

Protein conformational changes induced by adsorption onto material surfaces: an important issue for biomedical applications of material science

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Abstract. Protein adsorption on solid surfaces is a widespread phenomenon of large biological and biotechnological significance. Conformational changes are likely to accompany protein adsorption, but are difficult to evidence directly. Nevertheless they have important consequences, since the partial unfolding of protein domains can expose hitherto hidden amino acids. This remodeling of the protein surface can trigger the activation of molecular complexes such as the blood coagulation cascade or the innate immune complement system. In the case of extracellular matrix, it can also change the way cells interact with the material surfaces and result in modified cell behavior. In this review, we present direct and indirect evidences that support the view that some proteins change their conformation upon adsorption. We also show that both physical and chemical methods are needed to study the extent and kinetics of protein conformational changes. In particular, AFM techniques and cryo-electron microscopy provide useful and complementary information. We then review the chemical and topological features of both proteins and material surfaces in relation with protein adsorption. Mutating key amino acids in proteins changes their stability and this is related to material-induced conformational changes, as shown for instance with insulin. In addition, combinatorial methods should provide valuable information about peptide or antibody adsorption on well-defined material surfaces. These techniques could be combined with molecular modeling methods to decipher the rules governing conformational changes associated with protein adsorption.

Key words: protein, conformation, unfolding, aggregation, material surface, nanostructure.

1. The importance of protein-material interactions in cell biology and for medical or pharmaceutical applications

1.1. Protein-material interactions are key to understand the interactions between living cells and material surfaces.

In biological tissues, cells are attached to each other and to the extracellular matrix, a complex but precisely defined network of proteins and polysaccharides secreted by the same or other cells. In addition to its mechanical role, the extracellular matrix exposes signaling domains or adsorbs growth factors that influence the cell behavior. In most experiments, biologists try to reconstitute the cell micro-environment on plastic or glass surfaces. It is indeed well known that many cells spontaneously die (by apoptosis) when either a solid support or a suitable biochemical environment (presence of growth factors) is lacking. The cultured cells are not in direct contact with the solid surface, but instead bind specific molecular motives on the extracellular matrix adsorbed on the material surface. One well known example of these motives is the "RGD" amino acid sequence, found in many extracellular matrix proteins such as fibronectin, and which is the specific ligand of a family of integrin adhesion receptors in many higher eukaryote cells.

The way extracellular matrix molecules bind to the material surface is therefore of prime importance.

Quite often, both the extracellular matrix and the growth factors are initially provided to the cell culture in the form of "calf fetal serum", an undefined but rich mixture of proteins extracted from a fast-growing living being. Cell growth indicates that suitable matrix macromolecules have adsorbed to plastic. During cultivation, growing cells are likely to secrete other extracellular matrix components that can adsorb or replace the first ones. Another common cell growing technique consists in coating the plastic surfaces with "Matrigel". This hydrogel contains a mixture of polymers secreted by EHS tumor cells that are structurally similar to the basement membrane. Matrigel has attractive thermal properties that allow facile three-dimensional cell growth [1]. Matrigel is indeed liquid at low temperature and solidifies at 37°C. It is therefore easy to incorporate cells in the gel and plate them on solid surfaces. Because of the requirement for extracellular matrix, several kinds of plastic are manufactured, that help eukaryote cell growth by enhancing protein adsorption. The surface of commercial plastic materials are modified to enhance its hydrophobic or hydrophilic character or create immobilized positive or negative surface charges. In addition, biologists

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often use positively charged polymers, such as poly-L-lysine, to adsorb negatively charged cells to negatively charged surfaces such as glass.

Despite the fact that cells are not in direct contact with the underlying material, several reports indicate that they are sensitive to its nature. This is shown for instance by Sordel and coworkers, who studied CHO spreading on different material surfaces, using fibronectin as extracellular matrix [2]. In this work, the cell proliferation rate and cell morphology (round or elongated) were indicative of the influence of the material surface on intracellular signaling. The cell-surface adhesion was quantitatively measured by applying hydrodynamic viscous forces to detach the cells. On glass coated with poly-L-lysine or aminopropyl triethoxy silane (APTES), two surface treatments resulting in positive surface charge, the critical shear stress to detach 50% of the cells was similar, but 50% of the cells were elongated in the first case, and 80% were round in the second case. Conversely, there was a significant reduction of adhesion for cells grown on fibronectin- compared to APTES-coated glass (8 ± 0.2 Pa vs 3.9 ± 0.2 Pa), whereas no morphological change was observed. Furthermore, on silicon chips cleaned by O₂ plasma or treated with poly-L-lysine, cell adhesion was as high as on glass cleaned by NaOH or treated with poly-L-lysine, but cells were uniformly round. This indicates that different fibronectin and/or poly-L-lysine domains are exposed to the cells, depending on the underlying material, which triggers different cell behaviors.

