passivation against high adsorption of other proteins like Fn, important in blood clotting [103]. Passivation of a surface by HSA requires strong bonds between surface and protein without nevertheless inducing denaturation. In the last chapter induced desorption of HSA by TFE has shown this protein to be stronger bound to a hydrophobic surface (OTS) than to a hydrophilic one (quartz). The problem is that this hydrophobic surface induced conformational changes in HSA.

V.1 Proteins adsorbed on mixtures of -CF₃/-OH thiols.

The hydrophobic and hydrophilic mixed Self-Assembled-Monolayers (SAMs) were obtained by the competitive adsorption of thiols with different end groups. HS(CH₂)₁₁CF₃ and HS(CH₂)₁₁OH were mixed in different percentages. The monolayer structure of CF₃/OH mixtures was highly influenced by interactions of the tail group, -O-CH₂-CF₃. In all cases molecular mixing of the thiols was observed.

In this section it will be shown that Human Serum Albumin (HSA) adsorption and recognition by anti-body binding are dependent on the functionality of the mixtures and that the introduction of a slight hydrophobicity is important to maintain the conformation of adsorbed proteins.
V.1.1 Characterization of the monolayers

Contact angle measurements. Fluoryl and hidroxyl end groups give a large difference in the wetting properties of the resulting SAMs. -OH terminated thiols gave contact angle values <15° which is in accordance with literature [93]. For -O-CH₂-CF₃ terminated chains a value of 89° was found which is smaller than for a -CF₃ terminated thiol, known to give a contact angle of ~100°. It has been shown that water can penetrate and sense approximately 5Å of the outermost region of the thiol surfaces by formation of hydrogen bonds [94]. In the -O-CH₂-CF₃ monolayer the ether group is ~3 Å from the surface so on the region where water can interact via hydrogen bonding which can be the explanation for the decrease on the value of the measured contact angle.

Cosθ for -O-CH₂-CF₃ (graph V.1) shows a linear trend with concentration in solution, the contact angles are higher than expected. Calculating the fraction of each thiol on the surface via Cassie's [95] and Israelachvili-Gen [96] law, equation 43 and 44 respectively, it is shown a preferential adsorption from the -O-CH₂-CF₃ thiol for mixtures above 25% (table V.1). These two laws can only be used with certainty in cases where there is molecular homogeneity of the surface, so the calculated values can only show an approximated trend rather than real quantities. On the other hand the applicability of these equations to surfaces where hydrogen bonding and dispersion forces are mixed hasn’t been extensively studied [97].

\[
\cos \theta = \sum x_i \cos \theta_i
\]

\[
(1 + \cos \theta)^2 = \sum_i x_i (1 + \cos \theta_i)^2
\]

with \(x_i\) being the molar fraction at the surface \(\theta_i\) the corresponding contact angle on the pure surface.

Graph V.1 – Variation in \(\cos \theta\) for the different -O-CH₂-CF₃/ -OH ratios.
Further on was analysed the ratio of the integrated areas of the infrared bands (graph V.2b) and preferential adsorption of –CF₃ terminated thiols relative to the -OH thiol was obtained (table V.1). It is necessary to have in mind that calculating adsorbed amounts via integrated areas of infrared curves is only an approximate measure as orientation of the molecules relative to the surface normal, backgrounds and positioning of the samples play a role. Nevertheless to be more close to real values the calculated intensities are an average of three measurements.

From these results it is concluded that the surfaces used are richer in –CF₃ than –OH except for 25% mixtures.

<table>
<thead>
<tr>
<th>% mixture in solution</th>
<th>Area at 1178 cm⁻¹</th>
<th>% mixture in monolayer</th>
<th>Area at 1141 cm⁻¹</th>
<th>% mixture in monolayer</th>
<th>Results from Isr.-Gen/Cassie’s eq. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-O-CH₂-CF₃</td>
<td>0.062</td>
<td>100</td>
<td>0.020</td>
<td>100</td>
<td>100/100</td>
</tr>
<tr>
<td>75%</td>
<td>0.061</td>
<td>98</td>
<td>0.019</td>
<td>95</td>
<td>91/87</td>
</tr>
<tr>
<td>50%</td>
<td>0.039</td>
<td>63</td>
<td>0.013</td>
<td>65</td>
<td>69/62</td>
</tr>
<tr>
<td>25%</td>
<td>0.013</td>
<td>21</td>
<td>---</td>
<td>---</td>
<td>24/19</td>
</tr>
</tbody>
</table>

Table V.1 – Relative percentages of each compound in the monolayer by calculating the area under the IR bands at 1178 and 1141 cm⁻¹, and by Israillachvili-Gen and Cassie laws.

_Infrared discussion._ The –CH₂ stretching region (3000-2800 cm⁻¹) has been thoroughly studied in the literature. It gives information on the structural organisation of the alkyl chains [98,99]. The CH₂ stretches occur at 2850 cm⁻¹ (symmetric) and 2918 cm⁻¹ (asymmetric). By analysis of the peak positions for the –OH thiol and 25% mixtures an ordered monolayers maintained by van der Waals forces, closely packed for this range of concentrations is assumed (graph V.2b). The spectrum of pure –OH shows an additional peak at 2878 cm⁻¹. This peak has been assigned by Nuzzo et al [100] to the terminal -CH₂ bonded to -OH. We can see from the graphics that this peak vanishes for mixtures in solution above 50%. The variations in contact angle confirmed the –OH thiol presence on the surface (graph V.1). The two -OH characteristic peaks at 1058 and 1077 cm⁻¹ due to the small signal where also only visible until 50% mixtures (graph V.2a).

The -O-CH₂-CF₃/OH 50% and 75% mixtures behaved “non-ideally” as the -CH₂ stretching bands shifted with the increase of -O-CH₂-CF₃ on the surface, towards 2853 cm⁻¹ and 2925 cm⁻¹, for pure -O-CH₂-CF₃ (graph V.2b). This means we tend to a “liquid like” structure rather than a crystalline structure of the –OH and 25% mixed SAM.

The disorder in the monolayer is a result of the strong dipole associated to the CF₃-CH₂-O- end groups. This dipole is not compensated along the hydrocarbon chain which leads to local repulsion between side groups. For the case of 25% mixtures the monolayer structure is not affected by the introduction of –O-CH₂-CF₃. If this thiol is “diluted” on the surface the side
chains are enough separated to avoid local repulsion and the formed monolayer is organised with the closely packed trans configuration. As soon as we increase the percentage of -O-CH₂-CF₃ on the surface, the proximity of the chains and the formation of small clusters of molecules, leads to side chain repulsions.

Searching for other possible causes of disorder in the monolayer steric effects due to the higher diameter of -CF₃ (5.6 Å) when compared to -CH₂ (4.7 Å) can only play a role for a monolayer of pure -O-CH₂-CF₃. For mixtures the -OH thiol will adsorb also diminishing possible steric effects resulting from nearby fluorinated groups. This thiol is approximately 3Å smaller in length than -O-CH₂-CF₃ so when adsorbed together -CF₃ stays above the -OH group (figure V.1).

![Graph V.2](image)

**Graph V.2** - Graph b) shows -CH₂ symmetric and asymmetric vibrational bands for all mixtures and in graph a) it is plotted the 900-1350 cm⁻¹ region with the absorption bands typical for -CF₃ and pure -OH.

C-F and -C-O-C- stretches were assigned based on infrared spectra of 2,2,2-trifluorethanol [66] and 2,2,2-trifluorethyl methyl ether [101]. In the bulk molecules show a peak 1278cm⁻¹ and 1315cm⁻¹ due to asymmetric stretches from -CF₃ which shifted to 1282cm⁻¹ and 1317cm⁻¹ on the monolayer. The symmetric stretches appear at 1159cm⁻¹ and 1178cm⁻¹, bulk and surface respectively. The bulk -C-O-C- stretches are at 1141cm⁻¹ and 979 cm⁻¹, asymmetric and symmetric. These stretches are present in our monolayer at almost the same wavenumbers as in solution, 1141cm⁻¹ and 978cm⁻¹ (graph V.2a).
The relative intensities of the 1178 cm⁻¹ symmetric and 1282 cm⁻¹ asymmetric stretch of -CF₃ is roughly the same for all ranges of concentrations except for mixtures of 25% where the 1282 cm⁻¹ stretch is hardly seen. These results suggest that orientation of the -CF₃ group on the monolayer was maintained roughly the same for all experiments except for 25% mixtures, which form well packed monolayers. Due to the high intensity of the symmetric peak when compared to asymmetric stretch it is possible to consider the orientation of the -CF₃ group almost perpendicular to the surface.

![Image](image.png)

Figure V.1 – Sketch of mixtures of thiols based on the IR results. The -CH₃ vibrations showed that for 25% mixtures the thiols form well-packed monolayers. The -CH₂-CF₃ group remains above the -OH thiol being the differences in thickness between both groups of around 3 Å, measured by SPR. As soon as the amount of -CF₃ increases repulsion plays a role and the monolayer tends to disorganise.

