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Protein Interactions with Polymer Coatings and Biomaterials

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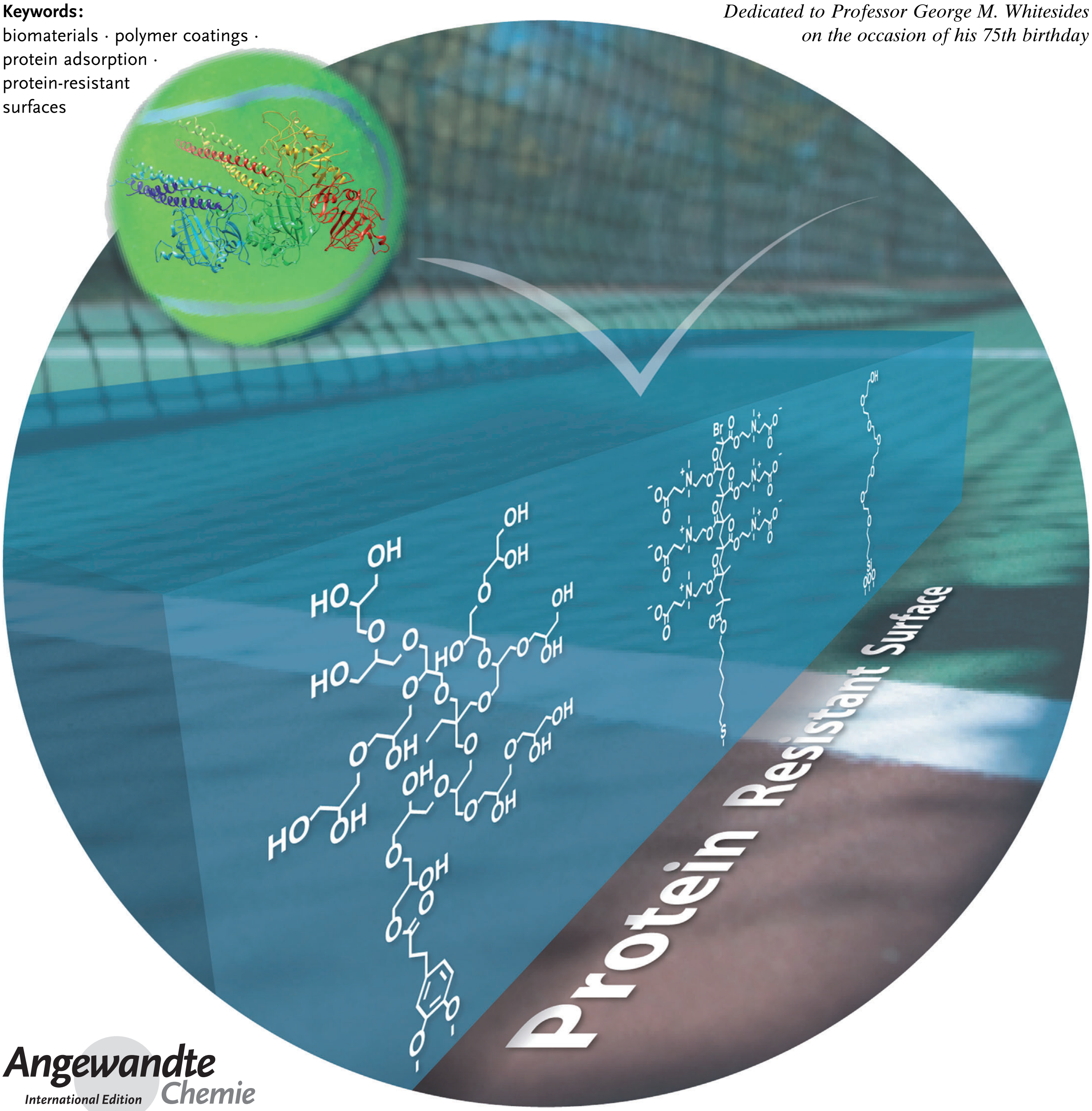
Dedicated to Professor George M. Whitesides on the occasion of his 75th birthday



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Protein adsorption is considered to be the most important factor of the interaction between polymeric biomaterials and body fluids or tissues. Water-mediated hydrophobic and hydration forces as well as electrostatic interactions are believed to be the major factors of protein adsorption. A systematic analysis of various monolayer systems has resulted in general guidelines, the so-called "Whitesides rules". These concepts have been successfully applied for designing various protein-resistant surfaces and are being studied to expand the understanding of protein–material interactions beyond existing limitations. Theories on the mechanisms of protein adsorption are constantly being improved due to the fast-developing analytical technologies. This Review is aimed at improving these empirical guidelines with regard to present theoretical and analytical advances. Current analytical methods to test mechanistic hypotheses and theories of protein–surface interactions will be discussed. Special focus will be given to state-of-the-art bioinert and biospecific coatings and their applications in biomedicine.

From the Contents

1. Introduction

Biomaterials and medical devices induce tissue responses upon implantation into living tissue or when they get into contact with human blood.[1] Within seconds, nonspecific protein adsorption occurs on implant surfaces (Figure 1) and can be quickly followed by the cascades of biological response, possibly including foreign body reactions.[2] This biological response may result in the production of a fibrous avascular capsule, which isolates the device from its target tissues, hinders the effectiveness of membranes and biosensors, and prevents drug release from delivery vehicles.[3]

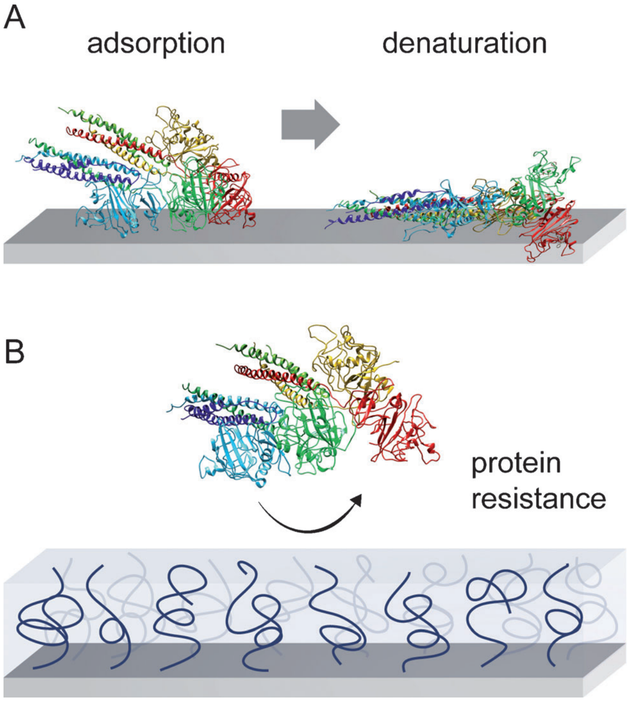

Figure 1. A) Dynamic adsorption and denaturation of proteins on a bare surface; B) protein resistance of polymer-coated surfaces.