1.2. Importance of protein-material interactions in medical implants and pharmaceutical systems. Besides cell culture, the behavior of materials in contact with biological fluid is of tremendous importance for biocompatibility in medicine and pharmacy. It has long been recognized that several negatively charged materials (glass, kaolin, celite, dextran sulfate polymers) induce fast blood clotting [3, 4]. The “contact activation pathway” of the plasma coagulation cascade is due to factor XII activation. Upon binding to negatively charged surfaces, Factor XII undergoes a conformational change that makes it 500-fold more susceptible to an activating proteolytic cleavage [5]. In addition, exposure of materials to the blood often triggers an innate immune response leading to inflammation, which is due to complement activation and subsequent reactions [6]. The material surface properties are critical, since the presence of OH or NH₃ groups are necessary for the covalent binding of C3b, which triggers several molecular and cellular responses [7]. Several complement components are involved in its activation: the C1 complex, the MBL-MASP complex or the ficolin-MASP complex, where MBL and MASP mean ‘Mannose Binding Lectin’ and ‘MBL-associated serine protease’, respectively. All of them are indeed large multimeric molecules associated with proteases, which are sensitive to the presence of closely repeated motives [8]. The enzymatic activity of the flexible C1 complex is induced when several domains simultaneously bind [9]. A similar activation mechanism holds for complement activation by the ‘lectin pathway’ [10, 11].

Conformational changes induced on these key proteins are therefore important issues for biocompatibility assays. As a consequence, the most effective technique to prevent contact activation of blood clotting, besides inactivating the protein cascade by EDTA or heparin, is to coat the material surface with protein-repellent polymers, such as poly-ethylene oxide or poly-ethylene glycol. It should, however, be noted that on the long term, these molecules become degraded, which abolishes their surface masking properties [12,13]. Furthermore, they do not prevent complement activation [14], which explains that the promises of PEG coatings expected from cell culture experiments did not hold in whole organisms [15]. Several other molecules (poloxamines, poloxamers, poly(2-methyloxazoline)-b-poly(dimethylsiloxane)-b-poly(2-methyloxazoline triblock co-polymers) are therefore under study [16, 17]. Conversely, surfaces that allow specific cell growth have also great medical applicability. For instance, reconstitution of the endothelial cell layer on artificial catheters is desirable because it ensures optimal blood compatibility [18, 19].

In protein biochemistry and in pharmaceutical formulations, material surfaces also play important roles. For instance, in protein crystallization trials or in high throughput screenings, many experimental conditions are tested in parallel. Reducing the size of the droplets, which is motivated by the cost and time of protein preparation, exacerbates the effects of the surfaces in contact with the protein solution. In pharmaceutical science, the role of material surfaces in the stability of therapeutical proteins has recently been questioned [20]. For instance, the material of prefilled syringes should fulfill two different roles, which put different limits on their design: long term protein compatibility and a good gliding of the plunger against the walls. It has been shown that model proteins such as albumin and lysozyme aggregate in the presence of silicon oil droplets used as lubricant [21, 22]. Implantable drug delivery systems, such as insulin pumps should also ensure long term protein stability and proper transfer into the body fluids.

1.3. Theoretical considerations on protein adsorption onto materials. Most biological phenomena occur in water, at an ionic concentration that considerably reduces the range of electrostatic interactions. As a result, proteins and materials interact by multiple short-range (Å) interactions (H-bonding, electrostatic, van der Waals or dispersive forces, hydrophobic effect), which are individually rather weak (a few k_BT or less) but sum up and give important adsorption energy for large macromolecules. In proteins, the internal energy that stabilizes their conformation state is rather large, typically 10⁴–10⁵ k_BT for a typical 300 amino-acid long protein. The energy possibly involved for extramolecular interactions at the surface of proteins is about 0.1–1 k_BT.nm⁻². Many protein domains have several conformations, and switching from one to another is often related to their function. The energy difference between these sub-states is much lower than the total folding energy, typically 1 to 10 k_BT, allowing fast transitions. The importance of protein flexibility in enzymatic activity and in protein recognition has been recognized for years [23, 24]. One should note that some protein domains are intrinsically

unfolded. The biological role of nonfolded protein domains is also the focus of considerable work in structural biology [25].

The classic view of Langmuir isotherm for protein adsorption on material surfaces assumes that some interaction energy is reversibly available to form a protein-material complex. The amount of adsorbed protein is limited by the available area on the material surface. In experimental conditions where the internal energy of the protein conformation is large, adsorption onto materials should not affect considerably the protein shape. On the contrary, when the internal energy of the protein is lower, more energy can be gained in the interaction with the material. Free energy takes into account an enthalpy term (energy associated with bond formation) and an entropy term (number of ways to realize a similar energy), which result from the replacement of protein-solvent interactions by protein-material interactions. This simple balance between the internal and external interactions was proposed by Norde to relate the protein behavior in contact with the material to their stability in solution: “flexible” proteins will more easily adsorb than “rigid” ones [26]. This classification is both clever and handy, because it relates protein adsorption on materials to two independent measurements: (i) the protein folding energy and (ii) the material surface energy. The protein folding energy in a given medium can be deduced from denaturation isotherms and the material surface energy in the same medium can be calculated from the drop contact angle. A three partners problem, protein, material and solution, is thus replaced by two simpler ones: protein and solution, material and solution. This simplifying approach however does not apply in more complex situations, where cooperativity takes place, for instance in the case of multidomain or multimeric proteins.

