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Keywords	protein adsorption; silica; MD; hydrophobicity; lysozyme (LSZ); bovine serum albumin (BSA)

Corresponding Author	Paul Mulheran
Order of Authors	Karina Kubiak-Ossowska, Barbara Jachimska, Mohammed Al Qaraghuli, Paul Mulheran

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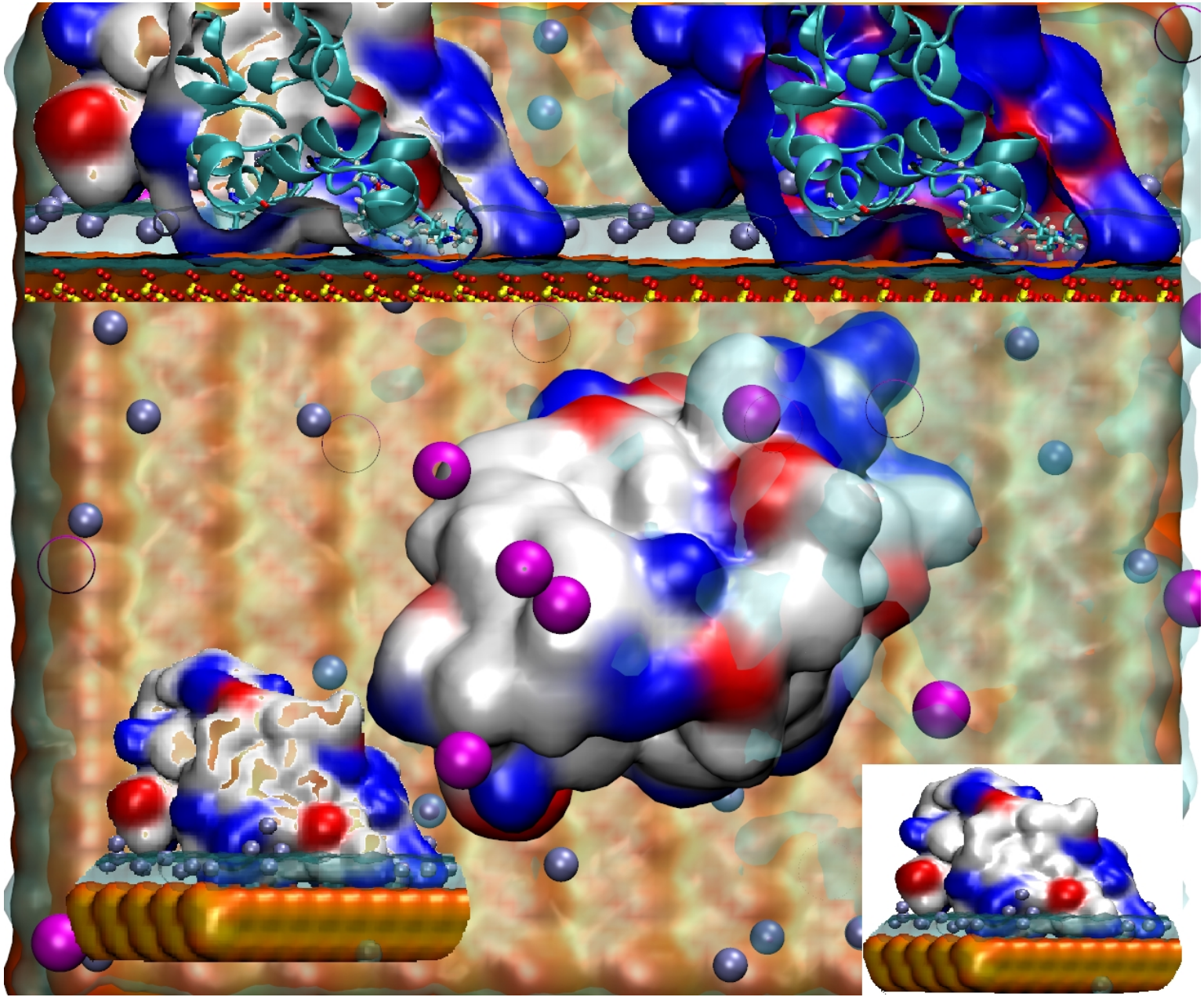

Protein interactions with negatively charged inorganic surfaces: simulation and experiment

Karina Kubiak-Ossowska[1], Barbara Jachimska[2], Mohammed Al Qaraghuli[3] and Paul A. Mulheran[3*]

[1]Department of Physics, University of Strathclyde, Glasgow, G4 0NG, UK

[2]Jerzy Haber Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, Cracow, Poland

[3]Department of Chemical and Process Engineering, University of Strathclyde, Glasgow, G1 1XJ, UK

*e-mail: paul.mulheran@strath.ac.uk

Abstract

Protein adsorption on charged inorganic solid materials has recently attracted enormous interest due to its various possible applications including drug delivery and biomaterial design. The need to combine experimental and computational approaches to get a detailed picture of the adsorbed protein properties is increasingly recognised, and emphasised in this review. We discuss the methods frequently used to study protein adsorption, and the information they can provide. We focus on model systems containing a silica surface, which is negatively charged and hydrophilic at physiological pH, and two contrasting proteins: Bovine Serum Albumin (BSA) and Lysozyme (LSZ) that are both water soluble. At pH7, BSA has a net negative charge while LSZ is positive. In addition, BSA is moderately sized and flexible, while LSZ is small and relatively rigid. These differences in charge and structural nature capture the role of electrostatics and hydrophobic interactions on the adsorption of these proteins, along with the impact of adsorption on protein orientation and function. Understanding these model systems will undoubtedly enhance the potential to extrapolate our knowledge to other systems of interest.

Keywords: protein adsorption, silica, MD, hydrophobicity, lysozyme (LSZ), bovine serum albumin (BSA)

1. Introduction

Adsorption of proteins onto various inorganic material surfaces is of significant importance, and can be applied in numerous disciplines including medicine and pharmaceuticals, where a new generation of drugs might be designed to be transported on nanoparticles for specific drug delivery (Fig. 1). Controlled protein adsorption might also help to improve containers for food storage; fast and efficient fuel production; and water purification that has a vital importance for billions of people globally. This potential impact has attracted growing interest from numerous researchers in both industry and academia. However, full understanding of the protein adsorption process is still under development due to the complexity of real systems, such as biological systems for medical applications. Bearing in mind the number of various proteins, the possible interactions between them

and the number of surfaces of interest, the topic appears very daunting. Therefore, simplified model systems are required for detailed study, enabling deep understanding and control that might then be translated to applications in more complex environments.