V.1.2 Protein Adsorption.

HSA was adsorbed on pure -O-CH₂-CF₃, pure -OH and 50% mixtures. The monolayers formed were discussed to be liquid-like for -CF₃ and tending to organise when decreasing the fluorine content on the surface, until a well packed monolayer for -OH and 25% mixtures is formed.

Protein adsorption on these surfaces was followed by SPS and TIRF. Fluorescence techniques are hard to quantify due to influence of the environment on the fluorophore quantum yield. So TIRF was used as a qualitative technique to follow the kinetics of adsorption (graph V.3). Usually I-labeling techniques are utilised to calculate adsorbed amounts. In this set of experiments the adsorbed amount was quantified by Surface Plasmon Spectroscopy and the TIRF curves were normalized by the obtained values.
The adsorption plateaus followed the order -OH > -O-CH₂-CF₃ > 50% but after rinsing the irreversibly adsorbed layer retained a different order in adsorbed amount, -O-CH₂-CF₃ > 50% > -OH.

Analysing the plateaus, -O-CH₂-CF₃ and mixtures of thiols reached a smaller level of adsorbed amounts of proteins than the single components and this roughly means, that a smaller amount of proteins wants to be in the vicinity of the surface. This shows the influence of hydrophobicity in the adsorption of proteins. As the hydrophobic amino-acids are buried inside the protein hydrophobic interactions are not the driving force for adsorption until the protein reaches the surface and changes conformation.

The irreversibly adsorbed layer retained an adsorbed amount consistent with the surface energy order (graph V.1), surfaces with lower energy usually retain higher amounts of proteins due to hydrophobic interactions between the amino-acids buried inside the protein and surface. Nevertheless this energy order, 50% mixtures retained an amount of adsorbed proteins near the amount observed by the -OH thiol. The surface structure played a more important role than the surface energy driving protein adsorption. One cause for this effect can be related to the fact that for 50% mixtures the protruding thiols will give origin to a steric instability for adsorption. The gaps between thiol molecules are on the order of 3Å (figure V.1). Another cause can be the presence of “arrested” water molecules in gaps formed between the thiols. Proteins would have difficulties to displace this water. Thus water molecules would mask the surface for adsorption of proteins which would function like a “cushion” for protein adsorption.

Graph V.3 – Protein adsorption on thiol surfaces followed by TIRF; (m) -OH thiol, (▲) 50%; (●) -CF₃. The adsorbed amounts were determined by SPR.
V.1.3 Protein Conformation. ELISA.

ELISA was used again as the screening test to look for conformational changes of the adsorbed proteins. The antibodies will recognise the assessable active epitopes of the protein (chapter II).

The pure components, -OH and -O-CH₂-CF₃, provoked the lowest adsorbed amount of antibodies when compared to mixtures of these two thiols (graph V.4). The introduction of hydrophilicity was important for the protein stability as 75% mixtures in solution (~95% on the surface accordingly to table V.1) had already a strong increase in antibody binding.

The way water structures itself around mixtures of hydrophilic and hydrophobic thiols can be decisive for the observed results as the only thing that was varied was the chemical distribution on the surface, less -OH new presence of -O-CH₂-CF₃. Water layers separating each protein from the surface is the diffusion medium for a protein approaching the surface. The structure of these layers is going to dominate the way of interaction as proteins themselves are surrounded by several layers of water. If the thiol surfaces are able to retain water, for instance in the gaps between thiols (figure V.1), disturbances in the water around proteins should be minimised - unfolding should not occur. This could explain the increase in functionality from HSA when adsorbed in mixtures of thiols. Another reason for the increased stability could be the α-helix retention by these mixtures in the adsorbed HSA molecules via the presence of TFE (-CF₃/-OH) units on the surface.

Graph V.4 – Results from antibody binding to HSA adsorbed on thiols mixed with different ratios in solution. The OD values were normalized by adsorbed amount for comparison terms.
V.2 HSA adsorbed on modified polyketone with $-\text{OH}$ and $-\text{CF}_3$ units.

In the previous section it was shown that HSA adsorbed onto thiols maintains it’s function better when hydrophobic and hydrophilic thiols are mixed. Thiols are interesting model surfaces to study the behaviour of adsorbed proteins but for applications they offer many restrictions, they need a metal surface for chemical-adsorption, which can be hard to manipulate. Polymers offer the alternative, the possibility to manipulate their mechanical properties – they form rigid to flexible surfaces, different ranges of solubility, a high variety and possible modification, makes them appealing surfaces. For the next section a polyketone was modified with $-\text{CF}_3$ and $-\text{OH}$ units where HSA was adsorbed.

V.2.1 The polymer

A polymer with TFE units was synthesised by chemical modification of polyvinylmethylketone (PMVK) commercially available (figure V.2). The synthesis was performed by Andreas Pawlik at Hüls. The reaction is not complete as shown by NMR (table V.2). This polymer retained $\sim$40% of the $-\text{C}=\text{O}$ groups unmodified.

![Chemical reaction diagram](image)

Figure V.2 – Reaction of polymethylvinylketone (PMVK) with trifluoromethyltrimethylsilane. It is obtained modified PMVK with TFE units, PMVK-TFE.

<table>
<thead>
<tr>
<th>Element</th>
<th>Measured concentration (%)</th>
<th>Expected concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>64.9</td>
<td>55.6</td>
</tr>
<tr>
<td>O</td>
<td>11.8</td>
<td>11.1</td>
</tr>
<tr>
<td>F</td>
<td>23.1</td>
<td>33.3</td>
</tr>
<tr>
<td>Si</td>
<td>0.2</td>
<td>---</td>
</tr>
</tbody>
</table>

Table V.2 – NMR results on PMVK-TFE.
V.2.2 CD of adsorbed HSA and Fin on PMVK-TFE.

HSA and Fin were adsorbed for two hours on PMVK-TFE and quartz, and the CD spectra of the irreversibly adsorbed layers was taken (graph V.5). It was shown in chapter IV that two hours adsorption induce already strong conformational changes in Fin and HSA adsorbed on quartz. So it is possible to compare conformational behaviours between quartz and PMVK-TFE.

![Graph V.5 – CD spectra of adsorbed Fin and HSA onto PMVK-TFE surface.](image)

The results show that HSA and Fin adsorbed on PMVK-TFE maintained approximately the same conformation as in solution, while on quartz denaturation was stronger. Problematic for both adsorbed proteins are the bands at 195nm, typical for the introduction of some random coil structure. Nevertheless from both results it is possible to say that there is a clear influence of the TFE group from the modified polymer on the proteins as they maintain a higher level of the α-helical structure. This was the same result as obtained for HSA adsorbed in mixtures of thiols: higher conformational stability when -OH and -CF₃ units are together.

V.2.3 Biological tests

The polymer PMVK-TFE interdigitated with polyethyleneglycol-polyurethane (PEG-PUR) (appendix B) was sent to a certified laboratory (BIOMAT) to test the biocompatibility of the material. PEG was chosen due to its well-known swelling properties [102,103]. In the last section the presence of water in the surfaces influencing the proteins conformation was discussed. PEG would allow the presence of water molecules on the polymer chains, like due to the difference in heights between thiols (figure V.1). The fluorinated groups would interact strongly with the water in their vicinity maintaining the α-helix structure in the proteins.
Samples coated with PVMK-TFE/PEG-PUR were tested against complement C activation, thrombin generation and thrombozyte adhesion. These are typical tests that search for the response of the biological system to a foreign material. They are all giving indications for phenomena like alteration of plasma proteins, thrombosis, damage of tissues, allergic reactions, etc. [91, 104].

The response of the tested materials is compared with a non biocompatible material, i.e. polypropylene, which represents the positive control. The results are plotted in graph V.6 and were taken from the laboratory report B0011/98-BIOMAT.

Graph V.6 – Results from the biological analyses made in PEG-PUR and interdigitated PVMK-TFE with PEG-PUR surfaces. The error bars in complement C activation are shown due to the high fluctuations offered by PEG-PUR in the measured samples.

The results show the improvement of the surfaces modified with TFE units against the positive control in the three tests. Comparing now the interdigitated network PEG-PUR/PVMK-TFE with PEG-PUR, the network offered improvements in terms of complement C activation and thrombin generation but an increase in adhesion of thrombocytes. This last test is dependent on the structure of the surface and both surfaces showed a relatively high roughness.

From these results it is shown that TFE units can improve a polymer chain in terms of biocompatibility. Nevertheless there must be further changes in the polymers to achieve total biocompatibility. It is important to remember that the modification of the polymer with TFE units was only achieved to 60% (table V.2). A higher percentage of TFE could be included in the polymer chains for further measurements and improvements.
V.3 HSA adsorbed on completely fluorinated surfaces.