This concept of protein flexibility is related to the fact that part of many proteins is indeed unstructured. The existence of large unstructured protein domains has recently been evidenced by NMR, for soluble proteins. It seems that unstructured protein domain, can fold upon contact with other proteins, as shown by the example of natively unfolded HIV-1 Tat protein and the tetramerization domains of the cellular tumor suppressor protein p53 [27]. It is therefore conceivable that the fine nanostructure of material surface can help folding protein domains that are unstructured in solution. For instance, mastoparan form an amphiphilic – helix in the presence of phospholipids [28]. Unfolded protein domains are therefore good candidates to be the main interactive part of proteins with material surfaces.

The formation of oligomers plays also an important role in protein adsorption. Insulin is a good example. This soluble protein exists in three forms, monomeric, dimeric and hexameric (Fig. 1), depending on its concentration and on the presence of Zn^{2+} ions that stabilize the formation of its quaternary structure [29, 30]. The different quaternary structures are associated with conformational changes of the monomer, especially in the C-terminus of the B-chain and the α -helical part of the A-chain (Fig. 1, boxed area). The insulin monomer, which exposes lateral hydrophobic residues, interacts more readily with hydrophobic surfaces, which leads to its denaturation [31–34].

Modification of critical amino acids in the B-chain, either decreases (Asp28 and/or Pro29, [35, 36]) or increases (Glu13Gln, [37, 38]) the stability of the oligomeric forms. In the case of protein mixtures, a situation often encountered is the kinetic competition between several proteins on the material surfaces, which results in the ‘Vroman’ effect [39]. Abundant proteins adsorb first, but they become displaced in time by less abundant ones that exhibit higher affinity for the surface. When artificial surfaces are exposed to whole blood serum, fibrinogen is initially adsorbed during the first minutes, then it is progressively replaced by other plasma proteins, high-molecular weight kininogen, Factor XII and plasminogen [40]. This has been nicely shown by Brash and co-workers, using radioactively labeled proteins to follow their binding on glass [41]. Since competition depends on the dissociation rate constant of the protein from the surface, it is of importance to characterize this parameter. Many experimental techniques indeed exist to do this, such as Quartz-Crystal Microbalance (QCM) and Surface Plasmon Resonance (SPR) [42]. In the interpretation of many experiments, it is often assumed that the surface structure of the material is stable. This is not always the case. It is well known that gold or silver surfaces react with free thiols in proteins, mediating covalent bonding. Stainless steel surfaces are also reactive, because they are protected by a passivation layer that involves redox and H bonding interactions with the solvent [43], and they may release ions [44]. Even glasses exchange protons (surface pKa) and may release some ions in water [45, 46]. The reorganization of polymers explains the large amounts of protein that can be adsorbed on certain hydrogels [47] or multilayered polyelectrolyte films [48]. It would therefore be important to also consider the reorganization of the material surface during protein adsorption, especially for “soft” surfaces.

Finally, one should realize that when a material surface has been covered with a given protein, its surface properties are changed, and this modifies the adsorption of other proteins remaining in solution. In many biological procedures, “non-specific” adsorption of cheap proteins such as bovine serum albumin, ovalbumin, casein is indeed used to “block” the surface and prevent the unwanted adsorption of valuable proteins. Conversely, large protein coats can form on certain material surfaces ([42] see also the case of insulin below), which shows that in this case, adsorption of a given protein may enhance its further adsorption. Adsorption kinetics therefore depend not only on the amount of protein adsorbed, but also on the precise adsorption protocol [49].

To conclude this first part, protein interaction with material surfaces is an important and complex problem, with many biological, medical and pharmaceutical applications. These interactions involve protein conformational changes, maybe coupled to a reorganization of material surfaces. It would therefore be of interest to detect and measure protein conformation when they are adsorbed on material surfaces. In this review, we are going to provide evidences that proteins may alter their conformation upon adsorption and summarize the available techniques to monitor these changes (part 2), and

to examine how the reported material-induced protein conformational changes are related to protein and material structures (part 3). Future investigations will be proposed in this review (part 4) and at the AMT 2010 meeting presentation.