In particular, protein adsorption has become the focus of modern nanotoxicology. Most nanoparticle surfaces will be immediately covered by a dense layer of proteins when injected into the blood stream and this "corona" will determine the response of the body to these particles.[4–8] Knowledge of the corona's composition and its temporal evolution[9,10] is necessary to understand its effect.[11,12] In addition, protein adsorption can be accompanied by slow protein denaturation. This results in an immunological recognition of the nanoparticles, which is caused by the denatured protein and not necessarily by the nanoparticle's material.[13,14] Additionally, protein adsorption is a kinetic phenomenon and the corona will change over time.[9,10] At an early stage, highly mobile proteins adsorb at the surface. Subsequently, a complex series of adsorption and displacement steps occurs, the so-called "Vroman effect", and leads to the replacement of the initially adsorbed proteins by other proteins with a higher affinity.[15–17]

In many cases, biomaterials get into direct contact with blood. This can occur in an intra- or extracorporeal fashion, depending on the application, for example as biomaterial

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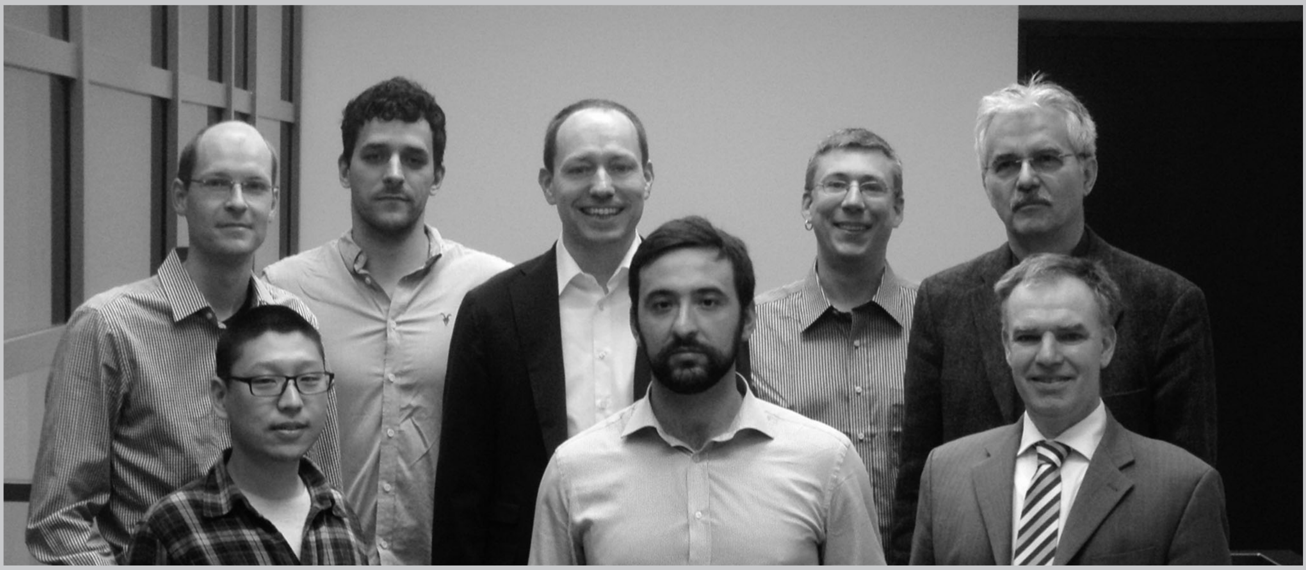
implants (e.g., heart valves) or as particulate formulations injected into the blood stream through dialyzers, tubings, and blood bags. However, the contact of blood with artificial (exogenous) surfaces could lead to thrombocyte adhesion and activation, which results in triggering plasmatic coagulation and/or activation of the complement system. It is a general consensus that these biological effects involve the adsorption of proteins on the exogenous surfaces. Since no biomaterial with absolute protein resistance is known, the application of biomaterials often requires simultaneous drug treatment, e.g., an anticoagulative therapy. This potentially causes side effects including hemorrhage, neutropenia, or thrombocytopenia, and the pharmacokinetic and metabolic interference of several drugs administered in multimorbid patients, which is not desirable.

Therefore, the prevention of nonspecific protein adsorption plays a key role in improving the biocompatibility of biomaterials. An efficient approach is the surface modification of biomaterials with polymeric coatings to make them "stealthy" with regard to body fluids and the surrounding tissue.[3] Decreasing the surface energy of biomaterial surfaces is a reasonable approach toward such "stealthy" devices. Fluorine-based coatings with low surface energy have been employed to decrease fouling with marine organisms.[18] However, in a physiological environment, even polytetrafluoroethylene (PTFE, Teflon) surfaces significantly adsorb proteins ($>1\ \mu g\, cm^{-2}$).[19] Therefore, the driving forces of protein adsorption must be understood in order to improve bioinert surfaces.

Furthermore, the rapid progress in the synthesis and characterization of functional polymer-coated surfaces strongly challenges the development of theoretical modeling tools for a quantitative description of the protein binding process. The theoretical understanding is a necessary prerequisite to develop rational design strategies to achieve an external control of both, binding thermodynamics and kinetics.[20]

Among all possible parameters, water-mediated hydrophobic and hydration forces were considered to be the major players in protein adsorption, a view that culminated in the concept of "Berg's law" or "Berg's limit". This concept describes which surfaces should or should not promote protein adsorption depending on their hydrophobic or hydrophilic properties.[21] Berg's limit is a quantitative definition of hydrophobicity/hydrophilicity: Surfaces are hydrophobic if their contact angle θ with water is bigger than a critical value of around 65°.[21] On hydrophilic surfaces with $\theta < 65°$, proteins should not be able to displace water from the surface and adsorb, whereas the opposite behavior is expected for hydrophobic surfaces. Although many experimental results can be explained by this approach, several drawbacks remain. First, by directing all attention to the surface–water interaction, little insight is provided regarding the competitive adsorption of different mutually interacting proteins. The second point is more conceptual: Berg's limit is typically derived by analyzing hydrophobic and hydration forces between large particles, which behave like flat surfaces from a theoretical point of view. However, the strength of these forces strongly depends on the length scales involved, with an important crossover in the nanometer regime where most proteins reside.[22,23] Thus, it is not clear how many of the results obtained for interactions on large surfaces are relevant for the adsorption on small particles.