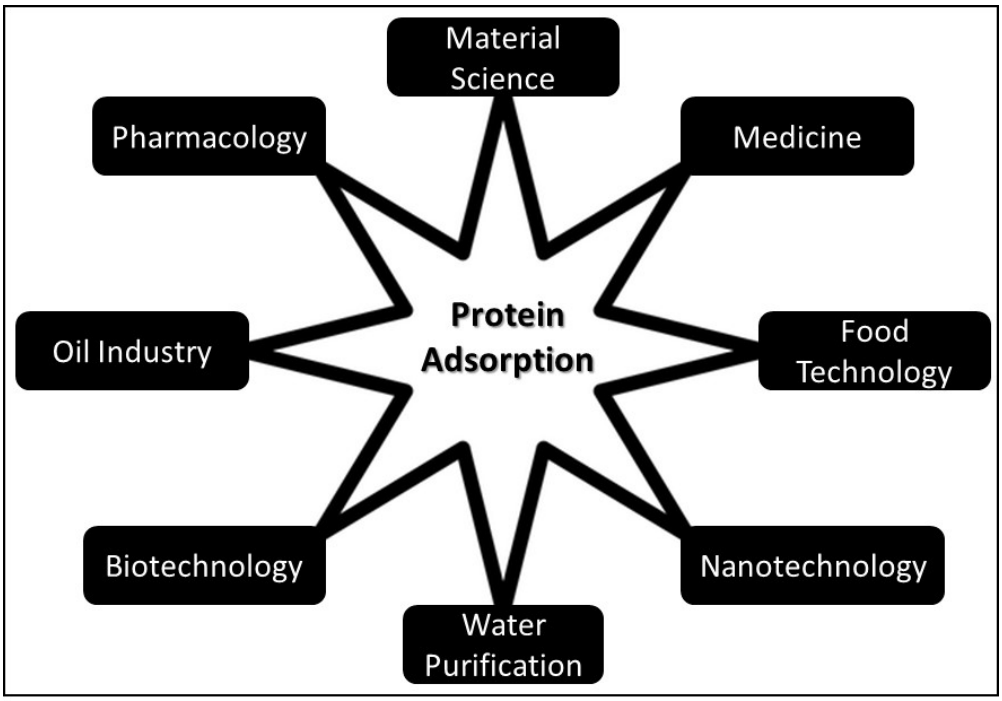

Figure 1. Applications that can benefit from improved understanding and control of protein adsorption.

Various types of surfaces have attracted high levels of interest, such as those composed of lipid bilayers [1], polymers [2-5] or copolymers [6], graphene [7-8] or graphite [9-10] and charged materials [11-12]. Amongst these, for their simplicity of use in experiments as well as ease of translation into theoretical modelling, silica-related materials are widely investigated as a model to understand the adsorption process [13-22]. The silica surface is biocompatible, and silica is classified by the US Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS), and is used in cosmetics and as a food-additive. It is also easy to handle and study using various experimental techniques. There is a wide range of literature that have focused on experimental data regarding silica itself, and for silica acting as a supporting surface for protein adsorption, as well as for mica where results can be extrapolated to silica surfaces [23-26]. Thus silica seems to be very promising model system for protein adsorption studies, which has allowed the creation of reliable and plausible models for Molecular Dynamics (MD) simulations [12,14-16,20,22,27-32]. Furthermore, numerous studies have focused on protein adsorption onto silica nanoparticles [27,33-36]. It is worth mentioning that for materials with surfaces that are less well-characterised, simulations are hampered by the lack of data for model validation. Consequently, only surfaces that are widely used in the laboratory (such as silica) are promising candidates for a computational approach.

Similarly to a credible model surface, protein candidates are also required to be studied both experimentally and through computational modelling. Of course, peptides are even simpler to manipulate than proteins, however, their simple structures and flexibility make data correlation

between systems challenging. Nevertheless, peptide adsorption studies have been used to validate the force field required for simulations of protein adsorption, to test new hypotheses in protein adsorption, and to propose new directions for studying protein adsorption [12,27,37-39]. A reasonable model protein needs to be easy to maintain and manipulate in the laboratory, inexpensive, and abundant in various biological systems, particularly in biological fluids. Therefore, a good candidate class seems to be albumin proteins that were adopted in early adsorption studies [40]. Among the studied albumins, Human Serum Albumin (HSA) is widely used along with its bovine equivalent, Bovine Serum Albumin (BSA); BSA is very similar in structure, function and sequence to HSA. Indeed, BSA adsorption studies using both experimental and computational approaches have recently attracted wide attention [5,8,14-15,18,22,26,30,41]. Another protein that is a good candidate for model studies, due to its size, stability, abundance and low price, is lysozyme (LSZ), which can be obtained from both human and chicken (hen egg white lysozyme, HEWL). Similarly to BSA, lysozyme adsorption has been extensively studied [23,42-43] and the hen egg white LSZ has been compared to the human counterpart for over 40 years [44]. Therefore, there is a wide knowledge allowing researchers to compare their findings and validate the computational models. Because of the relatively small size of LSZ, the computational costs of the simulations are comparatively low, hence it is one of the favourite protein models in theoretical studies.

The comparison of the adsorption process of BSA and LSZ is interesting due to the large difference in the proteins' size, stability of the structure (BSA is known as a flexible protein while LSZ is rather rigid), as well as nominal total protein charge and isoelectric point (pI). The BSA nominal charge is -17e at pH7 with isoelectric point at pH 5 [15], while the LSZ charge at pH7 is +8e with isoelectric point at pH 10 [29]. Interestingly, both proteins are easily adsorbed on a mica or silica surface [29]. Therefore, comparison of experimental and computational studies performed under similar conditions can give genuine insight into the underlying interactions and the competitive role of various factors, such as: protein internal flexibility, size, mobility, and hydrophobicity; the role of strong (electrostatic) and weak (van der Waals) forces; and the role of water in the layer above the silica surface. Based on physical understanding, the conclusions can then be extrapolated to other proteins and surfaces to understand the key factors governing protein adsorption.