It is seen in the previous section that the introduction of hydrophilicity associated to hydrophobic surfaces has an impact in preserving the structure of adsorbed proteins, like TFE alcohol in the recovery of denatured proteins (chapter III). The addition of too much TFE or IsoF to the protein solution is an adverse effect as it would increase the amount of helicity to values above native. To check for this possibility in this section completely fluorinated surfaces to adsorb HSA are studied. This protein was chosen as model due to its high helical content.

HSA was adsorbed for two hours on films of Polyethylenimine (PEI) complexed with perfluorinated acid, \( \text{OOC-}(\text{CF}_2)_n\text{-CF}_3 \), being \( n = 4, 7, 10 \) \([105]\). These polymers formed thin films on quartz surfaces deposited by the self-assembly process. The chemical structures of the polymers can be found in appendix C. After HSA adsorption the CD spectrum for each surface was measured (graph V.7).

Graph V.7 – CD spectra of HSA adsorbed in fluorinated surfaces of different surface energy.

The conformation of adsorbed HSA was maintained for the polymers with the shorter chains, PEI-C7/C4, but PEI-C10 showed a clear decrease of \( \alpha \)-helix structure.

Related to the observed results can be the total adsorbed amount of proteins (table V.3) as this factor has an effect on the conformation of adsorbed proteins (graph IV.5). Another reason can be the roughness presented by these surfaces resulting from deposition onto quartz. This can cause nucleation of proteins around defects, being \(-C_{10}\) the surface with larger amount of defects (AFM pictures not shown). The nucleation process would then induce the higher amount of conformational changes due to local concentration effects.
### Table V.3 – Surface energy and adsorbed amounts of the different PEI complexes used.

The adsorbed amount followed the surface energy increase, the higher is the energy the higher is the adsorbed amount. The surface energy variations can be explained by the chain length: $C_4$ is too small to form an organised layer as the dipole moment along a fluorinated chain is only compensated beyond 7 C [106] and so water will feel the $-CF_2-$ groups near the surface, exposed due to repulsion between side chains.

The surface structure influenced the conformation of the proteins adsorbed in PEI-complexes, as the chemical composition of the outermost $\bar{A}$ of the surface is the same for all surfaces, $-CF_2-CF_3$. Instead of inducing new $\alpha$-helices PEI $-C_{47}$ surfaces were able to maintain the conformation of HSA.

Four other fluorinated surfaces were used to measure the conformation of HSA. These surfaces were made by the complexation of polysiloxanes with perfluorinated acids [107] – see structures in appendix. These new polymers used to adsorb HSA differ from the last due to the change in the polymer backbone what resulted in surfaces of low energy. Results are shown in table V.4.

### Table V.4 – Surface energy, adsorbed amount and secondary structure analyses of HSA adsorbed in polysiloxanes complexes.

There is a difference on the complexation degree of the polymers (see appendix B), being the ratio of the amino functions to carboxylic groups 1:0.5 or 1:1. $PF_{125}$ and $PF_{85}$ differ on the length of the side chains ($12C$ and $8C$, respectively).

The surfaces which presented the higher complexation, 1:1, showed the highest $\alpha$-helix content with $PF_{85}$:1 introducing an $\alpha$-helix percentage above native, 74%. In all surfaces used, except $PF_{125}$:1, the adsorbed amount was similar so this parameter was not decisive for the protein conformation.

Again the outermost $\bar{A}$ of the surface has the chemical composition $-CF_2-CF_3$ so the surface structure, dictated by the degree of complexation and chain length, influenced the conformation of the proteins.
One can thus conclude that fluorinated surfaces in general maintain the conformation of adsorbed proteins but the final conformation is strongly dependent on the structure of the substrates were proteins are going to be adsorbed. This structure, influenced by the backbone, and dependent on the chain length and organisation of the chains, is going to dictate the way water and surface interact and as a result the induction of the α-helical structure.

To design a completely fluorinated biosurface it is thus important to pay attention to organisation of the interface where proteins are going to be adsorbed as this is decisive for the conformation adopted on the surface. These surfaces could also be interesting for surface passivation with HSA: the 2h exposure time doesn’t denaturate the protein contrarily to quartz and OTS (chapter IV) and hydrophobic surfaces form strong bonds with HSA what would avoid desorption.
V.4 Conclusions

In this chapter was searched for the influence of TFE units on the conformation of adsorbed HSA with the intention of looking for the design of a biocompatible surface. As a first approach were used mixtures of thiols $-$OH and $-\text{O-CH}_2\text{-CF}_3$ terminated. These mixtures are rather flat with a roughness of around 4Å due to differences in the thiols heights. For values above 50% there is a preferential adsorption of the fluorinated thiol while for values below 50% the OH chain is preferentially adsorbed. The monolayer becomes more ordered when this thiol is present.

HSA adsorbed on mixtures retains higher functionality than when compared with the single components with only $\sim$5% of $-$OH on the surface being responsible for introducing a change. The structure of the mixed thiol surface could be responsible for the observed conformational stability due to $\alpha$-helix preservation by TFE units or by the retention of water molecules due to the difference in heights between thiols. This interaction between water and surface is important for the response of the protein towards the surface as proteins themselves are surrounded by several layers of water.

The modification of PVMK with TFE was only achieved with 60% TFE units. CD measurements showed that this polymer retains almost the complete secondary structure of adsorbed HSA and FIn when compared to quartz under the same adsorption conditions. Specific biological tests confirmed the possibility of biocompatibility from this polymer as the introduction of TFE units showed improvements when compared with the unmodified polymer. Nevertheless new changes should be introduced like further fluorination of the polymer and decrease in surface roughness. This last parameter was essentially responsible for the high adhesion of thrombocites observed.

Searching for the $-\text{CF}_3$ effects on HSA secondary structure several fluorinated surfaces with different surface energies were used. It was shown that the induction of the $\alpha$-helix in the protein depends on the organization of the outermost 1-2 Å of the fluorinated surfaces. In general the conformation of HSA was maintained. For PF$_{85}$1:1 it was nevertheless possible to achieve 76% helix for adsorbed HSA, a value $\sim$10% above the native one. This result in fact shows that the organisation of the $-\text{CF}_3$ units is the one responsible for the $\alpha$-helix induction. This organisation distates the water structure on the interface like in solution the aggregation of TFE molecules affects the water structure. Proteins will then adsorb with a conformation according to what they sense at the interface: the chemistry and the water layer.
VI Folding properties of the amyloid peptide by -CF₃ groups.

β-amyloid is a small polypeptide with 42-43 amino-acids which in a non infectious form is represented by the conformation of an α-helix (appendix B). Due to an unknown process this α-helix structure is transformed into a β-sheet, the infectious form of amyloid. These β-sheets aggregate with the chains parallel to each other forming long fibrils which are the well known Alzheimer plaques [24,25,108,109].

Where does this polypeptide come from? There are several sources for the appearance of this peptide in the blood. The majority are genetically related where hereditary can play a role, loci in chromosome 1 and 14, mutations on the gene located on chromosome 21. From this gene derives APP (Amyloid Protein Percursor, see figure VI.1). It is a transmembranar protein with 130kDa where β-amyloid represents only ~3% of its amino-acid sequence. Amyloid is enzymatic cleaved from APP by enzymes which haven’t been identified yet and transferred to the blood. In our studies described on this report we will focus on β-amyloid with origin in APP. More recently it has also been found that there are cells that normally excrete this peptide to the blood stream.

After cleavage from APP the peptide transits into a β-structure and is transported to the extracellular space in the brain and in walls of cerebral blood vessels where it aggregates and precipitates due to the high hydrophobic character of the β-sheets. The brain is a chosen path as it is also a very hydrophobic area. It is here where the ordered aggregated molecules form rigid plaques which degenerate nerve endings and glial cells.

![Diagram](image)

Figure VI.1 – Different regions of the Amyloid Protein Percursor (APP). The largest part of the molecule is the extracellular domain, which contains a cysteine-rich region of approximately 200 amino acid residues at the N-terminus, and a region rich in acidic residues. The amyloid peptide is the transition region between the extra and intracellular medium.
VI Folding of Amyloid by -CF<sub>3</sub>

So to avoid the disease it is necessary to inhibit amyloid aggregation: either by blocking the cleavage of this peptide from APP or either by reconverting the peptide. This chapter will use the previous knowledge about the fluorinated drug effect on α-helix proteins, to try to understand factors which can contribute to the α→β transition. The motivation is related to the possibility of opening a path to inhibit amyloid aggregation as until now no cure to the disease has been found.

In chapter III it was said that the specific induction of helical structure by fluorinated alcohols is possible and done more efficiently on proteins that have “written” on the amino-acid sequence α-helix formation. Amyloid is one of these proteins.

VI.1 Amyloid structure.