The observation of strong protein adsorption in many types of charged hydrophilic gels, commonly used as polymeric coatings, is in clear contrast to the empirical concept of Berg's limit. Vogler, who suggested hydrophobic/hydration forces as the main player in protein adsorption,[21] observed this discrepancy and proposed to distinguish between proteins directly attached to a surface and those that are "confined" in



From left to right: C. Wischke, Q. Wei, T. Becherer, A. Lendlein, S. Angioletti Uberti, A. Neffe, M. Ballauff und R. Haag (missing from picture: J. Dzubiella). The author team is part of the Helmholtz Virtual Institute on Multifunctional Biomaterials for Medicine, which is funded by the Helmholtz Association of German Research Centers, the Freie Universität Berlin, and the Universität Freiburg since 2011 and includes around 40 scientists from different institutions, which explore protein–material interactions (spokesperson: Prof. Andreas Lendlein, HZG-Teltow).

its proximity. This ad hoc explanation, apart from requiring experimental evidence, might save the overall framework outlined by the concept of Berg's law but seems rather artificial. Also, it is not clear what difference it would make from a practical point of view to guide the design of biomedical coatings. In fact, it is difficult to see how proteins, whose distance from the biomaterial surface differs by only a single layer of water, can be distinguished by external agents such as microbes or antibodies. It needs to be discussed how they can change their overall interaction with the material, because no clear physical mechanism would allow these agents to probe such a tiny difference. Furthermore, the behavior of fluorinated surfaces cannot directly be evaluated in this model, as fluorophilicity opens another dimension, which cannot be categorized as "hydrophilic" or "hydrophobic".

In recent years, it has been recognized that the physicochemical processes behind protein adsorption are governed by a complex interplay of the conformational degrees of freedom of the (responsive) polymer and various polar and nonpolar interaction mechanisms.[20] The former include nonspecific electrostatic and ionic screening effects as well as more local noncovalent interactions such as salt bridges or hydrogen bonds. The polar interactions can be manipulated by a change in salt concentration or pH. Nonpolar interactions include van der Waals and solvent-mediated phenomena such as hydrophobic attraction[22] or hydration repulsion.[23] A quantitative description of these highly interconnected and collective mechanisms requires multiscale and coarse-graining modeling approaches for both, the solvated polymers[24–26] and the proteins,[27,28] to access all relevant time and length scales, while preserving chemical specificity. The wealth of hitherto developed tools has not converged to a full quantitative predictive machinery, but partial success has been achieved for individual systems, as we will discuss in the theoretical methods Section 2.5 below.

In search of appropriate coating materials, Whitesides and co-workers performed a systematic study of the structure–property relationship, comparing the different chemical structures of self-assembled monolayers and their efficiency to suppress protein adsorption.[29,30] Based on these studies, four molecular-level characteristics of protein-resistant monolayers were proposed and are nowadays recognized as the "Whitesides rules": 1) the presence of polar functional groups, i.e., hydrophilicity, 2) the presence of hydrogen bond acceptor groups, 3) the absence of hydrogen bond donor groups, and 4) the absence of net charge. Polymer coatings, which fulfill all of these rules, should provide an additional hydration layer that prevents protein adhesion to the surface.

According to the abovementioned empirical "Whitesides design rules", many types of bioinert molecules have been developed or employed that effectively decrease the protein adsorption on material surfaces including peptides,[31] peptoids,[32] oligo-/poly(ethylene glycol)s,[33] oligo-/polyglycerols,[34] zwitterionic polymers,[35] polysaccharides,[36] polyoxazolines,[37] poly(propylene sulfoxide)s,[38] and polyvinylpyrrolidones.[39] Although some of these polymer coatings such as polyglycerol and polysaccharides contain a large number of hydroxyl groups (hydrogen bond donors), which is in conflict with the third Whitesides rule, they perfectly fulfil the first rule due to their improved hydrophilicity. In this specific case, Berg's law is applicable. On that account, a general understanding of both bioadhesive and bioinert surfaces is required to be able to optimize coatings for their respective application.

In addition to these bioinert coatings, another approach are polymer surfaces that exploit the (un)controlled adsorption of proteins to shield the biomaterial and/or to promote its integration to enable biological contact of implant surfaces with tissue.[40] It is believed that the combination of biospecific molecules with bioinert coatings can increase the efficacy of biological communication.[3] Besides the zwitterionic phospholipid bilayer, natural cell membranes contain island-like carbohydrates and proteins. This concept was adapted for cell membrane-like polymeric coatings, which consist of a bioinert material with conjugated bioactive molecules that can selectively bind to target biomolecules in vivo. For example, arginylglycylaspartic acids (RGDs), which are bound to a bioinert coating, integrate with endothelial cells, whereas adjacent bioinert polymers prevent the adhesion of leukocytes and other cells.[41] Therefore, bioinert surfaces are an indispensable component for the design of biomaterials.

For many of the abovementioned applications, PEGylation was considered as the gold standard for protein-resistant surfaces. However, recent observations indicate that PEGylation is far from being an optimal candidate for biomedical applications. Even though PEG is a polyether and thus relatively stable under physiological conditions, it has been reported that degradation can occur in the presence of oxygen and transition metal ions especially at elevated temperatures, or in vivo in the presence of enzymes.[42,43] The thermal instability is of relevance for the fabrication and handling of PEG-grafted devices. Additionally, the enzymatic degradation of low molecular weight PEGs was shown to give toxic metabolites[44] and hypersensitivity reactions up to anaphylactic shocks have been reported in some cases.[45] Presently, it is still under discussion if these body reactions have to be assigned to PEG itself or rather to impurities from its synthesis, such as 1,4-dioxane, ethylene oxide, or formaldehyde. Several studies also detected antibodies (IgM and IgG) against PEGs in humans, which could be induced by repeated administration of PEG or PEG-drug conjugates.[46]

Therefore, in recent years, alternatives to PEG have received much attention. One class of polymers in this respect is polyglycerol (PG), which can exhibit either a linear or a branched structure.[47,48] These polymers are more resistant to heat[49,50] and oxidation[51] than PEG, and exhibit similar or higher levels of protein resistance.[52,53] Furthermore it has been shown that PGs are less thrombocyte activating than PEG.[54] Another class of remarkable alternatives are zwitterionic molecules.[35] As mentioned above, the water layer bound to the polymer chains is mainly responsible for the hydration forces repulsing protein adsorption.[55] Hydrophilic molecules, such as PEG and PG, achieve hydration through hydrogen bonding, which is weaker than ion–ion or ion–dipole electrostatic bonding. Zwitterionic molecules, which show good stability and perfectly combine the ion–ion or ion–dipole bonding with water and the rule of absence of net charge, are becoming a new benchmark of protein-resistant materials.

Understanding protein interactions with polymer surfaces is the key to obtaining the next generation of bioinert and biospecific surfaces. This review will therefore focus on methods to detect protein interactions with polymers on surfaces and in solution. It will also describe some recent developments in the area of bioinert and biospecific surfaces in the context of selected biomedical applications.