The main experimental and computational methodologies used to study proteins and protein adsorption are summarised in Table 1. The main difference between experimental and computational approaches is that in the case of the latter the biological system needs to be translated to a physical model for which simplified force fields can be used, while the former can study the system directly. Nevertheless, due to rapid development of algorithms and computer power since 1960s, the modelling methods have become an integral part of modern research. For example, Molecular Dynamics (MD) was initially used in 1959 for calculations of the movement of 32 hard sphere particles for a 1ns simulation duration [45], while now MD is routinely used to study material surfaces, proteins and water dynamics for hundreds of thousands atoms on a 100 ns timescale. Once the model is correctly parametrised and the results are validated, the computational approach can yield valuable insight into the studied system on the atomistic scale. Its limitations arise from the complexity of biological/experimental systems, and the relevant time scale of the studied processes. Although a milestone of studying a huge and complicated system has been recently achieved by all-atom modelling of HIV-1 virus capsid containing over 1,000 proteins for 1μs [46], typical simulations are still performed for single (or at most a few) proteins in the system. Therefore, the simulation results need to be extrapolated to reflect experiment. On the other hand, simplification of the studied system can provide full control and better understanding of the processes; once we understand a simplified model system, it is possible to gradually increase its complexity without losing this level of control and understanding.

Table 1. Most frequently used methods for studying protein adsorption mechanism and the information provided by them.

Method		Information
Experimental (In bulk)	Circular Dichroism (CD)	Structure, conformation, aggregation, folding
	Chromatography	Size, hydrophobicity
	Densiometry	Quantification of protein level (Density)
	Dynamic Light Scattering (DLS)	Size distribution, stability, aggregation, folding
	Fluorimetry	Conformation
	Enzyme Linked Immunosorbent Assay (ELISA)	Chemical composition
	Fourier Transform Infrared Spectroscopy (FTIR)	Structure, conformation
	Gel electrophoresis	Charge, stability, aggregation
	Isothermal Tritration Calorimetry (ICT)	Chemical composition, conformation
	MS	Chemical composition
	NTA	Stability, aggregation, folding
	Nuclear Magnetic Resonance (NMR)	Structure, magnetic properties
	UV-Vis Spectroscopy	Protein structure
	Viscosity	Protein shape, mobility, aggregation, unfolding
	Transition Electron Microscopy (TEM)	Stability, aggregation
	X-ray	Structure
	Z-potential	Effective charge
Experimental (On the surface)	Atomic Force Microscopy (AFM)	Surface imaging, surface roughness, viscoelastic and mechanical properties of surface and protein layer; protein orientation, conformation, aggregation; adhesion forces through molecular pulling experiments
	Attenuated Total Reflectance (FTIR ATR)	Surface properties, adsorbed protein structure, surface coverage, stability
	Confocal Laser Scanning Microscopy (CLSM)	Surface imaging (inc. adsorbed protein), protein aggregation
	Contact angle	Hydrophobicity of the surface, wettability, adhesion
	Elipsometry	Dielectric properties and refractive index of the surface, protein layer thickness
	Neutron Scattering	Surface coverage,
	Polarization modulation-infrared reflection-adsorption spectroscopy (PM-IRRAS)	Protein orientation, stability and conformation
	Quartz Crystal Microbalance (QCM) with energy dissipation monitoring (QCM-D)	Quantification of adsorbed protein mass, hydration, protein layer thickness, surface coverage, viscoelastic and mechanical properties

	Scanning Tunnelling Microscopy (STM)	Surface roughness, protein aggregation
	Small Angle X-ray Scattering (SAXS)	Size and shape of adsorbed proteins, distances between them
	Surface Plasmon Resonance (SPR)	Surface coverage, hydration
	Z-potential	Surface charge
	Reflectometry	Protein layer thickness
	Tensiometry	Surface tension
	X-ray photoelectron spectroscopy (XPS)	Chemical composition
	Transition Electron Microscopy (TEM)	Protein aggregation
	Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)	Protein aggregation
	Thermogravimetric Analysis (TGA)	Adsorption monitoring
Computational	Molecular Dynamics (MD)	Protein structure, stability, size and shape, mobility, aggregation, nominal charge, adsorption mechanism and interactions monitoring on atomistic level, hydrophobicity, charge maps, nominal charge, adsorbed layer thickness
	Steered MD (SMD)	Additional information on energetic barriers, mechanical properties

The other computational limitation arising from the relatively short (~100 ns) simulation times typically available, when compared to the time scale of biological processes, might be reduced by using accelerated methods such as Replica Exchange Molecular Dynamics (REMD), Coarse Grain Molecular Dynamics (CG-MD) or Steered Molecular Dynamics (SMD). In the case of MD adsorption studies, the proteins can be initially positioned near the target surface, so the time required for free diffusion in bulk can be negligible. Nevertheless, a fully atomistic, traditional MD simulation cannot be performed for sufficient time to be sure that all slow relaxation processes have finished, and for this reason, the majority of such simulations come with the caveat that they explore the initial stages of adsorption only.

The main limitation of the experimental methods is that full control over the system under study is never possible, as parameters such as ionic strength, pH, temperature, density, charge (or zeta-potential) are statistical values and are unknown locally. Moreover, there is no direct molecular-scale view of the system, and therefore, there is no guarantee that any measurement can provide full insight into the process of interest. As a result, in general, both experimental and computational approaches rely on some degree of interpretation; in the former due to the indirect nature of measurements, and in the latter due to the necessities of simplification. However, if both approaches are used to study the same system in a carefully controlled way, they can provide valuable and complementary insight, namely a molecular scale view of the protein behaviour supported by observable data. In combination, the whole is greater than the sum of the parts; the results from one approach might be used to validate the other, to direct new simulations or experiments, or to propose, confirm or reject particular hypotheses. The growing number of multidisciplinary groups suggest that the power of such a coordinated research programme has been widely recognised, and we shall expect further elucidation of protein adsorption phenomenon in the foreseeable future.

2. Role of electrostatics in protein adsorption

Various experiments have shown that protein adsorption is affected by the pH and ionic strength of the solution. Experimentally, the occurrence of maximum protein adsorption close to the isoelectric point of the protein is often observed [23,34,43,47-54]. To understand how the ionic strength of the system might affect protein adsorption, two components that influence the behaviour of the system might be considered: the interaction between the protein and the adsorption surface; and the interaction between protein molecules in the adsorption layer. Depending on the range of the ionic strength at which the adsorption process is carried out, various trends are observed: an increase in adsorption with an increase in ionic strength; and after exceeding a critical ionic strength, the opposite trend occurs.