Amyloid is a small peptide chain 15nm long in an extended configuration belonging to a protein with ~700 amino-acids (a.a.). It's sequence comprises part of the extracellular domain and extends into the transmembrananar domain of the protein (Figure VI.1). As a result of amyloid's location it's sequence shows two different regions: from a.a. 1-28 the peptide can form α-helices due to the presence of hydrophilic a.a., extracellular area, from 29-43 there is a strong hydrophobic region, intracellular, which is extremely insoluble (figure VI.2).

1  5   10  15  20  25
30  35  40

**Figure VI.2 – Amyloid's amino-acid sequence. Each letter corresponds to one amino-acid -appendix A (this representation is used by convenience)- and in red are represented the hydrophobic areas of the peptide.**

When inserted inside the APP chain amyloid folds with a helical structure in accordance with the a.a. sequence. What makes this protein turn into a stable β-sheet structure when cleaved out of APP remains uncertain. It is known that β-sheets are stabilized by hydrophobic forces so amyloid's hydrophobic region has to be an important clue for facilitating the transition.

VI.2 Amyloid aggregation.

Amyloid transition to a β-sheet structure makes it aggregate into long fibrillar structures due to hydrophobic interactions (figure VI.4). These aggregates become insoluble and as was said they end up precipitating in the brain. For studies with amyloid it is then very important to know the size of the aggregates in solution and after adsorption to a solid surface.
VI.2.1 Aggregation in solution.

The amyloid peptide was synthesized in the laboratory and received with a random coil structure. To convert this structure into a stable $\beta$-sheet it is necessary to incubate the protein for 3 days at 37°C. After the incubation process the peptide was used for all measurements described in this chapter.

The process of aggregation of amyloid in time was followed with light scattering at 37°C (table VI.1). The solutions used had an initial concentration of 0.05 mg/ml.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0 – 4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>18</th>
<th>36</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle averaged diameter (nm)</td>
<td>--</td>
<td>5.9</td>
<td>6.2</td>
<td>7.9</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

Table VI.1 – Variation on the sizes of the aggregates with time. Time 0 is recorded after the 3 days incubation.

From 0-4 h it was not possible to observe any aggregation due to the small size of the particles. After the particles reached the size of 30 nm they remain stable for three days. Normally this size of aggregates is observed at the early stages of fibril formation, and so they are called protofibrils [109]. Higher incubation times are needed to observe fibrils or with higher initial concentration a faster aggregation should be observed.

To obtain constant conditions all solution and adsorption measurements performed with amyloid and presented in the following report are with amyloid aggregated with the averaged diameter of 30 nm, so on the protofibril stage. Amyloid in the fibril stage tends to precipitate.

VI.2.2 Aggregation in solid support.

AFM was used to check for the size of aggregates formed by amyloid on a solid support (figure VI.3). A completely fluorinated surface was used as substrate. The picture shows that adsorbed amyloid aggregates into bigger size clusters. These new structures are bigger on the surface than in solution by approximately 7 times. Their height varied between 5-8 nm and looked to be formed from the association of spherical particles which is consistent with a nucleation controlled growth. This surface aggregation is similar to what is obtained in the brain cells. Other authors have found a similar result on a hydrophilic surface while in TEFLOM they obtained linear aggregates [110]. The surface used in figure VI.3, is also completely fluorinated, but it’s roughness avoids a linear growth of the peptide. The roughness of the polymer is also going to provide nucleation sites for the adsorption of the peptide.

The question that needs to be answered now is concerning the conformation of these aggregates in solution and after adsorption as this conformation is decisive for the activity of the disease.
VI  Folding of Amyloid by -CF₃

Figure VI.3 – AFM picture of PF₁₂₅ (A) and of Amyloid adsorbed in PF₁₂₅ (B). One clearly observes the formation of aggregates. These have a height of 5-8nm and a length of 200nm.

VI.3  Amyloid in different solvents.

The influence of hydrophobic solvents on proteins was studied in chapter III. It was shown with those studies that there is the possibility of inducing a β→α transition in large proteins by TFE.

As before to obtain a comparable behavior between alcohols the influence of ethanol on the amyloid peptide was measured (graph VI.1). The curves showed no variation on the structure until 50% of added alcohol, where 21% of α-helix was induced (see table VI.2). The α-helix present on the peptide was formed on the expenses of loss in random coil structure rather than on the destruction of the β-sheet. Ethanol is not capable of changing the β-sheet structures of the amyloid peptide: the size of the peptide when compared to the protein (42 a.a. against 1000) doesn’t determine the strength of the β-sheet.

<table>
<thead>
<tr>
<th>Ethanol (% vol/vol)</th>
<th>0</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>β-sheet</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>52</td>
<td>56</td>
<td>49</td>
</tr>
<tr>
<td>Other</td>
<td>40</td>
<td>42</td>
<td>43</td>
<td>41</td>
<td>23</td>
<td>24</td>
</tr>
</tbody>
</table>

Table VI.2 – Variation of the secondary structure of amyloid as a function of the ethanol content in vol %. The secondary structure values were calculated using the CONTIN algorithm (see appendix D).
Graph VI.1 – Titration of the amyloid solution \((C_{0t} = 0.05 \text{mg/ml})\) with different volumes of ethanol. There are no observable changes in structure until 50% \(v/v_{\text{sol}}\) of added alcohol.

Ethanol is the weakest in terms of hydrophobic character of all the solvents used. So as done previously the titration of amyloid solutions with TFE and ISOF (graph VI.2), strong hydrophobic solvents, was performed.

Comparing the curves representing variations in the helix and beta structures it is concluded that ISOF is more effective than TFE. TFE is able to induce maximal 26% \(\alpha\)-helical structure which remains constant beyond 20% \(v/v_{\text{sol}}\) of this solvent. The first 20% of alcohol added to the peptide solution is responsible for the 50% breakage of the total \(\beta\)-sheet and consequent transformation in \(\alpha\)-helix. With the next volumes of alcohol we observe the progressive destruction of the \(\beta\) structure accompanied by the increase in random coil.

ISOF is a stronger \(\alpha\)-helix inducer than TFE. For the first 10% \(v/v_{\text{sol}}\) of alcohol we observe in simultaneous the strong destruction of the \(\beta\) structure, reduction in random coil and formation of 45% \(\alpha\)-helix. Within the next volumes of alcohol the helix content increases to 68% due to the total destruction and progressive disappearance of the remaining \(\beta\)-sheet and random coil, respectively.

It is important to note that for both solvents only 12% \(v/v_{\text{sol}}\) has already an impact on the destruction of amyloid \(\beta\)-sheets. At this percentage of added alcohol amyloid has reduced its total \(\beta\)-sheet content by 50% and 80% for TFE and ISOF, respectively. It was seen before (chapter IV) that the alcohol likes to be in the vicinity of denatured proteins by exclusion from water probably by favoured environment offered by the proteins, next to it’s hydrophobic a.a.. This can tell why small amounts of alcohol have a strong impact on the peptide. For the big native globular proteins, HSA, Fin, IgG, small amounts of TFE produced only small effects on the secondary structure (graph III.4, III.5, III.6). When the concentration
of alcohol is large it associates forming hydrophobic clusters (chapter III) and the destruction of the β-sheets increases.

Graph VI.2 – Variation on the percentage of secondary structure of amyloid by titrating the solution with TFE and ISOF. The different symbols represent the four most common structures found within this protein, – α-helix, – β-sheet, – β-turn and – random coil.

It was discussed in section VI.1 and VI.2 that amyloid aggregates with the β-sheets parallel to each other forming fibrils. So one simple mechanism for the β → α transition by fluorinated alcohols can start with the separation of the β-sheets within aggregates into single β-sheet molecules followed by reconversion (figure VI.4).

TFE (see chapter III) and ISOF form hydrophobic clusters in water solutions. On the other hand these molecules like to be next to the peptide environment possibly by forming local alcohol aggregates (chapter IV). These hydrophobic clusters must be stronger than the hydrophobic force that maintains the amyloid β-sheet aggregates. Together they create an environment energetically more favorable to the peptide alone than in the aggregated sheets. Molecules of alcohol can surround peptide aggregates and penetrate in between aggregated molecules to separate them from each other. This is plausible as half of the amyloid structure is composed of hydrophobic a.a. which facilitates hydrophobic interactions between alcohol and peptide. Then would follow the breakage of the β-structure in single molecules and the transition to an α-helix (mechanism β → α transition by TFE is explained in chapter III). As ISOF is a stronger hydrophobic molecule than TFE it’s effect should be felt more effectively.

It is important to have in mind that CD gives an averaged signal of all the possible structures in solution and the mechanism proposed above requires a homogenous solution. So it is not
possible to exclude the fact that possibly some molecules are already α-helix while others are still being separated from the aggregates.

Figure VI.4 – Mechanism for the transition α-helix → β-sheet by TFE and ISOF. First we feature the destruction of the β aggregates and then the immediate transformation of single β-sheet molecules to α helix structure.