2. Methods for the Analysis of Protein Interactions

2.1. Overview of Physicochemical Tools to Study Protein Adsorption

In this section important quantitative physicochemical methods to detect and study protein interactions are summarized (Table 1), which allow estimating the capacity of surfaces to be bioinert or biospecific.

2.2. Selected Instrumental Methods for Protein–Surface Interactions

SPR and QCM are widely applied label-free methods for the investigation of interfaces due to their high sensitivity and their ability to measure in real time. ToF-SIMS is a powerful in situ method to probe the orientation and conformation of adsorbed proteins as well as to identify and differentiate specific proteins in mixtures. Therefore, these three methods will be highlighted here.

Surface Plasmon Resonance (SPR) and Quartz Crystal Microbalance (QCM)

SPR is an optical method that measures the change of the refractive index on metal surfaces. Measuring SPR requires the generation of surface plasmons (SPs). A setup capable of generating SPs is the Kretschmann geometry,[70] in which a glass prism is attached to a sensor chip coated with a thin layer of gold, which is in direct contact with the buffer solution in the flow cell (Figure 2 A). When directing light to the prism–gold boundary,[71] photons can interact with the free conduction electrons of the gold, thereby creating plasma oscillations. The quantum of this oscillation is called the plasmon. A resonance occurs when the plane-parallel vector component of the incident light matches the vector of the plasmon momentum, resulting in an extinction of the reflected light. Since the magnitude of the light vector components change as the angle of the incident light is changed, a resonance only occurs at a specific angle of incident light. The plasmon momentum in turn depends on the refractive index in the flow cell at the gold boundary, because its evanescent electromagnetic wave extends into the medium. Hence, changes in the refractive index in the flow cell cause a change of the plasmon momentum, thereby shifting the incident light angle at which SPR occurs. In an SPR experiment, the change in the angle of minimum reflected light intensity is measured. It is also proportional to the mass of the adsorbed molecules when the eluent is kept in contact.

QCM is an acoustic method that measures the change in mass adsorbed to the surface of a piezoelectric quartz crystal. The application of an oscillating electric field to the quartz crystal generates a transversal acoustic wave that propagates through the crystal and is reflected at its boundaries. Constructive interference of the reflected and the incident wave results in a mechanical resonance causing the amplitude of the oscillation to increase by a factor of 100 (Figure 2 B). The narrow frequency width for a resonance and the fact that the resonance frequency depends on the total oscillating mass enables the precise measurement of small perturbations at the interface, which, e.g., could be caused by adsorbed molecules.[72]

Although SPR and QCM provide highly time-resolved information on specific and nonspecific protein interactions, they cannot be directly applied to materials in the shape required for their application. Instead, gold-coated model sensor chips are typically used to enable electrical conductivity (QCM) or for the generation of plasmons (SPR). Additionally, the inertness of gold and the ease of surface modification are beneficial. In the case of SPR, silver may be the better choice with respect to its SPR characteristics but it is usually not considered due to its high oxidation tendency.[75] For QCM, a variety of different coatings on top of the gold layer is available, whereas the choice is rather limited for SPR. This can be attributed to the exponential decay of the evanescent field. By applying a coating on top of the sensor surface, which inevitably occupies the volume with the highest field strength, the sensitivity is considerably reduced. A commonly used definition for the decay length is the drop of the evanescent field intensity to 1/e, which is approximately half of the excitation wavelength.[76] Similarly to SPR, the decay length of QCM is defined as the distance at which the amplitude of the acoustic wave dropped to 1/e of its value at the surface.[72] Rigidly coupled mass extends the decay length and therefore does not cause a loss of sensitivity. For soft polymeric materials, however, decay is observed due to energy dissipation.

Despite the exponentially decaying fields, a linear relationship between the adsorbed mass and the relative signal is generally assumed for both methods. In the case of SPR, this may be true when measuring the protein interaction with a polymeric matrix, because the ligand and the analyte are ideally distributed equally throughout the matrix.[77] However, when the nonspecific adsorption is measured, the adsorbates can occupy a volume within a distance of up to several tens of nm from the surface, filling up the volume from bottom to top. Due to the decaying evanescent field, adsorbates sensed within the first 10 nm result in an almost three times higher signal than adsorbates at a distance of 300 nm.[76] This also implies that coatings of different thickness are hardly comparable with methods like SPR and QCM due to the different sensitivities depending on the distance from the surface. Therefore, depletion methods may be the first choice for comparing surfaces of different thickness as

Table 1: Common quantitative analysis methods for the amount of adsorbed proteins.

Methods	Real-time	In situ	Measured elements	LOD[a]	Advantages	Disadvantages	Ref.
UV/Vis spectroscopy	N	N	adsorption after chromogenic assay	1 μg mL^{-1}	cheap and fast, generally available	an extra chromogenic assay is needed due to the low protein amount on the surface; low sensitivity; needs solution conditions for staining; certain chemical components affect the chromogenic assay	[56]
Fluorescence microscopy	N	Y	fluorescence	1 ng cm^{-2}	good sensitivity; possibility to measure competitive adsorption	fluorescence quenching; expensive reagents	[57]
Isotopic labeling	N	Y	isotope	1 ng	high precision; possibility to measure competitive adsorption	toxicity; short half-life of the common ^{125}I label	[58]
SEC	N	N	molecular size	100 ng mL^{-1}	convenient	only available for solutions	[59]
Ellipsometry	N	Y	thickness	0.1 nm	cheap and fast; generally available	only available on flat surfaces with homogeneously adsorbed layers; difficult to set models for composite surfaces	[60]
DLS	N	Y	hydrodynamic radius	0.1 nm	hydrated size available; generally available	only applicable for particles (hydrated size)	[61]
XPS	N	Y	chemical components	10 ng cm^{-2}, 1 mol%	quantitative chemical composition of the surface layer	sample contamination; complicated quantitative analysis complex and expensive device	[62]
Neutron reflectivity	N	Y	structure of the surface	$2\pi/Q_{max}$	thickness, density, and roughness information of surfaces available; sensitivity to isotopes; sensitivity to light elements	difficult data interpretation; neutron facility needed	[63]
SAXS, SANS	Y	Y	chemical components	2–3%	full analysis of radial structure of particles; quantitative analysis of adsorbed proteins	difficult data interpretation; neutron facility needed	[64]
ToF-SIMS	N	Y	mass of the molecular fragments	0.1 ng cm^{-2}	extreme surface sensitivity; analysis of orientation and conformation of proteins; differentiation and quantification of protein mixtures	only low-mass fragments are detected; absolute amount of single protein is not available; expensive device	[62]
SFG	N	Y	specific chemical bonds	monolayer	analysis of orientation and conformation of proteins; working under ambient conditions	complex data analysis	[65]
FTIR	N	Y	secondary structure of proteins	2–3 wt%	full analysis of secondary structure of proteins	large amounts of material needed; complex data analysis	[64]
ITC	Y	Y	thermal analysis	1 wt%	access to thermodynamics of adsorption	involves analysis of data; large amounts of material needed	[64]
AFM	N	Y	force	1 pN	access to the force of protein adhesion; also for a single protein	proteins must be immobilized on the AFM tip; the absolute amount of proteins remains unknown	[66]
RIfS	Y	Y	reflexion of white light	0.3 ng cm^{-2}	high sensitivity, temperature insensitivity; real-time and in situ analysis	relatively poor time resolution; transparent substrates	[67]