The selected models in this review, namely the charged surface of silica and charged LSZ or BSA proteins, reflect the importance of electrostatics to the adsorption process. In the case of positively charged LSZ (nominal charge +8e at pH7) and the negatively charged surface, the situation seems to be straightforward, as illustrated by MD results displayed in Figure 2. Various experiments have clearly indicated that LSZ adsorption on silica and other negatively charged surfaces is strongly depend on the pH. Furthermore, the maximum adsorption (surface coverage), achieved between physiological pH and the LSZ isoelectric point (pH 7 to 10), is largely dependent on ionic strength of the solution [5,23,29,31,33-34,55-58]. It is worth noting that experimentally the effective (rather than nominal) charge of the protein can be measured as a function of pH and/or ionic strength.

The effective charge of a protein can be calculated from the electrophoretic mobility and hydrodynamic radius using the Lorenz−Stokes equation. Effective charge values are much smaller than theoretical values, indicating that 20−30% of the nominal charge is present on the surface of the LSZ molecule [23] or BSA [26,59]. The effect of lowering the surface charge by increasing the ionic strength can be explained by the co-adsorption of counter ions, which is often referred to as the ion condensation phenomenon. This can be visualised in the MD simulation results illustrated in Fig 2. A similar effect of counter ion condensation has been observed in the case of linear polyelectrolytes such as PSS, PAH or PAA [60-62], or branched [51,63]. These studies show how important it is to control both the influence of ionic strength and pH of the system because these parameters have a significant contribution to the effective charge of polyelectrolyte and biomolecular systems.

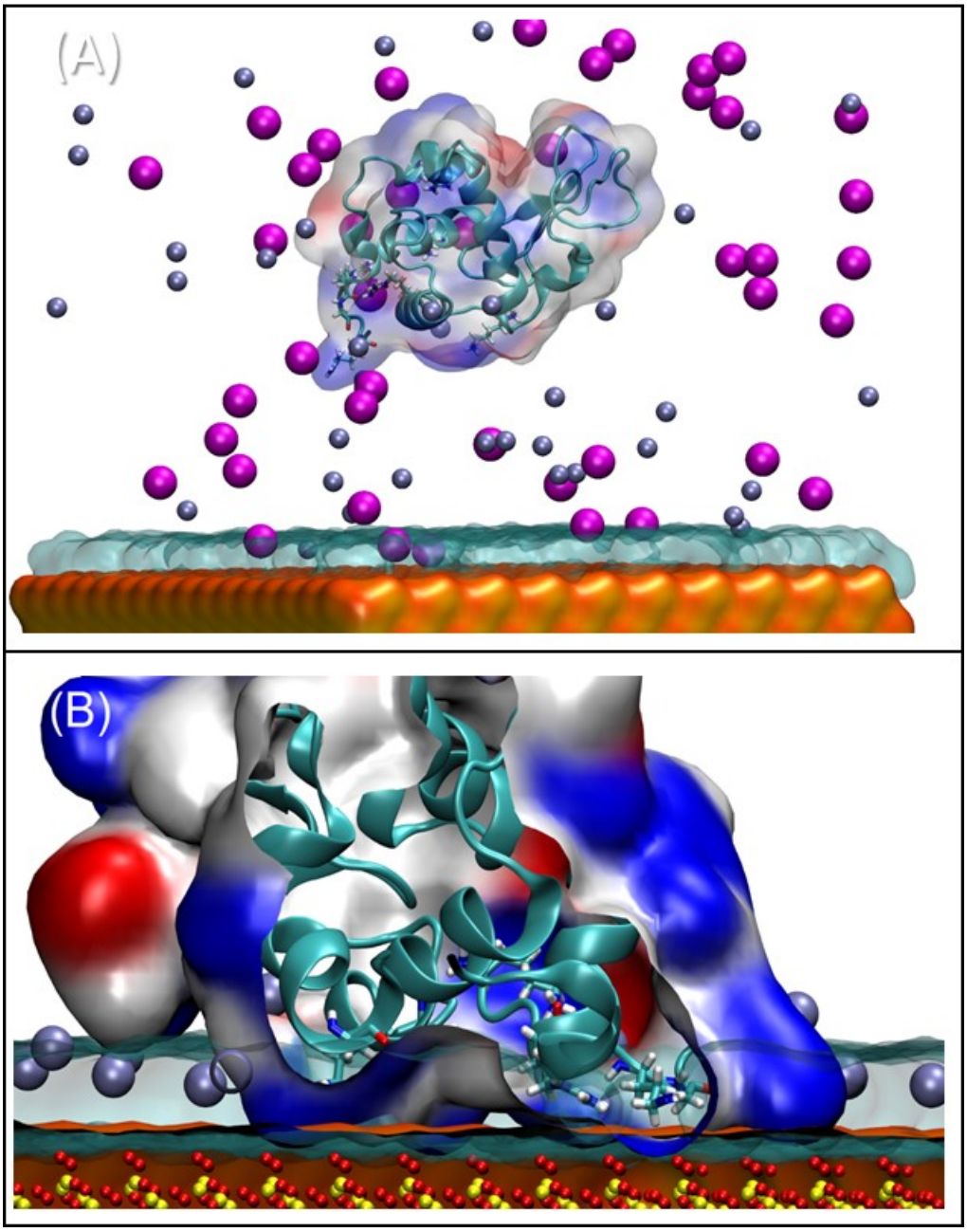

Figure 2. LSZ adsorption is driven by electrostatics. (A) Protein in the solution: the LSZ surface is shown as a ghost surface coloured by charge (positive – blue, negative –red, neutral – white), the secondary structure is shown by a cartoon representation (as defined in VMD [64]), and residues interacting with the silica surface (Lys1, Arg5, Arg14 and Arh128) are shown by licorice model coloured by atom type (oxygen – red, nitrogen – blue, carbon – cyan, hydrogen – white) . Solute ions are shown as VdW spheres: Na^+ ions are pink in colour while Cl^- are ice-blue. The surface water layers are shown as a blue transparent film, and the bulk water is not shown for clarity. The silica surface is shown as a quick surface representation coloured by name (oxygen – red, silicon – yellow). (B) LSZ adsorbed state: the colouring scheme is as above with two exceptions; the protein surface is opaque and the silica surface atoms are displayed as CPK spheres coloured by name.