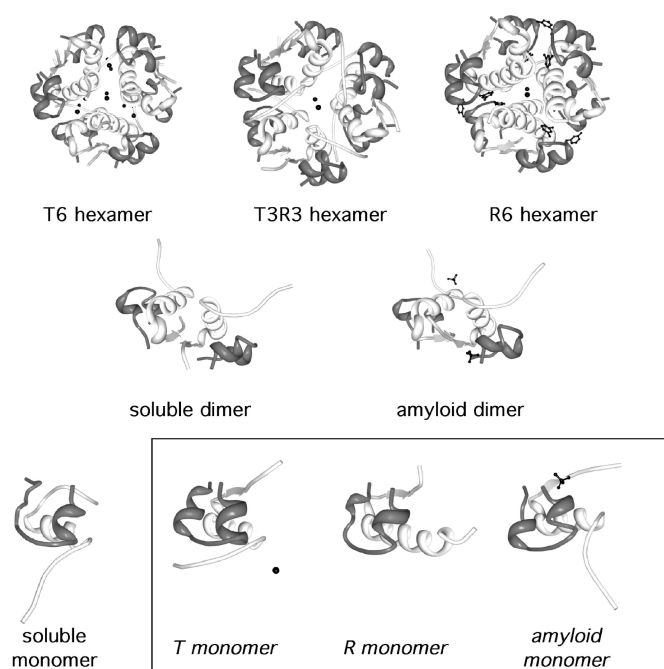


Fig. 1. The various conformations of human insulin in solution. Insulin is used as an example to illustrate the various conformations that a given protein can adopt, depending on its environment. From the top to the bottom and left to right are shown the following structures: T6 hexamer (PDB 2A3G, Ref. 133) in complex with two Zn^{2+} ions, T3R3 hexamer (PDB 1G7A, Ref. 134) in complex with four Zn^{2+} ions, R6 hexamer (PDB 1EV3, Ref. 135) in complex with four Zn^{2+} ions and phenols, Insulin dimer (PDB 1B9E, Ref. 136) of the B9 Ser→Glu mutant Insulin amyloid fiber (PDB 1GUJ, Ref. 137) obtained at pH 2. Insulin monomer in solution at physiological pH of the mutant (B1, B10, B16, B27) Glu B30 Δ , (PDB 1HUI, Ref. 138). In order to better visualize the differences between the different conformations, the structures of the insulin monomer within the T6 and R6 hexamer, and within amyloid fibers are also shown in the boxed area. The polypeptide chains are conventionally represented as ribbons, omitting the amino acid side chains. The A chain is shown in grey, the B chain in white and the other molecules present in the structure in black. All structures were displayed, scaled and oriented with the 'Protein Workshop' software freely available at the Protein DataBase, Ref. 139.

2. Evidences of material-induced protein conformational changes

2.1. Direct evidences of material-induced protein conformational changes. Given the nanometric size of proteins, it is difficult to visualize their conformational change directly in their liquid environment on a material surface. Two techniques have the necessary spatial resolution, atomic force microscopy and cryoelectron microscopy, but the sample is often dried or frozen. Fluorescence energy transfer between close fluorophores can also be used to evidence conformational changes at the nanometer scale, and this technique provides informa-

tion about changes in the distance between labeled protein domains. In the next paragraphs, we are going to review results recently obtained with these techniques that reveal the extent of protein conformational changes on material surface.

Large molecules containing several domains such as fibronectin [50], collagen [51] or myosins [52] have been imaged by AFM, which shows that this technique is able to resolve internal macromolecular movements, with a good time resolution [53–55]. Visualizing nanometric-size 'soft' objects requires the bare surface to be very flat. Most studies were therefore performed on mica, graphite or quartz. In addition, AFM imaging should be performed in solution, since drying is likely to severely perturb the protein conformation and arrangement on the surface. Another limitation of the method is that proteins should be firmly bound to the surface of interest, since lateral diffusion over the surface or interaction of soluble macromolecules with the tip of the probe prevents the interpretation of the images [51, 56]. As a result, when large amounts of protein are adsorbed on material surfaces, resolving individual proteins is not possible and the surface roughness instead reveals the presence of multiple layers of adsorbed proteins, as shown for immunoglobulins adsorbed on quartz [42] or lysozyme adsorbed on poly(ether sulfone) membranes [57]. Interestingly, by functionalizing the AFM probe or the surface with a molecule of interest, it is possible to probe its interaction with a given surface [58]. Using this technique, it was possible to show that poly(2-methoxyethyl acrylate) interacts more strongly with proteins in denatured conditions than in their native state [59]. From the retraction curves, the energy involved in their adsorption can be precisely determined, taking into account the pulling rate [60, 61]. For fibrinogen adsorbed on glass or mica, retraction forces are in the range 0.5–5 nN. Since the extent of molecular interactions in biological fluids is 1 Å, one can estimate the binding energy to be in the range 12–120 $k_B T$ [60]. This large value should be related to the extended shape of the fibrinogen molecule (3.5 × 35 nm). Cryo-electron microscopy [62] is widely used to image complex macromolecular structures such as motor proteins, proteasomes, ribosomes or viruses [63, 64]. Tomographic methods are used to reconstitute in three dimensions the electronic density of the objects, and the resolution is good enough for molecular structures of protein domains obtained at atomic resolution to be mapped inside the structures, which gives important cues about their relative orientation [65, 66]. Concerted changes of macromolecular assembly can therefore be resolved by this technique [67]. The structure of individual adsorbed fibronectin molecules in frozen samples has been observed by cryo-electron microscopy [68], which exemplifies the spatial resolution of the technique.