Table 1: (Continued)

Methods	Real-time	In situ	Measured elements	LOD[a]	Advantages	Disadvantages	Ref.
SPR	Y	Y	oscillation of electrons	0.2 ng cm^{-2}	high sensitivity; real-time and in situ analysis	expensive sensors; limited types of surfaces, limited film thickness	[68]
QCM	Y	Y	frequency of a quartz crystal	1 ng cm^{-2}	good sensitivity; real-time and in situ analysis; different surfaces are available; dissipation related to the shear viscous loss can also be measured	only measurement of hydrated mass; energy dissipation affects mass calculation	[69]

[a] LOD = limit of detection. The exact LOD relates the quality of machines, the type of measured proteins, and the measurement conditions. The values in this table are only crude approximations. Abbreviations: Y = yes; N = no; UV/Vis = ultraviolet-visible; SEC = size-exclusion chromatography; DLS = dynamic light scattering; XPS = X-ray photoelectron spectroscopy; SAXS = small-angle X-ray scattering; SANS = small-angle neutron scattering; ToF-SIMS = time-of-flight secondary ion mass spectrometry; SFG = sum frequency generation; FTIR = Fourier transform infrared spectroscopy; ITC = isothermal titration calorimetry; AFM = atomic force microscopy; RIfS = reflectometric interference spectroscopy; SPR = surface plasmon resonance; QCM = quartz crystal microbalance; Q_{max} = maximum momentum transfer.

well as for the absolute quantification of protein adsorption especially in the case of thick coatings.[78]

The mechanical way of sensing in QCM adds yet another degree of complexity to the quantification since the dissipative properties of the adsorbates prevent a linear relationship between frequency and adsorbed mass. A linear relationship, however, as given by the Sauerbrey equation, is assumed for rigidly coupled adsorbates. In contrast, adsorbed protein layers, which include the mass of the associated water,[79,80] do not follow the oscillation rigidly. In general, higher water content causes a decrease in viscosity, which in turn results in an increase of dissipation. Since the Sauerbrey model does not take this energy dissipation into account, the calculated masses may be considerably smaller than the real mass. The changes in dissipation, however, can easily be determined by switching off the oscillator and measuring the exponentially decaying amplitude of the oscillation.[81] Given the dissipation, the resonance frequency, and optionally complementary methods, different models for quantification are available. Due to the complexity of this topic, the reader is referred to the excellent review by Reviakine et al.[82] It should be added that the sensitivity of QCM to dissipative effects has both advantages (more information) and disadvantages (complex modeling). On the one hand it complicates quantification, but on the other hand it is the key advantage over optical biosensors, which lack sensitivity to structural changes.

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS is a mass spectrometry technique for detecting adsorbed molecules on solid surfaces. A pulsed ion beam is used in SIMS to remove molecular fragments from the surface of the adsorbed proteins. These secondary ion fragments are accelerated by an electrical field to the same kinetic energy with the velocity of the ion depending on the mass-to-charge ratio. The ToF detector is used to measure the velocity, which is used to determine the mass-to-charge ratio.

Due to its high surface and chemical sensitivity, ToF-SIMS was employed to discriminate between different adsorbed proteins and quantify the composition of protein mixtures.[83,84] However, the data interpretation for adsorbed protein films is always challenging, because SIMS is only valuable for small fragments with $m/z \leq 500$.[85] Even single peptides often cannot be detected in their complete form. To decode the information in the low mass fragmentation, the statistical procedure of principal component analysis (PCA) is combined with the characteristic peaks for a given system.[86] Furthermore, ToF-SIMS is sensitive to the conformation, positioning, and orientation of the proteins on the surface.[87] This is due to the very shallow sampling depth of SIMS (10–15 Å),[88] which is smaller than the physical dimension of many proteins. Thus, only the outermost amino acid residues of adsorbed proteins may be sampled under proper conditions.[85] Overall, ToF-SIMS is a developing technology which reveals new details of adsorbed proteins on the surface.

2.3. Characterization of Protein Adsorption on Particles in Solution

The adsorption of proteins to polymeric surfaces can be studied by using colloidal core–shell particles with a well-defined polymer shell. Evidently, colloidal particles with a typical size of approximately 100 nm exhibit a large surface even at a relatively low particle concentration so that a concomitant high amount of protein can be adsorbed in small volumes. Thus, protein adsorption to colloidal particles can be studied by a number of methods devised for the characterization of particles in general. With the help of a recent review on this topic,[64] we will briefly delineate three main methods and their results.

Isothermal Titration Calorimetry (ITC)

ITC utilizes the enthalpic effect generated by the adsorption of proteins on nanoparticles in solution. As a result, this method gives some direct thermodynamic information that is not available on planar surfaces. This obvious advantage of ITC for the study of protein adsorption has led to a large number of studies,[89–96] which were recently reviewed.[64] In