MD simulations, both in the case of LSZ and BSA, have shown the effect of excess on delaying the adsorption. This is due to the creation of a counter ion layer at the surface (as apparent in Fig. 2A), which additionally complicates the electrostatics in the system and screens long-range interactions [5,31]. The strong dependence on pH and ionic strength are a clear indicator that LSZ adsorption on negatively charged surfaces is indeed driven by electrostatic forces. Nevertheless, when LSZ is neutral or negatively charged, at pH 10 and higher, adsorption also occurs [29,57] and cannot be simply explained as driven by electrostatics only. Other MD simulations have revealed that the electrostatic interactions are the dominant factor, however, they are modulated by hydrophobic forces [28-29,32,65]. This could be because both charged patches and the pattern of hydrophobic and hydrophilic residues at the protein surface play a crucial role. Such effects might be hidden at low and physiological pH due to the dominant role of stronger interactions, while it becomes noticeable above the LSZ isoelectric point. Similar conclusions have been made for other surfaces with characteristics similar to silica [66-67]; this effect is discussed further below.

Calculations give a direct insight into the most important residues anchoring the protein of interests to the surface [22,28,32,68-72]. The main residue anchoring LSZ on the silica surface is Arg128, while other positively charged residues located nearby, such as Arg5, Arg14 and Lys1, can create additional contacts with the surface and stabilise the LSZ adsorption [22,28,32,68-74]. The described scenario is in a good agreement with the observation that proteins containing numerous arginine residues and a small number of aromatic residues adsorb better on silica than others [27]. Interestingly, hydrophobic residues might also contact the surface to avoid interactions with the solvent water [28,32,68]. Simultaneously, aromatic ones are able to establish interactions with the surface via water-mediated contacts [73]. This is another indicator that in protein adsorption the pattern of charged, hydrophilic and hydrophobic residues plays an important role even for oppositely charged objects, and it is even more important in the case of similarly charged object, as in the case of BSA adsorbing on silica.

Due to BSA's charge at pH7, a simple charge consideration would indicate that it should be repelled from the silica surface, reducing the probability of adsorption. However, experimental evidence demonstrates that BSA does adsorb on negatively charged surfaces [14-15,26,41]. This can be rationalised by the fact that proteins are not homogeneously charged hard spheres, and that the charged groups are irregularly distributed on a particular protein surface. The charges usually appear in patches, as shown in Figures: 2 and 3 A, C, E. Moreover, the internal flexibility of the protein domains allows these patches to alter their location, producing a dipole moment that fluctuates in direction and magnitude. In addition, protein rotations and translations introduce further aspects to the changing dipole of the protein in the electric field above the charged surface [15,29-31]. The system complexity is compounded by the diffusion of the counter ions above the surface, which provide screening of the fluctuating electric fields arising from the surface and protein. Moreover, as illustrated in Figures 2 and 3, the water molecules can create well-organised layers on the surface that additionally modify the system electrostatics and create a barrier for the adsorbing protein [22,29-32,68-70,75-76].

In general, and despite the same polarity at pH7, BSA can adsorb onto hydrophilic and negatively charged surfaces [5,8,14-15,18,22,26,30,41]. Experimental investigations over a wide pH range indicate that the adsorption might surprisingly still be dominated by electrostatics [15,26]. Indeed, simulations at mild ionic strength and physiological pH have confirmed that the driving force is electrostatics [15,30].

As mentioned above, BSA is a flexible protein, composed by three domains which are further divided into A and B subdomains [77-80]. Both subdomains and domains substantially differ in the

total amount of positively and negatively charged residues so that, as illustrated in Figure 3, the charge distribution is heterogeneous. Moreover, the negative net surface charge is balanced by Na^+ counter ions, and as can be observed in MD simulations, the sodium ions create an additional electrostatic layer within the ordered water layers present on the silica surface to neutralise the charge of the surface itself (Figure 3). Therefore, at large distances from the surface, there is no time-averaged electric field, and the BSA protein, is free to diffuse and rotate as it would in bulk water alone. Adsorption might occur only if the favoured subdomain IIIB, which is one of the less negatively charged subdomains, is exposed towards the surface and with the protein dipole moment pointing towards the surface. Simultaneously, the distance between the protein and the surface needs to be small enough for the protein to feel the weak fluctuating electric field within the ionic screening layer of the surface. If the exposed part of protein is not optimal, the BSA might desorb and re-adsorb (Figure 3 A - D); in the most favourable orientation BSA is anchored to the silica surface via both negatively and positively charged residues. Positively charged Lys537 and Lys535 interact with the surface (directly or *via* water mediated contacts) while negatively charged Glu494, Glu541 and C-terminal Ala583 (with a partial negative charge) interact with the Na^+ ionic layer. The negative residues locally disturb surface ion layer and, therefore, positively charged residues are able to reach the surface directly. Recent systematic experiments performed for the closest BSA analogue, namely HSA and its variants, have confirmed a universal, electrostatic driven mechanism of albumin adsorption [21,81].

3. Role of hydrophobic interactions in protein adsorption

It is widely recognised that adsorption to hydrophobic surfaces can often cause loss of the secondary structure of the protein [41,82-86]. Sethuraman *et al*. showed correlations between changes occurring in the secondary structure and the properties of the adsorption surface and the degree of hydrophobicity [87]. The degree of change of the secondary structure is closely related to the internal structural stability of the protein [88]. Hydrophobicity affects both the amount of adsorbed protein, the thickness of the adsorbed layer and its structure. Consequently, properties of the adsorbed protein layer can be explored using water contact angle with the surface [26,89-94]. The value of the wetting angle of functional layers is of significant application importance because it is directly related to cell adhesion [95-97].

As mentioned above, negatively charged LSZ (at pH > 10) adsorbs on negatively charged silica, although the adsorption is less effective than at pH conditions resulting in positive LSZ charge, nevertheless it is still substantial. The dominant electrostatic forces might overwhelm the effect of hydrophobic interactions at low pH, while at high pH the short-range forces come more to the fore [28-29,32]. The list of the most important residues for LSZ adsorption contains mainly arginine and lysine residues, which, according to hydrophaty scale introduced by Kyte and Doolittle, are strongly hydrophilic [98], and therefore prone to interact with surface water layers. Consequently, adsorption of LSZ at low and middle pH is the result of both electrostatic and hydrophobic interactions, which can be de-convoluted only at high pH [28-29,32].