Fluorescence Resonant Energy Transfer (FRET) is an imaging technique that provides information about the distance between donor and acceptor fluorescent molecules. The intensity of the resonant energy transfer depends on the inverse sixth power of the distance, which makes FRET very sensitive to distance changes in the nanometer scale. This technique was used to probe internal conformational changes

in fibronectin on material surfaces [69] and in cell culture [70]. In these studies, the large fibronectin molecule was labeled with both donor and acceptor fluorophores so that the FRET signal gave indications about the extension of the molecules. Fibronectin associated to the cell body exhibited FRET whereas fibronectin present in focal adhesion did not, indicating a more extended structure at sites where fibronectin interacted with the material and was likely to be subjected to large cell forces. These conformational changes were confirmed by successive FRET measurements using fibronectin modified with GFP [71]. An attractive feature of FRET (that was not used in the reported studies) is the good time resolution achievable, which allows probing conformational changes at a sub-second time scale. A difficulty may come from the possibility of resonant energy transfer to certain material surfaces.

From these examples, it can be seen that FRET, cryo-electron microscopy and AFM give evidence for protein conformational changes upon adsorption on material surfaces, at least for large proteins. FRET and cryo-electron microscopy separately provide excellent time and spatial resolution, respectively. In addition AFM allows to estimate binding energies.

Another line of evidence comes from conformation-sensitive antibodies. Antibodies indeed recognize part of the surface (epitope) of the molecule (antigen) they interact with. Remodeling protein conformation therefore exposes different structural determinants that can be recognized by different antibodies. Such antibodies have been used to discriminate between the inactive and active conformations of a protein [72], to neutralize viruses [73] and to differentiate between the aggregated or monomeric form of proteins [74–76]. When proteins are adsorbed on material surfaces, some epitopes are masked, which reduces the binding of the corresponding antibodies [77–79]. Conversely, some internal protein epitopes may become exposed upon adsorption onto material surfaces, which increases the binding of the corresponding antibodies [80]. Monoclonal antibodies are therefore useful to determine the domain of interaction between proteins and material surfaces and to estimate the degree of unfolding of adsorbed proteins. Generalization of this technique to any given protein is nevertheless hampered by the difficulty to obtain or generate several conformation-sensitive antibodies. Physico-chemical methods, in contrast, are applicable to all proteins (see 2.3).

2.2. Indirect evidences of material-induced protein conformational changes. Conformational changes of extracellular matrix proteins adsorbed on material surfaces can also be monitored by their consequence on cell adhesion, spreading, proliferation and differentiation state. A good example is provided by fibronectin, a large modular protein containing repetitive domains widely used in cell cultures. Two of these domains expose an RGD motive, which binds and activates specific integrin adhesion receptors in cells. Several other domains are able to form intermolecular disulfide bridges that result in polymeric fibrils [81, 82]. The structure of fibronectin

layers thus depends on its density [83]. In addition, fibronectin interacts with other extracellular matrix proteins and modulates their cellular activity. For instance, fibronectin reveals secondary integrin binding sites on thrombospondin [84]. It is therefore not surprising that the effect of fibronectin on cells depends on the underlying material surface. Using different material surfaces, several groups showed that the strength of cell adhesion, measured for instance by applying hydrodynamic shear stress is uncorrelated with changes in cell morphology, round, spread or elongated, which reflects the activity of the actin cytoskeleton [2, 85]. Furthermore, cell differentiation depends on the biochemical structure and on the mechanical properties of the extracellular matrix [86–88].

Another line of evidence comes from protein aggregation induced upon contact with material surfaces. Since aggregated proteins are released into solution, this implies that materials, besides reversibly adsorbing proteins, are able to catalyze a conformational change resulting in protein self-assembly. The best-documented example concerns insulin, which forms aggregates when it is exposed to hydrophobic surfaces under agitation [33]. Insulin consists of two polypeptides with essentially alpha-helical tertiary structure linked by disulfide bonds. In physiological conditions such as phosphate buffer saline and 37°C, insulin conformation is stable and monomers self associate into dimers and hexamers, stabilized by Zn²⁺ ions. Nevertheless, in the same bulk conditions, insulin becomes unstable in the presence of hydrophobic surfaces [31]. Experiments conducted at different protein concentrations further showed that the insulin monomer is the molecular species that leads to aggregation, since higher protein concentrations reduce aggregation in the presence of hydrophobic surfaces, presumably by promoting insulin dimerization and hexamerization [32]. We reproduced these experiments and demonstrate that the nucleation reaction initiating aggregation takes place at the material surface (Ballet et al. unpublished). Furthermore, the aggregates released in solution bind thioflavin T, which suggests that insulin aggregates are stabilized by intermolecular beta-strands (see below, Subsec. 3.1). This points to the similarity between insulin aggregation at hydrophobic surfaces and the formation of insulin amyloid fibers, which can be obtained at low pH and high temperature (pH 2, 63°C) [89, 90]. Glucagon [91] and islet amyloid polypeptide [92] also aggregate on tantalum oxide coated quartz surfaces and mica surfaces, respectively. Material surfaces may therefore have profound effect on protein stability, compromising the long-term storage of therapeutical proteins.