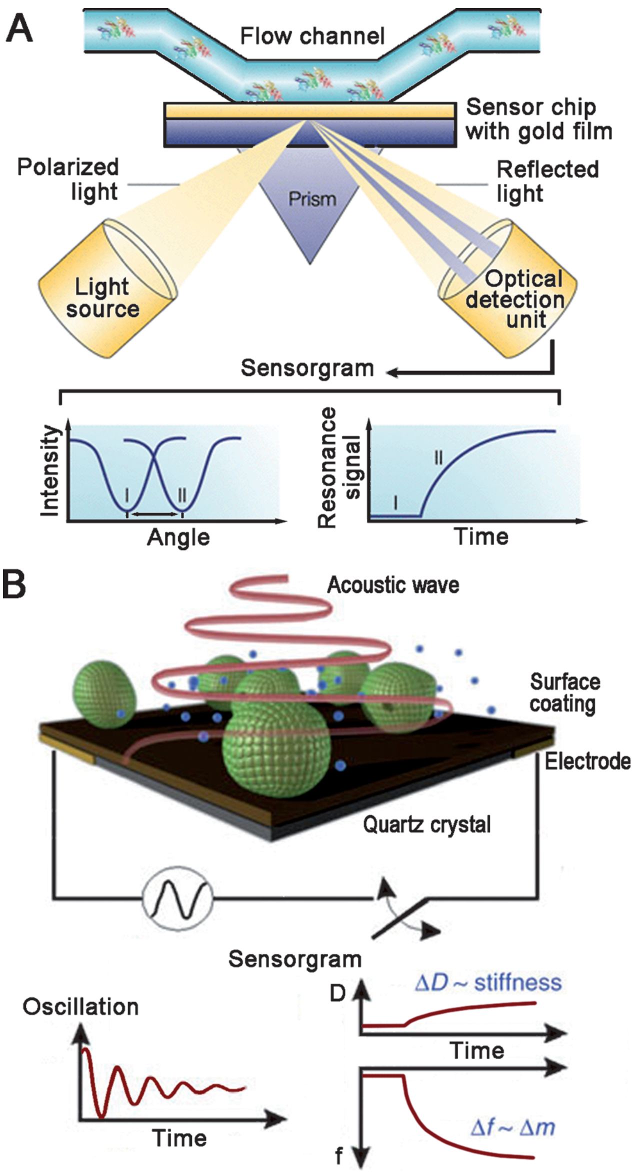


Figure 2. A) Schematic representation of a surface plasmon resonance (SPR) measurement utilizing a Kretschmann configuration. The laser beams are reflected from the back-side of the sensor except for the angle in which the metal absorbs and turns the energy into a plasmonic wave. This resonance angle depends on the mass of the material at the surface. The angle shifts from I to II in the lower left diagram, when proteins are adsorbed on the surface. This change can be monitored in real time (lower right diagram). Reprinted from Ref. [73] with kind permission of the Nature Publishing Group. B) Schematic representation of a quartz crystal microbalance (QCM) measurement. The piezoelectric quartz crystal oscillates and generates a resonant shear acoustic wave in the sensor, when an alternating electric field is applied. The resonance frequency of oscillation and energy dissipation shift, when proteins are adsorbed on the surface, which changes the mass and viscoelasticity of the surface layer. The green balls symbolize proteins and the small blue balls correspond to acoustically coupled water. "f" is the frequency, and "D" represents the energy dissipation. Reprinted from Ref. [74] with kind permission of the Nature Publishing Group.

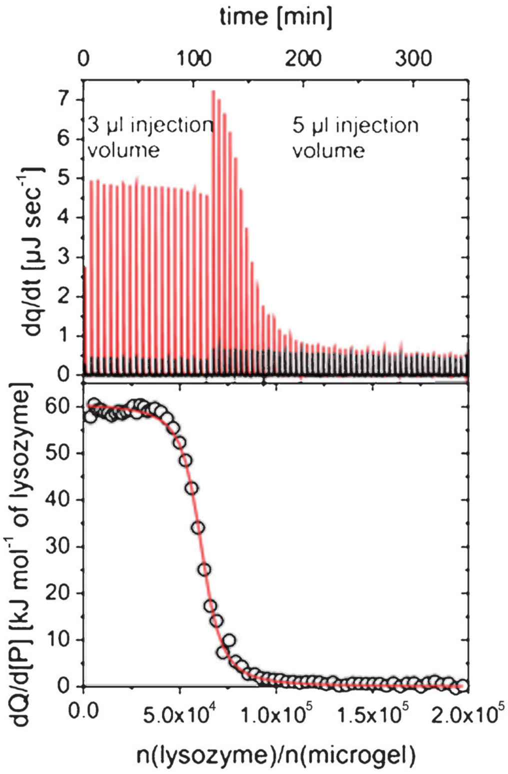


Figure 3. Analysis of protein adsorption by isothermal titration calorimetry (ITC). Upper part: The ITC signal of the binding of lysozyme to microgel particles at 298 K in a buffer solution. The black spikes are the heat of dilution of the protein and the colored spikes are the change of heat corresponding to each injection of protein into the dispersion of particles. Lower part: the integrated heat Q of each portion of lysozyme (circles) injected to the dispersion of the particles. The heat of dilution has already been subtracted from the overall signal. The solid line represents the fit based on the Langmuir isotherm. Reprinted from Ref. [94] with kind permission of The Royal Society of Chemistry.

general, ITC measures the evolution of the heat Q when a small portion of protein in aqueous solution is mixed with a solution of particles. Figure 3 displays an example of such a measurement. The heat of dilution of the protein must be determined in a separate experiment and subtracted from this data. Integration of the raw ITC data gives the heat ΔQ as a function of the molar ratio between the protein and particles. Clearly, a prerequisite of this method is that equilibrium can be reached. This is not the case, e.g., for hydrophobic particles, on which an irreversible adsorption occurs. Since the equilibrium is the basis for the subsequent thermodynamic analysis, this point must be carefully checked for each case under consideration.

Since the evaluation of ITC data is usually based on the Langmuir adsorption isotherm, a given number of adsorption sites are assumed for a given protein. The result of this analysis is the adsorption constant K and the number of adsorption sites N. An evaluation of ITC data independent of these rather stringent assumptions was developed recently.[96] Here, the resulting heat of adsorption is directly fitted to a model, which takes the main driving forces for adsorption, namely electrostatic and hydrophobic interactions, into account (see Section 2.5).

Additionally, the dependence of the measured heat of adsorption on the temperature is important. General thermodynamics state that the enthalpy of binding is derived from the van't Hoff equation through

$$\left(\frac{d\ln K}{dT^{-1}}\right)_p = -\frac{\Delta H_{\text{bind}}}{R}. \qquad (1)$$

Hence, ΔS_{bind} can also be extracted from the temperature dependence of the free energy:

$$\frac{d\Delta G_{\text{bind}}}{dT} = -\Delta S_{\text{bind}}. \qquad (2)$$

However, recent investigations by ITC on, e.g., protein–protein interaction or protein unfolding have revealed apparent discrepancies between the enthalpy directly measured by ITC and the binding enthalpy derived from the application of van't Hoff's law to the deduced binding constant.[97,98] It was proposed that in case of protein adsorption, the enthalpy measured by ITC also contains other contributions, which are not directly related to the adsorption equilibria. Hence,

$$\Delta H_{\text{itc}} = \Delta H_{\text{bind}} + \Delta H_{\text{res}} \qquad (3)$$

in which only ΔH_{bind} is caused by the binding of the protein to the particles. ΔH_{res} represents unrelated equilibria, that is, processes that proceed independently of the binding process. For example, it was demonstrated that this residual enthalpy was due to the protonation of lysozyme bound to an acidic microgel.[95] Another example is given by the adsorption of RNase A to a spherical polyelectrolyte brush.[92] Here it was found that the adsorption heat is very small and that the heat measured by ITC is related to other processes. This point is clearly in need of further evaluation and demonstrates that the results furnished by ITC may defy direct and simple interpretation.