VI.4 Reversibility of the process (α↔β).

The amyloid peptides are in their original form folded mainly as α-helices. By an unknown process these peptides are converted into β-sheets. There exists evidence of this “switch phenomenon” by which after one molecule is converted into β-sheet it can induce β-sheets in all the other molecules in the surroundings [25]. So for the treatment of prion and amyloidosis diseases it is necessary to have stable reconverted molecules.

In the previous section the infectious form of amyloid (β-sheet) was transformed into a non infectious (α-helix) structure by adding TFE and ISOF to the solution. So the next question concerns the stability of the formed α-helix.

The solutions with different volumes of TFE were measured over three days giving always the same CD spectra. The formed structures were stable with time. This means that the TFE environment blocked the β-sheet reconversion. Several authors have used TFE to simulate the
VI Folding of Amyloid by -CF₃

hydrophobic/hydrophilic environment of the phospholipids. In this environment the α-helix of amyloid is naturally stable.

To check for the reversibility of the process was proceeded with dilution of the solution containing 50% TFE in vol. until 5%. The same CD spectra was measured for the whole range of dilutions. This result shows the formation of a stable and irreversible structure against dilution. So when a β-peptide is reconverted to the non-infectious form, α-peptide, it remains unchanged. Both structures, α-helix and β-sheet, are stable in the amyloid peptide. As amyloid, IgG protein has also an a.a. sequence typical for helices but it folds as a stable β-sheet by TFE (chapter III). The stability of the TFE-IgG solutions was tested in time and they proved to be also stable. Contrarily dilution of the 50% v/vₜₜol TFE to 5% (table VI.3, graph III.6b) showed a final structure which has recovered it’s initial β-sheet. The amount of α-helix and β-turn decreased to values near the initial ones. The structure resultant from TFE environment is not a stable conformation for this protein. The process is reversible. It was found another example in literature of a reversible process. Goto et al [111] used a small chain from the IgG molecule with a random coil conformation. Then the sample was treated with TFE for the coil→α transition. After they proceeded to dilution which reconverted back the peptide to a random coil structure.

<table>
<thead>
<tr>
<th>% v/v TFE</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>β-Turn</th>
<th>Random Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>54</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>50</td>
<td>35</td>
<td>1</td>
<td>27</td>
<td>39</td>
</tr>
<tr>
<td>25</td>
<td>12</td>
<td>38</td>
<td>13</td>
<td>36</td>
</tr>
<tr>
<td>12.5</td>
<td>6</td>
<td>36</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>58</td>
<td>10</td>
<td>33</td>
</tr>
</tbody>
</table>

Table VI.3 – Variation on the secondary structure of IgG after diluting the solution containing 50% TFE with buffer. The structures were calculated using the SELCOM algorithm (see appendix).

With this set of experiments it is shown that the α-helix induction into stable structures by TFE is a property of the peptide or protein under study and not of this alcohol. The alcohol just “helps” the conversion.

The energy of proteins has to be maintained at a minimum for the achievement of structural stability. In the particular case of amyloid one observes a jump from a minimum of energy (β-sheet) to another minimum (α-helix) which is still stable in the absence of alcohol. It is known that the energetic difference between folded and unfolded conformation is on the order of 20kcal/mol, so proteins are said to be metastable. Facing this fact it is very easy to observe changes in the conformation of proteins: the transport of substances in blood, the binding to cells, the α→β transition of amyloid, all these phenomena require low energetic costs.

The problem, why after cleavage the amyloid peptide folds into a β-sheet is far from being solved with these results. What is important to remark is the existence of two metastable
structures, the α and the β. When this peptide constitutes part of APP its stability is dependent of the other a.a from the total protein chain and on the phospholipid of the membranes, as amyloid is situated between the intra and extracellular medium (figure VI.1). All these factors contribute to the α-helix stability. The TFE environment makes also stable helices so in a way it simulates the real environment surrounding the peptide. If TFE is able to reconvert β→α it is possible that there are other molecules circulating in the blood that can create the unstable environment that does the inverse, α→β transition. After transition into β molecules can induce β in all other molecules by switching the α structure [25]. Schladitz et al [112] observed that a monolayer of random coiled amyloid when compressed at the air/water interface forms β-sheets, showing that environment and external parameters are important.

VI.5 Amyloid interaction with solid surfaces.

The mechanism of α-helix induction by TFE was explained to be indirectly due to the hydrophobic effect and directly to the binding of TFE molecules to hydrophobic a.a.. If hydrophobicity alone plays the role in the β→α transition the next step is to incubate amyloid on a completely fluorinated surface. For that was used PEI-C₄, polysiloxane-C₁₂ (PF₁₂₅₁:0.5) and PUR-CF₃ polymers (description of the structure is made in appendix C). These polymers have in common an interface of low surface energy due to complete coverage with fluorinated, -CF₃, groups. In chapter V it was also shown the possibility of increasing the α-helical content of adsorbed HSA induced by fluorinated surfaces.

Amyloid was allowed to adsorb for 2h in PEI-C₄ and PF₁₂₅₁:0.5 at 37°C. The CD spectra of the adsorbed monolayer was measured in transmission. Important is also to control changes in the amyloid solution before and after incubation to check if there was an influence of the fluorinated sample on the solution medium. If the adsorbed layer has changed conformation and these proteins are allowed to exchange with proteins from solution it would be possible to observe changes in the solution medium, unless the desorbed amount is so small that the averaged CD signal wouldn’t be able to discriminate changes. After adsorption of amyloid for 2h the samples were placed 24h against water to see if the adsorbed molecules would change conformation by long exposure to the surface. The CD spectra and secondary structure estimations are plotted in graph VI.3.

- Amyloid in solution before and after contact with the polymer films.

Analysing the results for PF₁₂₅₁:0.5 it is seen that changes in solution conformation before and after incubation are small. This is shown by the small differences on the graphs, same peak positions and small changes in [9]. Calculating the secondary structures by two different algorithms shows that the β-sheet content of the solution before and after incubation was constant by the SELCOM algorithm and increased slightly with CONTIN.

In the case of PEI-C₄ the solution after being in contact with the sample increased the intensity of the band near 200nm. This band has been assigned to a ππ* transition (see chapter II). Twisting of the β-sheets leads to a stronger ππ* coupling which explains the increase in intensity. Calculation of the secondary structure for PEI-C₄ showed a variation in the β-sheet content of ~4% based on estimations using the SELCOM and CONTIN algorithm.
From these results it is concluded that after putting an amyloid solution in contact with a PF$_{125}$:1:0.5 and PEI-C$_4$ surface the amyloid structure in solution organizes differently, increased twisting of the $\beta$-sheets, but the amount of $\beta$ structure remains approximately the same.

- **Adsorbed film of amyloid.**

Comparing then adsorbed layers the results were slightly different. The surface structure introduced differences on the adsorbed amount and conformation of adsorbed proteins relative to the solution for both polymers. If we look at peak positions in both samples we observed a shift in the peaks at 197nm relative to solution. This means that the adsorbed layer has changed conformation relative to the solution.

Calculating the secondary structure a small increase on the $\beta$ content of the adsorbed proteins relative to solution for PF$_{125}$:1:0.5 was obtained. This can be then explained by the increase in the size of the aggregates. It was seen by AFM (figure VI.3) that the size of the aggregates on the PF$_{125}$:1:0.5 surface can reach 200nm while in solution they have only 30nm. PEI-C$_4$ on the other hand increased the $\alpha$-helical content by ~10% relative to solution, estimated by both algorithms. The high roughness of this polymer didn’t allow to make an AFM picture reliable for comparison with PF$_{125}$:1:0.5.

The differences between polymers concerns the backbone and length of the fluorinated chains and as a result there are differences on the properties of the interfacial layer; packing, roughness and surface energy. Taking the surface properties into account PEI-C$_4$ has a higher roughness and surface energy than PF$_{125}$:1:0.5. The higher roughness will block aggregation into fibrilar structures and the higher surface energy allows the peptide higher conformational changes to adapt their structure to the surface and so a higher possibility of inducing a $\alpha$-helix.

The differences in chain length of PEI-C$_4$ and PF$_{125}$:1:0.5 resulting from the different number of carbons of the side chains from these polymers, -C$_4$ and -C$_{12}$ respectively, can also affect the structure of the peptide. It is then possible that the peptide feels the backbone, the charged PEI, in the case of -C$_4$. In the case of PF$_{125}$:1:0.5 the chain length is big enough to avoid sensing of the backbone from the peptide (in chapter IV was observed that the presence of an oxygen in a thiol chain near the surface makes the contact angle decrease ~20%).