2.3. Experimental tools to investigate protein conformation at the surface of materials. Several physical and biochemical methods exist to study protein unfolding in bulk solution, but not all of them are amenable to study proteins adsorbed on material surfaces. Circular dichroism spectroscopy, for instance, is widely used to characterize protein conformational changes in solution, but this technique is not sensitive enough to be applicable for monomolecular protein layers. Here we review the experimental tools that have indeed been used so far to detect protein conformational changes

at the surface of materials. Physical tools consist of IR and UV spectroscopy to characterize bond remodeling and Quartz Crystal Microbalance in Dissipative mode (QCM-D) to estimate the visco-elastic properties of the adsorbed protein film. These techniques are sensitive to both the total amount of protein and to their folding state. To separate both contributions, a precise quantification of the mass of protein adsorbed to the material surface is necessary, which can be provided by another technique, surface plasmon resonance (SPR). In addition, several fluorescent dyes have the property to intercalate in protein structures depending on their folding state, which makes them sensitive probes to conformational changes.

Conformational changes are known to affect amide bands (1700–1600 and 1500–1400 cm^{-1}) which makes IR spectroscopy attractive to monitor protein conformational changes. Experimentally, the IR spectrum of a protein of interest adsorbed on a material surface is measured by ATR-FTIR and compared to the spectrum of the protein in solution obtained by FTIR spectroscopy. This technique has been applied to evidence conformational changes during fibronectin adsorption of self assembled monolayers deposited on an IR-transparent germanium crystal [93], IgG adsorption on polyurethane [94] and lysozyme on different self assembled monolayers [95].

Tryptophan, tyrosine and in a lesser extend phenylalanine intrinsic fluorescence is a sensitive probe of protein conformational changes because the intensity and Stokes' shift of the fluorescence depends on local environment of the fluorophore. In addition, fluorescence polarization and/or lifetime measurements probe the rotational diffusion of the fluorophore, which makes intrinsic protein fluorescence also attractive to detect the formation of protein aggregates. This property has been used to characterize fibronectin and fibrinogen denaturation by silicones [22].

QCM-D measures the mechanical mass and viscosity of the thin layer at the surface of the crystal, and is thus sensitive to changes in water content and protein conformation. This technique has been successfully applied to the adsorption of glucagon to tantalum oxide [91]. Simultaneous changes in the resonance frequency (DFN) and the dissipation factor (DDN) at each N^{th} overtone are measured and analyzed using the Kelvin-Voigt model, which relates the observed shifts in frequency and dissipation to the thickness, density and viscoelastic properties (shear modulus and shear viscosity) of the forming layer. Compared to SPR signals (see below), QCM-D dissipation signals are able to detect conformational changes of adsorbed protein or protein aggregates, since they are sensitive to the mechanical properties of the protein film plus the associated water molecules.

Since the SPR signal is sensitive to the refractive index of the adsorbed layer, this technique allows real time measuring of association and dissociation kinetics of a protein of interest on material surfaces. This technique nevertheless requires a transparent sample, whose surface chemistry should be modified to mimic that of the material to be studied.

Another possibility to detect conformational changes is to use dyes that, upon binding to unfolded protein, give rise to a fluorescence or absorbance signal. Two such drugs have

been widely used to detect amyloid fiber formation in solution, Thioflavin T and Congo Red, but their sensitivity makes them useful to detect protein unfolding on material surfaces (Ballet et al. unpublished). Thioflavin T and Congo Red are small flat molecules that bind parallel to the long axis of amyloid fibers, most probably by inserting in channels between rows on amino acids, on both sides of the β -sheet [96]. This oriented interaction between Thioflavin T and aggregated proteins leads to fluorescence emission polarization, revealing that amyloid fibrils are oriented within an aggregate such as "spherulites" [97]. In the case of Congo Red, its binding to amyloid-like structures result in a shift of the absorbance peak. An isosbestic point is therefore present, but its position depends on the protein under study. Thioflavin T and Congo Red interact more strongly with intermolecular β -sheets in amyloid fibers than with intramolecular ones in native proteins, since the latter are more distorted and irregular and the former longer. A β -sheet indeed needs to contain at least 5 strands (strand to strand spacing 4.7 Å) to match Thioflavin T or Congo Red sizes (15.2 Å or 18.8 Å). The usefulness of Thioflavin T and Congo Red is limited to proteins that form amyloid-like aggregates on material surfaces. Recently, the environment-sensitive Sypro Orange fluorescent dye has been used to determine the thermal protein stability in bulk solution (Fluorescence-based Thermal Shift Assay) [98, 99]. As proteins unfold due to increasing temperatures, hydrophobic regions become more exposed to the dye, resulting in a large increase in fluorescence. The derivative of the fluorescence signal with time gives a very sensitive measurement of the unfolding temperature, which even allows resolving individual protein domain conformational changes. This technique should also be applicable to adsorbed proteins and would provide a measure of stability change associated to adsorption.