Finally, ITC can be used to determine the enzymatic activity of bound proteins.[99] No colorimetric assay is needed since ITC directly measures the heat of the chemical reaction catalyzed by the enzyme. ITC therefore provides valuable information on the tertiary structure of adsorbed proteins and shows to which extent the enzymatic activity may be lowered or enhanced by adsorption.

Small-Angle X-Ray Scattering (SAXS)

Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) can be used to measure the radial density profile of spherical particles.[100] In general, small-angle scattering measurements are sensitive to the contrast of the objects under consideration, i.e., the difference between the scattering length density of the dissolved object and a dispersing medium, like water. SAXS is more suitable than SANS to study the adsorption of proteins to particles since proteins generally have excellent contrast. The measured scattering intensity $I(q)$ (q: magnitude of scattering vector; $q = (4\pi/\lambda)\sin(\theta/2)$, λ: wavelength of radiation, θ: scattering angle) of spherical particles is (see the review in Ref. [100])

$$I(q) = B^2(q) \qquad (4)$$

in which the scattering amplitude is given by

$$B(q) = 4\pi \int_0^\infty [\rho(r) - \rho_m] - \frac{\sin qr}{qr} r^2 dr. \qquad (5)$$

Here $\rho(r)$ is the radial electron density and ρ_{m} is the respective quantity of the dispersing medium. The central result of SAXS as applied to protein adsorption on particles is the radial density of the adsorbed proteins, which gives full information on the process of adsorption. Despite this valuable information, SAXS (and SANS) have not been applied very often to this subject and the number of related papers is limited.[101–104] SAXS has also been used to follow the adsorption of BSA into spherical polyelectrolyte brushes. As a result, time-resolved SAXS gives comprehensive information for an extended study on the dynamics of protein adsorption.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR is a valuable tool to investigate the secondary structure of proteins in aqueous solution[105,106] as well as in the adsorbed state.[107–110] The protein secondary structure is analyzed on the basis of the adsorption pattern of the characteristic amide bands, i.e., the amide I and II vibrational modes of the protein amide groups that appear in the IR regime between 1500 and 1700 cm^{-1} (Figure 4). These are the most sensitive vibrational modes to determine the content of α helix and β sheet, in other words, the secondary structure of the proteins. This method also works for adsorbed proteins and is among the most valuable tools to investigate possible distortions of the secondary structure by adsorption.

Figure 4 shows an example of such an analysis, and compares the spectra of adsorbed hemoglobin with the native protein. Here bovine hemoglobin was adsorbed to a spherical

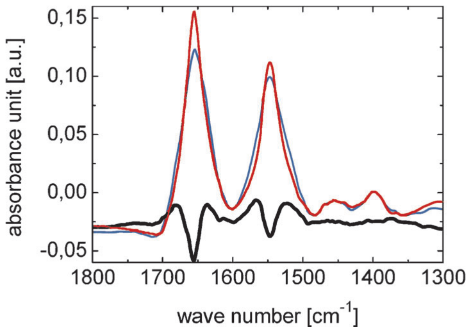


Figure 4. FTIR spectra of native hemoglobin before adsorption (red line) and after adsorption to a spherical polyelectrolyte brush (blue line). The IR absorption is plotted in the amide I (1600–1700 cm^{-1}) and amide II region (1480–1575 cm^{-1}). The spectra are scaled to the same concentration of bovine hemoglobin. The difference spectrum of adsorbed and native protein (black line) shows that the adsorption leads to structural changes of the secondary structure of hemoglobin. Reprinted from Ref. [102] with kind permission of The American Chemical Society.

polyelectrolyte brush bearing chains of poly(styrene sulfonic acid).[102] The different spectra provide further insight into the conformational changes caused by the interaction with the spherical polyelectrolyte brush (Figure 4, red line). There is a significant change in the secondary structure induced by the adsorption. The maxima of the amide I bands are located at 1656 cm^{-1} in both cases. However, the lower intensity for adsorbed hemoglobin is due to a significant loss in α-helical structure during adsorption. This is accompanied by the formation of a β sheet structure (cf. signals at 1682 cm^{-1} and 1636 cm^{-1} of the difference spectra), which is absent in native hemoglobin. The helical content decreases from 71 % to 34–42 % depending on the amount of adsorbed protein. In addition, 10 to 13 % of β sheet structure is formed upon adsorption. Thus, FTIR spectroscopy provides information on the change of the secondary structure that is not available by other methods (at least not for proteins on surfaces).

2.4. Biochemical Tools

The biochemical analysis of the adsorption of single or multiple proteins as well as complex protein mixtures remains a widely applied approach to study biomaterial–protein interaction. Compared to polymer films as test substrates, micro-/nanoparticles with large surface areas can be beneficial for mechanistic studies in terms of sensitivity and bound quantities, but may also be faced with distinct experimental challenges. For instance, massive colloidal stabilization at high protein concentrations and a large interparticulate volume of medium containing unbound protein may impede a quantitative separation of substrate and medium as needed for their subsequent separate analysis. The dynamic movement of small particles may affect the kinetics of protein adsorption and particles may be less suitable for surface-based analytical techniques. Additionally, it should be considered that the surfaces of particles may exhibit distinct differences to bulk substrates prepared by other methods. Examples include the use of surfactants for particle preparation/stabilization or the predominant arrangement of one type of repeating units of copolymers at the aqueous interface during particle preparation. the case of copolymers, which are composed of chain segments, with some of them being more hydrophilic, which leads to their predominant arrangement at an aqueous interface during particle preparation.

In addition to using diluted protein solutions for mechanistic studies, it is particularly interesting to apply application-relevant concentrations, e.g., physiological concentrations, for predictive studies of competition and exchange. For example, at cell culture plates with different proposed binding capabilities, fibronectin only showed differences in adsorption at low protein concentrations, but these differences were insignificant at higher concentrations.[111] While the supernatant of polymer samples with the nonadsorbed protein fraction is easily accessible, higher precision may be assigned to a characterization of the adsorbed protein fraction. Immunochemical analysis with polyclonal antibody-based staining allows the parallel determination of one or several proteins clustering directly at the surface.[112] Additionally, clues for the conformation of one or several adsorbed proteins may be provided when monoclonal antibodies are available for cryptic epitopes that are only exposed upon unfolding.[113]