- **Adsorbed film of amyloid 24h in water.**

After placing the adsorbed film of amyloid in water for 24h there was no desorption of proteins as concentration was maintained constant. Rather unexpectedly was observed an increase on the $\beta$-sheet content of ~8% and ~14% for PF$_{125}$:1:0.5 and PEI-C$_4$ respectively. When in contact with solution the peptides on the surface can exchange with $\beta$-peptides from solution. When in contact with water there are no exchange molecules and mobility on the surface is the only effect that can bring molecules together in order to refold. Now it is possible to imagine that the adsorbed molecules find an unstable conformation on the surface, as the surface by itself is not powerful enough to induce a total transition. Then what would happen is the search for the stabilization of surface-peptide and peptide-peptide interactions.
VI Folding of Amyloid by -CF3

If the later are stronger refolding would be more favorable. As a result an increase on the β-sheet structure content is measured.

Graph VI.3 – The curves a) and b) represent the CD spectra of amyloid's solution before (Δ) and after incubating the fluorinated sample (■); and a layer of adsorbed amyloid (√) after incubation and after 24h in water (○), for PF1251:0.5 and PEI-C4. The final adsorbed amounts in PF1251:0.5 was 1.6*10^3 mg/cm^2 and in PEI-C4 1.3*10^2 mg/cm^2. The curves are all normalized by concentration and path length. A fit for the spectra of adsorbed layers was introduced as is easier to discern differences. The original curves are plotted in the inserted graphs.

Curves c) and d) represents the variation on the secondary structure, calculated by CONTIN (—, --) and SELCOM (—, —) algorithms, of amyloid in solution (A), after incubation (B), adsorbed amyloid (C) and after remaining 24h in water (D). The higher % are refered to β-sheets and the lower to α-helices.
VI Folding of Amyloid by -CF₃

Only ~10% reconversion was observed so the chosen fluorinated surface does not change the energy enough to make the protein pass the energetic barrier from a stable β-conformation to another stable α-conformation, as does TFE in solution. Two causes can be pointed out:

- first in solution there is a peptide aggregate surrounded by fluorinated molecules which are free to move and that want to interact with the hydrophobic a.a. of the peptide, as this is energetically more favorable than the aqueous environment. On solid support we have a static system from which peptides can “escape” by aggregation or exchange with solution.

- second the peptide feels the surface at the peptide-surface interface being the rest of the aggregate the new surface for the next approaching molecules.

One possible way to design a future experiment would be to work with small colloids of fluorinated polymers. This would increase the interacting surface area between polymer and peptide (figure VI.5) and on the other hand would provide a surface sterically repulsive to the formation of large aggregates.

![Interaction area](image1)

![Adsorption Desorption](image2)

**Scheme I**

**Scheme II**

**Figure VI.5** – Schematic representation of surface area influence on the stability of aggregated molecules. In scheme I the peptide would only interact via adjacent a.a close to the surface. Molecules to desorb or aggregate in case of unstable situation next to the surface. In scheme II molecules are surrounded by the surface and unable to “escape” so the interaction area will be much larger.

To test further the influence of the surface on an amyloid solution was used a free standing film of a polyurethane modified surface with -CF₃ groups. This film is not optically transparent so it is impossible to use it to measure CD in transmission and check the conformation of the adsorbed monolayer.
Nevertheless this polymer film was placed in a solution containing amyloid for 1 hour and the CD signal of this solution before and after incubation measured. The results are plotted in graph VI.4.

![Graph VI.4](image)

**Graph VI.4 – Influence of a polyurethane free standing film modified with CF₃ groups on amyloid solution before and after placing the film in the solution for 1 hour. The concentration of the amyloid solution was 0.05mg/ml.**

Direct analyses of [θ] shows that the structure of the β-sheets in solution changed conformation, as observed before for PF₁₂₅ and PEI-C₄, but with PUR-CF₃ the changes were more pronounced. Estimation of the secondary structure before and after incubation showed an increase in the β-sheet structure of amyloid in solution of ~20%. This high variation on the β content was not observed with any of the previous samples.

With this result it is shown that the environment has a strong impact on amyloid towards the transformation process into β-structures: in this particular case the process was accelerated. The size of the aggregates before and after interacting the polymer film with the peptide were measured and there was no size variation. So the new β-sheet structure induced was on the early stages of aggregation.

The mechanism of this transformation is rather puzzling as the surface has to exert a long range influence on the peptide! Is there a chain reaction providing communication between surface→peptide near surface→peptide solution?!
VI.6 Conclusions.

The process of aggregation of amyloid in solution was followed for a concentration of 0.05mg/ml. The aggregates reached a constant diameter of 30nm. It was shown that it is possible to destroy these aggregates by solvents which reconvert the peptide into an α-helix conformation. This process is irreversible for amyloid which means that after transformation into an α-helix this structure remains stable. Contrarily stable β-sheet proteins like IgG are reversibly changed when in contact with TFE. From this result it is concluded that the peptide has two stable conformations α-helix and β-sheet. Fluorinated alcohols are very powerful solvents that help this peptide to overcome the energetic barrier for the β→α transition. TFE or ISOF would be ideal substances for curing the disease if they were not so toxic. A way to overcome this problem would be to chemically modify TFE/ISOF into non-toxic molecules. It is important here to refer that some fluorinated hydrocarbons possess anesthetic properties in humans and animals and so they are non-toxic [113,114].

In order to search for the -CF₃ impact in amyloid was used three different modified surfaces containing highly fluorinated interfaces to adsorb amyloid aggregates. PF₁₂₅₁:0.5 and PEI-C₄ surfaces were not powerful enough to destroy the aggregates and lead to a β→α transition. Nevertheless ~10% helix was introduced by PEI-C₄. When samples were placed in water for 24h rearrangements of the peptides on the surface played an important role and a higher content of β structure was finally introduced on the samples.

A solution of the amyloid peptides exposed to the PUR-fluorinated polymers has altered its conformation. There was an increase in the content of β-sheet in solution of ~20% with one hour of incubation while the normal process of reconversion in solution would take three days. The environment proved here to be very efficient on the chain reaction leading to reconversion of peptides and showed once more that external factors can be on the origin of the α→β transition in the blood. It is interesting to refer that nothing is known concerning this mechanism and the only thing proved is the possibility of one β-sheet peptide introducing β-sheet in a α-peptide. Where does this reaction start?

For a future approach it is possible that -CF₃ modified surfaces have a potential for blocking amyloid aggregation. It is necessary nevertheless to introduce a system that has a higher interacting area with the peptide as on flat surfaces peptides are allowed to rearrange and to exchange with solution. Colloidal particles could be an option because of their high surface are and shape.
VII - Conclusions

Fluorinated alcohols have a potential to the recovery of secondary structure of proteins. There is a strong correlation between the measured ellipticity and the physical chemical properties of the buffer/TFE mixtures showing that the water structuring in presence of TFE is the driving force for folding of proteins. The higher or lower recovery in the structure of proteins is dependent on the conformational state of the proteins, more denatured proteins fold with higher rates than native ones as they are more dependent from the H-bonds with water.

The TFE/water behavior was divided in four different concentration regions which are related to protein structure:

A - region where alcohol and water forms homogeneous mixtures (% w/v<sub>sol</sub> < 30) with slow recovery of the α-helical structure of HSA and Fin (native and denatured), and no impact in IgG;
B - transition region from 30 - 40% where alcohol molecules start to aggregate and the rate of recovery from the secondary structures has a turning point;
C - region of high alcohol clustering where proteins are still soluble (40 - 80%) and the induced ellipticity is greater, IgG suffers an α→β transition;
D - region where the alcohol/water properties behave like pure alcohol and proteins start to precipitate due to the lack of water molecules in solution.

Two model surfaces were used, hydrophilic quartz and hydrophobized quartz (OTS) to detect the protein structure variations by Circular Dichroism. This technique proved to be very sensitive to the structural changes of adsorbed proteins as it can detect multilayers, time or concentration dependence of conformational changes.

When adsorbed proteins denature, refolding was induced by taking advantage of TFE properties. TFE mixed with buffer diffuses and remains near the surfaces containing adsorbed HSA, with a rate dependent on the protein denaturation. As a result this protein reacts by diffusing faster, refolding and desorbing with a higher content of α-helical structure. The protein remaining on the surface is “Partially Folded”, with regions recognizable by the antibodies. There is recovery of the function of the protein as the new reinduced α-helices are present on places where old α-helices were before denaturation.

Based on the TFE properties several approaches were proposed for the design of biosurfaces that stabilize the conformation of adsorbed proteins:

1) Mixtures of -CF<sub>3</sub> with -OH thiols.

These thiols formed mixed monolayers where the organization is dependent on the amount of adsorbed -OH. It was revealed that the functionality of HSA is dependent on the thiol composition: mixed monolayers resulted in the best HSA recognition by antibody binding. This result shows the strong impact of small variations on the surface chemical composition on the protein functionality: an introduction of 5% hydrophilicity resulted already in an increase in the functionality of HSA.

2) Modification of PVMK with TFE units.