To conclude this second part, several techniques are available to monitor protein conformational changes on material surfaces. Unfortunately, X-ray diffraction and NMR, which would have provided ultimate resolution, are not sensitive enough to provide structural information on adsorbed material. The available physical means give partial information about the state of proteins, they should therefore be combined to determine whether conformational changes take place, and to what extent. In addition, the available techniques are difficult to interpret in the case of protein mixtures or when different protein conformations may be present. Fluorescent dye binding is attractive but requires careful study to check that they do not interfere with the protein adsorption process and to understand where they bind.

3. Structure-function relationships in protein-material interactions

3.1. Influence of the material surface: roles of surface chemistry and topology fine structure. At the material surface level, the details of surface chemistry and topology are likely to play a role on protein adsorption and conformation. Generally speaking, parameters that influence material stability in an electrolyte, such as the presence, thickness and

porosity of a surface oxide layer or the diffusion of atoms at the material-electrolyte interface are also relevant for protein interaction. For instance, it is known that gold and silver surfaces readily form covalent bonds with free thiol groups exposed by cysteine amino-acids. In addition, material surfaces can be complex, as exemplified by polycrystalline materials, where the grain crystalline orientation, their size, the structure of grain boundaries make the surface heterogeneous.

A recent report points to the importance of surface porosity on complement activation [100]. The complement system is a set of proteins that activate each other by partial proteolysis. It is generally activated by repetitive patterns found on pathogens, which trigger the innate immune response. The distance and orientation of the complement proteins is essential for their enzymatic activity. Using nanoporous aluminium oxide, the authors showed that more complement proteins adsorbed on the 200 nm than on 20 nm pore-size material, which in turn released more activated soluble complement components. It should be noted that the C1 component of the complement is a multimeric protein complex, whose size is about 20–30 nm, which suggests that the surface accessible to C1 is simply higher in the case of 200 nm pore size materials. Similarly, blood clotting is a physiological process involving a proteolysis cascade, that can be triggered by protein adsorption on materials. It has been reported that a nanocomposite material made of silica polyhedral oligomeric silsesquioxane (POSS) and poly(carbonate-urea)urethane (PCU) is less thrombogenic than the pure materials [101]. The thrombo-resistance properties of this material seem to originate from the presence of nanometric POSS cores embedded in the PCU matrix, again pointing to the importance of size matching between the material surface and the interacting proteins. In the latter case, one can hypothesize that individual Factor XII molecules bind to these cores but are too far away from each other to become activated. In addition, many studies point to the influence of surface topography on cell adhesion, besides surface chemistry [102–105], which suggests that it has a strong, but still uncharacterized, effect on the extracellular matrix protein conformation.

3.2. Influence of the protein detailed structure: comparison of mutant forms. Another approach to understand material-protein interactions is to compare different isoforms of the same protein, or different mutants. Measuring differences in protein adsorption can help determine amino-acids interacting with the material surface, or that play key roles in protein structure.

Along this line, Belcher and co-workers pioneered a combinatorial approach based on phage display techniques to uncover the many facets of peptide-material interactions. Phage display is a clonal selection technique using bacteriophages. These bacterial viruses are engineered to expose a peptide or an antibody fragment at their surface in fusion with the bacterial coat protein. The sequence of this peptide is encoded by the DNA sequence contained in the bacteriophage. A large collection of such nanometric particles is generated, each one exposing a different peptide at its surface, and is screened for

its affinity to a given molecular or material bait. The purified bacteriophages are amplified in bacteria and screened again. After repeating several times this enrichment procedure, a few bacteriophages are obtained that bind with high affinity with the bait. Sequencing their genome reveals then the sequence of the fused peptide. This method has been used to discover peptide sequences that bind to semi-conductor surfaces depending on their composition and crystalline orientation [106], or to ZnS or CdS quantum dots [107], or to conductive Chlorine-doped polypyrrole (PPyCl) polymer surfaces [108]. In the latter article, the involvement of specific amino acids in binding PPyCl was studied, showing that the peptide composition, but not the sequence order or the amino acids, was important, and pointing to the role of an aspartate amino-acid close to the C-terminus in PPyCl binding. Along this line, these results could be further extended using molecular biology techniques to systematically explore variants of a peptide obtained first by the phage-display technique.