The impact of short-range interactions is more visible in the case of BSA adsorption, which is negatively charged for pH>5. BSA adsorbs on silica by its IIIB subdomain [15,26,30], which possess a total charge of -2e, while subdomain IIIA has total charge +3e [15,30]. Analysis of the BSA demonstrates that subdomain IIIA is less hydrophobic (hydrophaty index -40) than subdomain IIIB (hydrophaty index -16), and comparing with other subdomains, IIIB is simultaneously the most hydrophobic with low negative charge [15]. Detailed analysis of the BSA adsorption revealed by MD

simulation strongly suggests that hydrophobic interactions should not be ignored. To reach a relatively stable (final) adsorption state, the BSA needs to contact the model silica surface via a well-defined part of its surface, namely by strongly hydrophilic residues: positively charged Lys537 and Lys535, negatively charged Glu494, Glu541 and polar and neutral residues such as Thr495, Thr539, Thr580 (slightly hydrophilic) and Gln542 (strongly hydrophilic). The slightly hydrophobic and nonpolar Ala583 plays small role in the adsorption process due the fact that this is C-terminal residue (so possess a small negative charge). It has been demonstrated that another possible adsorption site (the initial one found by MD simulation), composed of a mixture of positively and negatively charged residues (Lys573, Glu570) supported by Thr580, Val569 and Val576, does not lead to a stable adsorption. In this case the protein centre of mass (COM) is ~10 Å further from the surface than in the stable adsorption state, and the BSA rapidly desorbs from this state to reorient and adsorb in the most preferred fashion [15,30]. This indicates that selection of the correct protein surface part is crucial for stable adsorption [15].

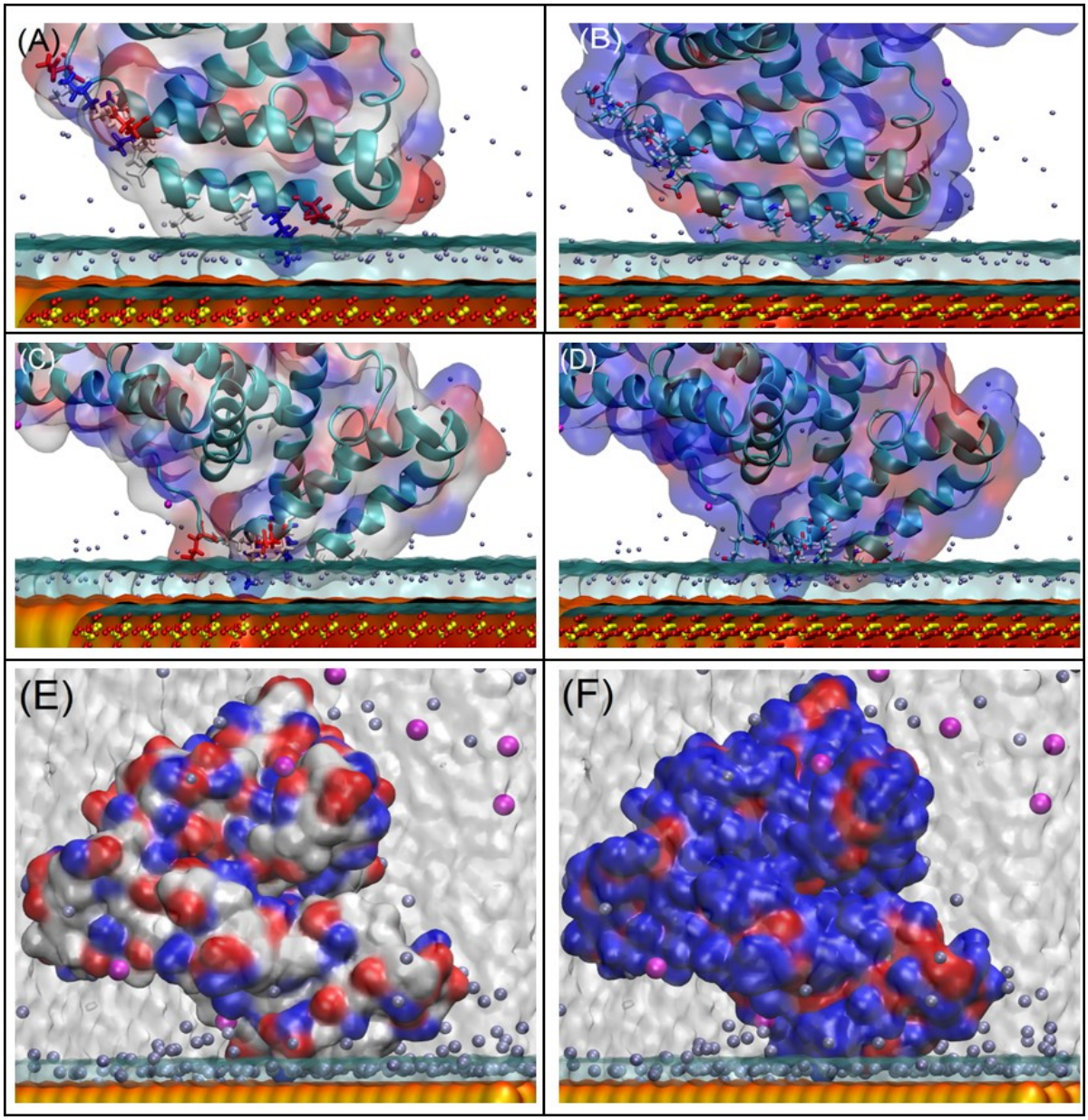

Figure 3. BSA adsorption is driven by electrostatics and modulated by hydrophobic interactions. Colouring scheme follows the one introduced in Fig. 2. **(A) & (B): Close view of initial adsorption state**.

The BSA surface is shown as a ghost surface coloured by charge (A) and hydrophaty index (B) defined in [98]. Hydrophobic residues are indicated by red while hydrophilic by blue colour. Residues involved in interactions with SiO_2 during initial (Val569¸Glu570, Lys573, Val576, Thr580) and final (Glu494, Thr495, Lys535, Lys537, Thr539, Glu541, Gln542, Thr580 and Ala583) adsorption state are shown by licorice and coloured by charge (A) or atom type (B). **(C) – (F): Final adsorption state.** Similarly to (A) and (B), (C) and (E) indicates the protein surface charge while (D) & (F) indicates hydrophobicity. To keep clarity the ions are shown as CPK representation in (C) and (D) which show a close view. (E) and (F) show a far view of the final adsorption state. The solute ions are shown as VdW spheres and bulk water is shown as a continuous grey film.