The presence of TFE units in the modified polyvinylketone polymer surface showed increased biocompatibility when compared to the unmodified one. The biological tests revealed the possibility of using this polymer for biological applications but further modifications are demanded. This polymer has only 60% of it’s chains modified with TFE units, so a further improvement should be the further fluorination of the polymer.
3) Use of complete fluorinated polymers.

Variations in the polymer backbone, complexation degree or length of the fluorinated chains had an impact on the conformation of the adsorbed protein. The outermost Å of the surface exposed to HSA adsorption had all the composition –CF₂–CF₃ but nevertheless there were variations in the α-helical content from 35% to 76%, above native. For enzyme immobilization or passivation of a surface with HSA these polymers could be suitable: strong bonds formed between surface and protein together with the possibility of maintaining the structure of adsorbed proteins.

The last application of the special properties of –CF₃ was to look for the conformation transition of amyloid. This peptide has shown to be able to form stable irreversible α-helices when exposed to fluorinated alcohols, like TFE or IsoF. One of the objectives to cure the disease is the blocking of the β-amyloid aggregation or the transition into stable α-helices. The fluorinated alcohols can not be used due to their toxicity but there are non-toxic fluorinated compounds, already used in mice with anesthetic properties. Further investigation is demanded.

The fluorinated surfaces used could only induce ~10% α-helix in the adsorbed amyloid. It is necessary to increase the surface-protein contact area in order to have a higher number of –CF₃ groups exposed to the peptide aggregates.
Appendix A

The amino-acids have all the general structure represented below by the Fisher projection (A, B). Draw C shows the 3D distribution of the amino-acid atoms, with Cα representing the carbon connected with the chemical group which is going to define each amino-acid, the R group.

Un-ionized form of an amino-acid

Dipolar ion (or zwitterion) form of an amino-acid

The 3D picture shows the typical bond lengths of -C=O and N groups. They form rigid and planar structures with the -C=O and -N-H bonds in the same plane. -Cα is out of plane and can rotate with an angle φ and ψ if bonded to -N or to -C=O, respectively. The allowed values for φ and ψ are dependent on the secondary structure assumed by the peptide and can be predicted by the Ramachandran plot.

The R groups differentiate the 20 different existent amino-acids in. They are known by the following abbreviations:

<table>
<thead>
<tr>
<th>Amino-acid</th>
<th>Three-letter abbreviation</th>
<th>One-letter symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine (apolar)</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Arginine (basic)</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>Asparagine (polar/uncharged)</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Cysteine (Polar/uncharged)</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Glutamine (polar/uncharged)</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Glycine (apolar)</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td>Histidine (basic)</td>
<td>His</td>
<td>H</td>
</tr>
<tr>
<td>Isoleucine (apolar)</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>Leucine (apolar)</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Lysine (basic)</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Methionine (polar/uncharged)</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td>Phenylalanine (aromatic)</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td>Proline (apolar)</td>
<td>Pro</td>
<td>P</td>
</tr>
<tr>
<td>Serine (polar/uncharged)</td>
<td>Ser</td>
<td>S</td>
</tr>
<tr>
<td>Threonine (polar/uncharged)</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td>Tryptophan (aromatic)</td>
<td>Trp</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine (aromatic)</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Valine (apolar)</td>
<td>Val</td>
<td>V</td>
</tr>
</tbody>
</table>
Appendix B

Brief description of the structure of the different proteins used in this work. By knowing how they look like it is easier to imagine how would they react in contact with a surface or with a foreign molecule like an alcohol.

B.1 Human Serum Albumin (HSA)

HSA [115] protein is relatively large, with multidomains formed by one single peptide chain. Due to its high abundance in the blood it has many physiological functions: the transport and distribution of substances like phospholipids which otherwise would be insoluble, ions like Ca$^{2+}$, pharmaceuticals like aspirin and for maintaining the colloid osmotic pressure in the blood. It is “heart-shaped” with the two halves from the “heart” maintained by salt bridges and hydrophobic interactions, it is flexible due to the high possibility for binding to molecules and forms basically $\alpha$-helices (67%). Changes in pH provoke the expansion of the protein to conformers like $F$ and $E$, which can possibly be found when this molecule adsorbs to solid surfaces.
B.2 Immunoglobulin (IgG)

IgG protein [27], with defense properties, is synthesized in response to the presence of a foreign substance. It is Y shaped with two kinds of peptide chains, a light (L) and a heavy (H) chain. These chain are connected by three intermolecular disulfide bonds which maintain the structure of the peptide. Each IgG recognizes only one binding site of a foreign molecule and this is done via the two Fab (antigen binding fragment) regions at the end of the peptide. The Fab area is defined in the drawing by \( V_H \) and \( V_L \) (variable heavy and light chain regions) being \( V_L \) similar to \( V_H \). It is the variability of \( V_H \) and \( V_L \) which differentiates antibodies. The other end, Fc (complement fixation), does not bind the foreign substance, but mediates the protective response against the intruder, like the approach of macrophages which contain receptors for Fc.

The specificity of these molecules makes them very attractive for use in key–lock system like ELISA, described in chapter II.
B.3 Fibrinogen (Fin)

Fibrinogen \([116]\) is a rather important protein in the chain reaction leading to coagulation. If activated it converts into fibrin, catalyzed by the enzyme thrombin. Fibrin polymerizes spontaneously following clotting. If fibrinogen is adsorbed into a solid surface, due to conformational changes, it can provoke also the activation of the coagulation processes. On the other hand the platelets have receptors for this molecule which can bind and also lead to coagulation.

The structure of this protein can be divided into two identical parts: each has three pairs of non-identical polypeptide chains held together by 11 disulfide bridges. Thrombin acts in the central part of the fibrinogen by cleaving four arginine-glycine peptide bonds and originating the referred above fibrin.

---

B.4 Amyloid

Amyloid is a small polypeptide chain 40-43 amino-acids long, belonging to a protein with \(~770\) amino-acids (APP, figure 6.1). APP is a transmembranar protein where the amyloid peptide chain occupies the region between the intra- and extra-cellular medium. Due to its "border" location it has a great number of hydrophobic amino-acids.
When inserted in APP this peptide forms two small α-helices with the structure pictured below. This structure was designed [117] accordingly to its amino-acid sequence where the hydrophobic regions correspond to the represented random coil.

This structure is representative of the non-infectious form of this peptide: as it is soluble in the blood it is possible to eliminate it. By an unknown process these peptide molecules switch into a β-sheet conformation which tends to aggregate. This new structure corresponds to the infectious form of amyloid. These aggregates form long insoluble fibrils that precipitate in the brain causing Alzheimer disease.
Appendix C

C.1 PVMK-TFE interdigitated with PUR

Structure of polyvinylmethylketone-TFE (PVMK-TFE) interdigitated with a polyurethane (PUR). The formation of the PUR comes from the reaction of polyethyleneglycol (PEG) with an isocyanate. The surface is modified with an aminosilane which by reacting with the isocyanate, giving an urea, covalently bonds the polymer to the surface. PVMKTFE remains interlinked with the formed polyurethane.

C.2 Completely fluorinated polymers.

PEI-C$_{4/7/10}$
Polyethyleneimine formed fluorinated complexes by reaction with fluoracetic acid of different chains lengths (Thünemann). This work used complexes formed with $n = 4, 7, 10$. 
Another fluorinated polymer resulted from the reaction of polysiloxanes with fluoroacetic acid. Here $n = 8, 12$. The degree of complexation can be varied by changing the temperature. The polymers used had 1:1 or 1:0.5 complexation.
Appendix D

CONTIN
Provencher et al. [53] fitted the CD spectrum, \( cd(\lambda) \), of an unknown protein by a linear combination of CD spectra, \( R_i(\lambda) \), being \( R_i(\lambda) \) represented by a much larger number of reference spectra rather than just one set of 4 spectra like \( b_k(\lambda) \). They used 18 reference CD spectra of known proteins where the fractions of the different structures were calculated based on X-ray data. This way they increased the number of variations possible for combinations of structures. In their work they applied the method of ridge regression (or constrain statistical regularization) to control instabilities from the curve fitting of equation 38, involving many structural parameters. With the introduction of a coefficient \( \gamma_i \), which is to be determined, equation 38 can be written as:

\[
\text{cd}(\lambda) = \sum_{i=1}^{N_S} \gamma_i R_i(\lambda)
\]  \hspace{1cm} (47)

and the secondary structure of proteins is calculated from the relation for known \( \gamma_i \):

\[
f_k = \sum_{i=1}^{N_S} \gamma_i f_{ki} \hspace{1cm} k = 1, \ldots, n_i
\]  \hspace{1cm} (48)

Here \( N_{CD} \) is representing the number of CD spectra existent on the data set, \( n_i \) is the number of secondary structures and \( f_{ki} \) is the fraction of the \( k \)th conformation of the \( i \)th protein. The \( \gamma_i \) values can be determined from \( N_S \) experimental ellipticities by minimizing the following relation,

\[
\sum_{j=1}^{N_S} \left[ cd(\lambda)_{\text{cal}} - cd(\lambda)_{\text{obs}} \right]^2 + \alpha_r^2 \sum_{i=1}^{N_{CD}} (\gamma_i - 1/N_{CD})^2
\]  \hspace{1cm} (49)

with the constraints from equation 48. The second term is called the regularizator and its relative strength is determined by the regularization parameter \( \alpha_r \). With \( \alpha_r = 0 \) this equation is simply reduced to the least square solution with low sensitivity towards noise. With increasing \( \alpha_r \) the second term on the right side of the equation tends to stabilize the solution by keeping \( \gamma_i \) small, i.e., near \( 1/N_{CD} \), unless the solution is tending to the spectra of reference proteins from the set that fit well with the unknown spectra, resulting in a smaller first term in equation 49.