In the case of hydrophobic surface-induced insulin aggregation (cf 2.2), the amyloid fibers released from the surface are quite similar to those obtained in solution at pH 2, which allows to gain benefit from the detailed structure-function studies on insulin fibrillation under the latter conditions. Both chains of insulin contribute to amyloid fiber formation [109]. Furthermore, short peptides (6 amino acids) from the A or B chain are able to form fibrillar aggregates on their own [110] and, under fibril forming conditions, they are protected from proteolysis and slowly exchange hydrogen with the solution at the amides along the peptide main chain [111]. More recently, an eight amino acid long peptide issued from the B chain, LVEALYLV, was shown to either inhibit or accelerate insulin aggregation, depending on the concentrations used [112]. The LVEALYLV peptide forms crystals where it is packed in parallel beta-sheets. These beta sheets run in opposite directions along the 3D structure, interacting in an anti-parallel manner through hydrophobic and electrostatic interactions. Based on these findings and on X-ray analysis of the fibrils, a model of insulin aggregation was proposed, that suggests that the insulin LVEALYLV segments will form two parallel leaflets of anti-parallel beta sheets, forcing the rest of the structure to also adopt an extended β -sheet structure [112]. It is tempting to speculate that hydrophobic surfaces would mimic one of the leaflet and help the other one form, the surface facing the solution then acting as a template to grow other layers. Further experiments are required to test this hypothesis, but nevertheless, it is clear that pinpointing the minimal structure able to aggregate on hydrophobic surface will shed light on the molecular mechanisms of this intriguing behaviour.

3.3. Modeling protein-material interactions. The availability of many protein molecular structures and the comparison of very large numbers of orthologous protein sequences generated by genome sequencing has given the opportunity to study protein-protein interactions to a sufficient level of detail so that predicting softwares could be developed. The current methods to predict protein interaction from 3D mole-

cular domains are based on the surface complementarity between proteins [113, 114], or on the solvent accessible area of amino-acids and on their general propensity to be part of interaction domains [115–117], or on the local desolvation energy [118]. A good evaluation of the accuracy of these methods is given by the “Critical Assessment of PRredicted Interactions (CAPRI)” experiment, which consists in comparing prediction models to experimental models of protein complexes provided by crystallographers and before publication. The coordinates for the individual proteins are given as a starting point for predictions. Between 2000 and 2005, 17 protein complexes were tested, and acceptable to good predictions was made on 12 of them. The 5 protein complexes, where predictions failed, not surprisingly involved large conformational changes. As a consequence, protein flexibility is now incorporated in prediction softwares [119]. It should however be noted that besides the correct predicted structures, many incorrect ones were also generated, which undermine the usefulness of these softwares, as for now.

Several softwares were also developed to predict protein disordered structures, that are likely to play an important role in adsorption [120–126]. None of them are fully reliable as yet and they should be combined to improve the predictions [127]. In addition, softwares exist to predict the propensity of protein stretches to form amyloid aggregates. The PASTA algorithm calculates the energy gained when parallel or antiparallel β -sheets are formed between the same stretch in a protein sequence [128]. The TANGO algorithm scans protein sequences for segments that are likely to simultaneously satisfy the following three properties: (i) to adopt a β -sheet secondary structure, (ii) to be buried (hydrophobic) and (iii) not to have any net charge (to avoid electrostatic repulsion or complementary electrostatic interaction) [129]. The Zyggregator method computes an ‘intrinsic aggregation propensity’ and a ‘local stability’ profile along the sequence, taking into account the physicochemical features of a seven amino-acid long stretch [130]. Regions of large ‘intrinsic aggregation stability’ and low ‘local stability’ map those involved in the formation of amyloid intermolecular structures. All these methods are able to correctly predict the output of point mutations, but they are not directly applicable to the case of multimeric or multidomain proteins. They point to the presence of ‘gatekeeper residues’ in protein sequences that prevent aggregation [131] or modulate the aggregation rate [132].

On the other hand, material surfaces are also described in great details. The relevant parameters for molecular interactions in solution involve chemical parameters (bulk structure, crystalline orientation, chemical homogeneities, redox and H-bonding properties, surface charges) and topological ones (atomic steps, surface roughness, grain size and boundaries). For a given material, electromicroscopy, XPS, AFM provide insights into the surface properties, down to the nanometer scale. It is therefore possible that softwares will be developed in the future to predict protein interaction with well-characterized material structures, such as silicon, graphite or mica surfaces that have no or well-characterized surface defects.

In conclusion, molecular modeling of protein-material interaction is not yet possible. Nevertheless, the pace of progress in protein modeling is extremely rapid, and protein interaction predictions should be available in the future, that are accurate enough to model their interaction with materials. In order to validate the algorithms, it will be necessary to carry out experiments with different model proteins and with protein mutants adsorbing on a set of well characterized surfaces. Protein mutants would help defining the interaction region and different material surfaces would pinpoint the relevant physicochemical parameters.

4. Conclusions: drawing a roadmap for future investigations

Although it is satisfactory, from an intellectual point of view, to derive the rules on protein material interaction from a set of examples studied in great detail, this is not technically practical, given the urgent need of practical information for biological, pharmaceutical and medical purposes. New methods should therefore be developed to monitor protein conformation in situ, even at a low resolution. They could be based either on the well-known optical properties of proteins or on the development of new molecular sensors of protein conformation. Fluorescence resonance energy transfer is a technique ideally suited to resolve nanometer changes in protein structure. In addition, the systematic study of peptide material interaction or antibody-material interaction, made possible by the phage display techniques, should provide very interesting insights into the relevant parameters. These approaches are especially interesting to study the effect of material surface defects, or when adsorbed proteins interact together, because no predictive protein model is as yet available on these issues.

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