Comparison of both adsorption sites reveals that two conditions need to be fulfilled: (i) a good balance between positively and negatively charged residues; and (ii) correct hydrophilic characteristics [15]. Negatively charged residues attract Na^+ ions, and locally disturb the ionic layer. Adsorption is strong because residues interact simultaneously with the surface (directly or throughout tightly bounded water layers), while other residues can interact with surface screening ions to stabilise the adsorption state. In the first instance, electrostatic condition is fulfilled in both adsorption states, although initially through two charged residues, but then with four in the final state. Secondly, the hydrophobic condition is well met in the final adsorption state only, where all residues involved in interactions with the silica surface are strongly hydrophilic (five residues), hydrophilic (three residues) and only one is slightly hydrophobic accordingly to the hydrophaty index [98]. In contradiction, in the initial adsorption state, two strongly hydrophobic residues (valines), two strongly hydrophilic (lysine and glutamic acid) and one slightly hydrophilic (threonine) are involved in the protein – surface interactions.

From the above discussion, it appears clear that even if adsorption is predominantly driven electrostatics interactions, the hydrophobic effects needs to be taken into consideration to lead to complete understanding of the adsorption mechanism. Therefore, both electrostatics and hydrophobic effect should be carefully considered when protein adsorption is investigated. The system tends to minimise the energy of both long-range and short-range interactions, and depending on the particular system studied and experimental conditions applied, the long-range interactions either dominate to make the effect of the short-range forces invisible, or the domination is reduced and short-range effects became more important. This fact can be generalised to different kinds of proteins and surfaces, even if the adsorption is less specific than in the case of silica, as observed on hydrophobic surfaces [99].

4. Functionality of surface-adsorbed protein

Spicer *et al*. have presented the role of protein in nanotechnology for biomedical applications, enabling the treatment, diagnosis, and prevention of disease. Nanoparticles entering biological systems are almost covered with biofluids [100]. Thus, to develop a selective delivery of nano-objects to particular compartments of the body, it is crucial to understand phenomena involved in conformational changes and the displacement of proteins at the interface [101-103]. From the nano-medical viewpoint, the phenomena of competitive binding and protein displacement the Vroman effect, [104-105]) are also extremely important in this regard [106]. Dawson and co-workers have characterised the thermodynamics and kinetics of the binding of polymeric nanoparticles to proteins in human plasma using a broad range of analytical techniques including calorimetry, chromatography,

and surface plasmon resonance (SPR) [107-108]. They reported the formation of two types of protein coatings on the surface of nanoparticles: a soft corona and a hard corona. Dawson and co-workers also found that nanoparticle size and surface chemistry have significant effects on the compositions of the nanoparticles-protein coronas. In a more recent study, it has been concluded that plasma-derived protein coatings on the surface of polystyrene nanoparticles and silica nanoparticles are sufficiently long-lived that they, rather than the nanomaterial surface, are likely to be what the cell recognise. Note that the interaction of proteins with nanoparticles depends on their surface curvature. Very small nanoparticles have been shown to suppress protein adsorption in some cases. The key unanswered questions are whether the observation and hypotheses of Dawson and his co-workers on the interactions of proteins with relatively large and hard nanoparticles are applicable to small and softer organic nanostructures with sizes comparable to those of proteins.

In general, functionality of the adsorbed protein depends on maintaining the secondary and tertiary structure of the immobilised protein, as well as its orientation on the surface. For instance, if an enzyme is adsorbed at its active site, sterically blocking substrates from access it, this will undoubtedly lead to substantial reduction of activity.

It has been demonstrated through a range of experimental techniques that the active site of silica surface-adsorbed LSZ is exposed to the bulk [109], making it accessible to substrates. Possible activity reduction is due to changes of the secondary structure around the active site rather than the adsorption orientation [109]. On the other hand, numerous computational studies report that LSZ conformational changes upon adsorption are minor, and the protein adsorbed with active site exposed to the bulk should maintain its activity [28-29,31-32,69-70,74], although we should recall the caveat about the extremely short timescales accessible to traditional MD simulations. Nevertheless, this observation is supported by the experimental work of Saha *et al.*, who immobilised LSZ on silica, and maintained 98% of the free enzyme activity [56]. Similar observations have been made for other surfaces as well [110-111].

While various studies conclude that the active site of adsorbed LSZ is exposed to the solvent, the discrepancy in conclusions regarding activity level of adsorbed LSZ requires further investigations. The source of this discrepancy might originate from the fact that in computational models single protein adsorption is usually investigated [28-19,31,68-70,74]. In contrast, experimentally observed activity reduction might be caused also by protein-protein interactions on the surface [109]. Such an effect might be deduced from MD simulations for multi-protein adsorption where the region around the LSZ active site is demonstrated to be involved in protein-protein interactions [71]. Consequently, both experimental and computational approaches might be used to explain the observed features, as long as the studied models are comparable, and any labels or other chemicals have not been added in the experiment. As it has been demonstrated, attachment of even a small fluorescent label can significantly change the interaction properties of LSZ, and results of such experiments should be interpreted by modelling the structures with and without the labels [112-114].

By using various experimental techniques it has been clearly indicated that both adsorbed mass and BSA structure on the silica surface strongly depends on pH [14,26]. Nevertheless, at physiological pH the adsorbed mass can reach a maximum, and the protein structure is notified as compact and triangular [15,26]; similar results have been recently obtained for HAS [115]. Computational studies indicated that when adsorbed on silica, BSA indeed maintains its compact triangular structure very well. Moreover, the adsorption site location at the bottom of subdomain IIIB leaves most of the active sites, namely the Sudlow binding sites I and II as well as numerous fatty acid binding sites [79], exposed to the solvent [15,30]. It indicates that BSA activity might not be affected

by surface immobilisation, nevertheless experimental evidence supporting the computational predictions is desired.