SELCOM
The other method developed by Sreerama and Woody [54] again assumed a set of linear equations relating the CD spectra with the secondary structure. They used a set of 15 proteins and 2 \( \alpha \)-helix polypeptides as reference spectra. Their mathematical representation is described by the matrix equation:
\[ F = XC \]  \hspace{1cm} (50)

where \( C \) is the \( m \times n \) matrix of all CD data from the reference spectra; \( F \) is the \( l \times n \) secondary structure data matrix; \( X \) is an \( l \times m \) matrix relating the spectra to the structure. With \( n \) representing the number of proteins, \( l \) being the secondary structure elements and \( m \) the number of wavelengths used to represent the CD spectra. The spectrum, \( cd \), that is going to be analyzed is then included in the matrix \( C \) and one obtains a new \( C_1, m \times (n+1) \) matrix. All proteins in this matrix are then arranged in increasing order of the root-mean-square (RMS) distance (\( \delta \)) from \( cd \), the spectra to be analyzed. The spectra which are least like the spectrum of interest are systematically deleted which increases the speed in finding a solution. A new \( C_2 \) matrix is formed with \( cd \) placed in the first row. The \( F \) matrix also originates the \( F_2 \) matrix. Equation 48 becomes:

\[ F_2 = XC_2 \]  \hspace{1cm} (51)

The elements of the first row in \( F_2 \) are unknown and correspond to the protein to be analysed. An initial guess for the different structures present, \( f_0 \), is made. This guess is based on the known structure which has the secondary structure nearer the spectra to be analysed, \( cd \). The equation 49 is ready to be solved for \( X \). Then it is possible to calculate the new structure \( f \):

\[ f = Xcd \]  \hspace{1cm} (52)

The matrix \( C_2 \) was decomposed using the single variable decomposition algorithm as the product of three matrices:

\[ C_2 = USV^T \]  \hspace{1cm} (53)

where \( U \) and \( V \) are unitary matrices, \( S \) is a diagonal matrix, and the superscript \( T \) is the transpose of the matrix. Combining equations 52 and 53 one obtains:

\[ X = F_2VS^{-1}U^T \]  \hspace{1cm} (54)

with \( S^{-1} \) being the inverse of \( S \). Only the first few diagonal elements of \( S \) are required to reconstruct the matrix \( C_2 \) to within experimental error and not all proteins contribute significantly toward the solution. A set of solutions \( f \) are obtained by varying the number of proteins considered (\( N_{CD} \) from 3 to \( N_{CD}+1 \) for analyses and the number of singular values (\( Ns \) from 1 to \( N_{CD}-2 \) or 7, whichever is the smaller) used to construct \( X \). Only those solutions which satisfy the conditions below are considered.

\[ |f_k - 1.0| \leq 0.05 \quad \text{and} \quad f_k \geq -0.025 \]  \hspace{1cm} (55)

For each value of \( N_{CD} \), the number of proteins retained in the basis set, a set of solutions meeting the above criteria was obtained corresponding to various values of \( Ns \). For each value of \( N_{CD} \), the single solution with \( \sum_k f_k \) was retained. These solutions are then averaged to obtain the final solution, \( f_i \). The solution \( f_i \) now replaces \( f_0 \) in the matrix \( F_2 \) as the second approximation to the unknown structure. The process is repeated until the RMS difference
between the successive solutions was less than 0.0025. The RMS deviations ($\delta$) and correlation coefficients ($r$) were calculated using:

$$\delta = \sqrt{\frac{1}{N_{CD}} \sum (x_i - y_i)^2} \quad (56)$$

$$r = \frac{N_{CD} \sum x_i y_i - \sum x_i \sum y_i}{\sqrt{(N_{CD} \sum x_i^2 - (\sum x_i)^2) (N_{CD} \sum y_i^2 - (\sum y_i)^2)}} \quad (57)$$

where $x_i$ is the fraction of protein secondary structure from X-ray analyses and $y_i$ is the calculated secondary structure. For each $y_i$ calculated a new $r$, and a $\delta$ are obtained.
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[117] Extracted from the Protein Data Bank
Nomenclature

a – activity
A - absorbance
A_{sol} – absorbance of solution
A_{ad} – absorbance of adsorbed proteins
b_k – set of reference CD spectrum
C – concentration
C_0 – bulk concentration
D – diffusion constant
D_{AB} – diffusion of A in a stationary medium B
D_{TFE} – diffusion constant of TFE
dp – depth of penetration
E – electrical field
f – correction factor
f_k – structural fraction with conformation k
F – force constant
F_1 – fluorescence
F_m – maximum force reached by a platin ring when pulled out of a liquid
I – light intensity after passing a medium
I_a – adsorbed light
I_{LR} – intensity of left/right hand polarized light
I_0 – light intensity
k – Boltzmann constant
k_w – wavevector
K – ball constant
l – thickness
L – length of the crystal
m – mass
n – number of residues in a peptide chain
N – number of reflections
N_{cd} – number of CD spectrum existent in data set
N_i – number of secondary structures
Q – flow
r – radius
r – instrumental factor
r_r – correlation coefficients
R – gas constant
Rey – Reynolds number
R_r – mean ring radius
t – time
T – temperature
u – reduced mass
v – velocity
V – volume
v/v_{sol} – volume of alcohol over total volume of solution
z – distance
Nomenclature

$\alpha$ - alfa helice
$\alpha_r$ - regularization parameter
$\beta$ - beta sheet
$\delta$ - RMS deviation
$\varepsilon$ - extinction coefficient
$\phi$ - quantum yield
$\gamma$ - surface tension
$\gamma_i$ - phase difference
$\gamma_s$ - shear rate
$\eta$ - refractive index
$\lambda$ - wavelength
$\mu$ - viscosity
$\mu_{\text{ext}}$ - electric dipole transition moment
$\nu$ - frequency
$\theta$ - angle
$\theta_{\text{critical}}$ - critical angle
$\theta_{gi}$ - angle at grazing incidence
$[\theta]$ - molar ellipticity
$[\theta]_C$ - mean residue ellipticity of a complete random coil
$[\theta]_H$ - mean residue helicity of a complete helice
$\Delta \rho$ - density variation
$\Gamma$ - adsorbed amount
Acknowledgements

Help, stimulation, discussion, interest, fun are fundamental words in a work environment. To all of people who contributed not with words but with actions I would like to say many thanks. Of course not only from work lives a person so a special gratitude will go also to those that made these three years funny, distracting, happy, stressful, relaxing, enjoyable...simply marvelous!

To Prof. Dr. Helmut Möwald I want to express my sincere appreciation for great input and stimulation. Thanks for the opportunity, it was an unique experience.

Prof Manuel Coelho was an important key for my presence in Berlin and for that he deserves already my gratitude. All my thanks also for all the support given.

To Dr. Hubert Motschmann I have to say thanks for the interesting discussions.

Dr habil Horst Hermel my sincere gratitude for all the help and interest shown in my work.

To Prof. Dr. Stroeve I want to say thanks for helping me in the field of self-assembly monolayers and for the nice time I had in america. It was really important.

All my group, Sue-Min Bae, Gunter Moller, Randolf Teppner, Christian Radügue and last Michael Harke (just because you are last doesn’t mean you are less important, just opposite), I want to say thanks for the nice time we had.

And many other people like Irina Estrela-Lopis, Henning Krass, Stefano Leporatti, Andreas Fery, Tilo Pompe, Andre Moreira, Karin Riske, Antonio Valencia, Tina Tedeschi, Sergio Moya, Sebastien Bourbon, Michael Metersdorf,...sorry for my temperamental moods!

Special thanks also to Roland Wagner for all we discussed about chemistry and much much more...I came to learn the difficulties that man have in understanding women! It is so easy Roland!

I think I should mention some of the people I organized parties, dinners, poker nights because that was really formidable! I will leave here a challenge to Irina, Gunter, Karin and Henning: lets do it again! But this time we make it different, we only invite 2134 people!

Last I want to give a huge hug to my Parents for their lovely support and special care! It is really extraordinary all that comes from them!!!