5. Adsorbed protein orientation and monolayer packing

LSZ can be configured in three different orientations on a surface, namely side-on, edge-on (also called between) and end-on [29,116], as corroborated by QCM-D. However, recent studies have reported only the side-on orientation [28]. This discrepancy might also arise from coverage dependence: at low coverage LSZ adopts side-on orientation and, perhaps intuitively, increasing surface coverage leads to a more standing orientation in the crowded protein layer, with edge-on or end-on orientations [116]. Interpretation of the experimental results might depend on the adopted adsorption model [29]. This disagreement might be judged by MD simulations results, where most of the publications report either side-on or edge-on orientation on silica surface, or both mixed together [28-29,31-32,68-70]. The situation appears similar with other comparable surfaces [67]. The protein dynamics on the surface cannot be studied experimentally with single protein resolution, and due to long time scales is not easily accessible with the computational approach. On the other hand, there are strong indicators that LSZ as well as BSA adsorb irreversibly on silica surface [15,22,29,72]. From SMD simulations, the adsorption energy was predicted to be ~1.2 eV in the case of BSA [22], and ~0.9 eV - 1.2 eV in the case of LSZ [72]. The computationally predicted adsorption energies suggest that spontaneous desorption is not very likely on a time-scale of hours, as commonly observed in experiments.

Both adsorbed proteins might diffuse laterally across the surface due to low energy barriers, which can be much smaller for surface diffusion than desorption. The calculated energy barrier for protein diffusion across a model silica surface for LSZ is ~0.4 eV [72],, when compared to BSA (0.2 eV) [22]. These values could explain why LSZ diffusion was not observed in 100ns MD simulations, in comparison to BSA simulations that have shown some details of the diffusion mechanism (without any need for desorption). In the case of BSA, it is enough to temporarily lose the direct interaction of Lys537 with the surface to slide on the water layer, before re-establishing the anchoring interaction [15]. For LSZ to diffuse across the surface, it needs to break only one direct contact of Arg128 [72], albeit at twice as high an energy barrier. The differences in diffusion energy barriers is rationalised in terms of the hydrogen bonds that must be broken and reformed as diffusion occurs.

The adsorbed protein orientation, and to some extant mobility and clustering [72,117], can be used to interpret the structure of an adsorbed surface layer in terms of packing fraction, and it should be noted that the shape of most protein molecules deviates from a spherical shape. Various proteins resemble elongated spheroids, with conformational stability verified by measurements of circular dichroism. On the other hand, using viscosity measurements, one can obtain essential parameters of the system regarding both shape and changes in the conformation of protein systems [26,43,59]. This allows the calculation of the specific conformation of the protein in the suspension, approximated by a prolate (elongated) or oblate (flattened) spheroid shape. The conformation of the protein can be strongly influenced by ionic strength and pH of the solution. Due to the lack of a universal theory, the exact structure of biomolecules in solutions cannot be unequivocally determined from viscosity measurements alone. Complementary data are necessary, such as the hydrodynamic radius of molecules (containing shape information) and their electrophoretic mobility, enabling estimations of the correction counter ion condensation degree, etc. On this basis, the conformational stability of the protein can be controlled through choice of solvent properties.

Experimental studies confirm that LSZ belongs to proteins with high conformational stability [43], whereas BSA occurs in several conformations depending on environmental conditions [26,77-78]. The conformational stability of the protein will affect the structure of the protein layer adsorbed on the surface. One of the models utilised to determine the structure of the adsorbed layer is the random sequential adsorption (RSA) model, which assumes that particles adsorb randomly to the substrate. If the process continues, the surface coverage increases until it saturates. This saturation threshold is referred to as the jamming limit that is reached for hard sphere at a coverage of Θ_{jam}=0.547; this is much smaller than the coverage obtained for a regular hexagonal packing of the spheres in two dimensions (Θ=~0.91).

For charged particles, the adsorbed maximum saturation coverage Θ_{max} may be substantially below the jamming value. These deviations are caused by the fact that electrostatic repulsion between the adsorbing proteins can be crucial, particularly for smaller ones. The jamming limit is approached at high ionic strength, where screening effects are significant. The distance between nearest neighbors decreases with increased ionic strength due to the corresponding decrease of the Debye length, which determines the range of the screened Coulomb repulsion between the particles.

The RSA model can be applied for simulating adsorption of particles of various shapes. This phenomenon was demonstrated previously [60] by using the RSA approach for predicting adsorption of spheroidal particles at a solid/liquid interface. These theoretical results are relevant for modeling irreversible protein adsorption whose shape deviates from a spherical one [60,118]. Using the RSA model, it is possible to determine adsorption kinetics, the structure of mono-layer and the jamming coverage, the latter being the parameter of primary practical interest. The electrostatic interaction among adsorbed particles influences not only the maximum coverage but also the structure of adsorbed particle monolayer characterised by a pair correlation function. The correlation function is accessible experimentally by direct imaging of adsorbed particles using optical microscopy [119] or AFM [120-123]. From the analysis of the pair correlation function in the limit of low coverage, it is possible directly to estimate the range and magnitude of lateral interaction between particles. By AFM imaging of protein, it appears that adsorbed protein can flatten substantially and spread laterally on the surface [120]. However, this post-adsorption deformation process will not be instantaneous, and one expects that the surface area occupied by the adsorbed molecules can increase over time [124-125]. This makes the interpretation of protein layer formation challenging, bringing us full circle to the time scale issues discussed above.

Summary

From the above discussion, it is apparent that there has been good progress made in understanding protein adsorption at inorganic surfaces in recent years, using a combination of experiment and (primarily MD) simulation. While we have focused on adsorption at a silica surface, its negative charge and hydrophilic character at physiological conditions makes it a useful model for many other materials of interest including metals and metal oxides. The work has demonstrated the importance of electrostatics to the understanding of adsorption, supplemented by the role of hydrophobicity and short range interactions such as hydrogen bonding.

Nevertheless, there is still much to be accomplished. The primary challenge appears to be the one related to timescale. Timescale challenges for simulation, extrapolating from 100ns to experimental timescales, are clearly significant. There are also inevitable challenges with experiments,

concerning to how we can address the life time of, for instance, a medical implant *versus* the readily achievable duration of hours on a laboratory bench?

Other consequential phenomena are related to these timescale issues. Competitive adsorption may well occur in biologically relevant environments, where rapidly diffusing proteins that initially adsorb might be displaced at a later stage by other proteins. This is particularly important for applications where desired surface functionality needs to be imparted reliably. Examples include: cell adhesion or anti-microbial activity; drug delivery; as well as therapeutic and diagnostic applications of nanoparticles.

In order to progress further so that we can fully control and exploit protein adsorption at material surfaces, it is apparent that continued integration between modelling and experiment is essential. The reality of experimental data requires the interpretation that simulation can provide, and the insight of simulation to design new systems indeed needs experimental verification. Given the growing prevalence of experimental and modelling collaborations, we can optimistically look forward to further developments in this branch of science that underpins technology of great societal importance.

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