In many cases, the desorption of proteins is assisted, e.g. by detergents,[114,115] to make them accessible for further analysis in solution. One important method in this respect is electrophoresis, and here especially the corresponding 2D and/or Western blot techniques.[116,117] It should be considered that different methods including comparative instrumental analyses of surface bound proteins, e.g., by XPS and biochemical analysis of desorbed proteins in solution, may provide substantially different results.[118] This is due to incomplete desorption and/or the limited capability of surface-based analytical techniques to access porous polymer structures. Alternatively, the hydrolysis of bound proteins can be performed under acidic or basic conditions. Subsequently, the degradation products, in this case individual amino acids, can be quantified.[119,120] Similarly, mass spectrometric analysis of adsorbed proteins[121] can also be performed after tryptic digestion, in which a fragment analysis may allow the detection of the adsorbed proteins from complex mixtures.[122]

Since surface coverage by proteins is often fast, classical biochemical methods to analyze proteins in solution (supernatant) such as chromophoric reactions (e.g. bicinchoninic acid assay, Bradford assay, Lowry assay)[123] may not be preferred to kinetically monitor the adsorption/exchange processes, but may rather be suited to analyze the equilibrium. Any of these methods may be only applicable if single proteins are used or if no distinction between mixed proteins is required. The individual quantification of specific proteins in mixtures requires either their separation (e.g. by gel chromatography), their direct selective biochemical detection (e.g. with antibodies,[111] aptamers[124,125]), or indirect detection (after labeling).[126–128]

The binding of large fluorescent probes to proteins may, however, alter their physicochemistry and also potentially change their conformation and bioactivity. That may be overcome with the help of radiolabels,[129] although the modification conditions might also lead to changes in the adsorption behavior. In addition to the detection of tryptophan and tyrosine-derived inherent protein fluorescence, fluorescent probes are a preferred and strongly expanding technique for selective protein quantification.[130] Posttranslational intracellular labeling[131] or solid-phase-assisted coupling approaches[132] may allow accessing proteins with site-specific labels and thus enable control of the quantity and position of fluorescent probes bound to the protein of interest.[133] Alternatively, labels can be attached to specific antibodies for immunochemical protein staining.

Platelet Activation

An important question is how the identified protein adsorption pattern affects the behavior of biomaterials in vivo, which is often not predictable. Biochemical assays of protein adsorption must be complemented with a functional analysis, which should also consider cell-based responses and the triggering of immunological cascade reactions that particularly occur upon material contact with blood. The released soluble factors and the activation status of cells may be biochemically assessed as well.

The adhesion of thrombocytes to material surfaces and the initiation of the coagulation cascade are mostly considered to be mediated by proteins bound to the surface. Many different proteins have been associated to this process, including fibrinogen,[134] vitronectin, the Von Willebrand factor[135] as well as unfolded albumin.[136,137] Interestingly, thrombocyte adhesion is only partially affected by the amount of protein bound to the surface, whereas the type of proteins and their conformation seems to play a greater role. The binding of thrombocytes might, however, be tolerated if not connected to an activation of thrombocytes.

When blood-derived test media are to be used, different aspects of hemocompatibility may be studied after a specific pretreatment of the blood collected from typically healthy donors. Platelet-rich plasma (PRP) treated with trisodium citrate as an anticoagulant is suitable to study platelet–biomaterial interaction and thus the thrombogenic potential of the biomaterial.[138] Complement activation in a mimicked physiological condition can be studied when hirudin is used as anticoagulant for whole blood.[139] For the widely used heparinized whole blood, relevant interlaboratory differences in terms of concentration and type of employed heparin should be noted, because they can affect the comparability of the results. In all cases, the blood collection and handling need to be well standardized to avoid the pre-activation of coagulation or the loss of cellular functions.[140]

Thrombocyte activation is related to the release of vasoconstrictors (thromboxane, platelet-derived growth factor, serotonin), mediators for platelet aggregation (fibrinogen, Von Willebrand factor, thrombospondin, adenosine diphosphate, platelet activating factor (PAF)), and promoters of platelet adhesion (fibronectin, Von Willebrand factor), which all support coagulation. Subsequent clotting could lead to thrombosis and infarction distal to a vascular implant, representing a major danger in biomaterial applications. However, it is impractical to study protein release from thrombocytes to estimate thrombocyte activation in vivo because of the short release time, local occurrence, and low concentrations.

A better alternative is to look at the changes in the thrombocyte phenotype during activation with regard to size and shape, i.e., from a doughnut-shaped to a flat outspread form. The size and shape of single thrombocytes is connected to specific activation states, which are classified from 0 to VI. Therefore, the average size of a thrombocyte adhered to a biomaterial gives valuable information on its degree of activation.[138] Furthermore, the overall thrombocyte-covered area of a biomaterial after contact with full blood can be evaluated. A major step for allowing a faster routine screening would be an automated identification of single thrombocytes on a surface and the corresponding thrombocyte-covered area, which has recently been addressed by optical microscopy. It could be shown that the employed method for data evaluation is highly effective when a two-step filter was used that includes 1) the background subtraction by the rolling ball technique and 2) a watershed segmentation algorithm.[141]

Another option to visualize the activation state of thrombocytes utilizes the immunostaining of proteins on thrombocytes. The pattern of proteins on the surface of thrombocytes changes according to their state of activation, e.g., nonactivated thrombocytes are covered with albumin, while activated thrombocytes are not. Therefore, in contrast to activated thrombocytes, nonactivated thrombocytes can be stained by antibodies to albumin. In contrast, fibrinogen can be detected primarily on activated thrombocytes. Other proteins that can be evaluated include myosin, actin, α-actinin, tropomyosin, and vinculin.[142]

Plasmatic coagulation can be induced by biomaterials in a thrombocyte-independent pathway ("contact activation pathway"), though one should keep in mind that only the activation of both pathways leads to a fast coagulation. The contact activation is routinely measured in clinical chemistry laboratories evaluating the coagulation of blood from patients. The most common techniques are the measurement of the prothrombin time (PT) evaluating the extrinsic coagulation system as well as the (activated) partial thromboplastin time [(a)PTT] representing the intrinsic coagulation system.

Complement Activation

Immunological responses to biomaterials can potentially prohibit or restrict the applicability of a material in vivo. Other than antibodies emerging to repeated administration of, for example, PEGylated substances,[143] the complement system is part of the innate immune system, which can react to biomaterials in pathways originally connected to the defense against microbial intruders. The activation of the complement system can lead to inflammatory responses against biomaterials and protein adsorption plays a major role in this respect. Therefore, the test for complement activation is an important functional test of the biological performance of biomaterials. For a more comprehensive overview of the complement system, the reader should consult the literature specialized in this field.[144] About 30 different proteins participate in the complement activation, in which the proteolysis of component 3 (C3) to component 3b (C3b) is the key step. This cleavage occurs through different pathways, of which only two seem to play a role in the context of biomaterial-induced complement activation.[145,146] The C3b formation is part of either the classical pathway and mediated by C3 binding to the biomaterial surface, or the alternative pathway, which occurs by spontaneous hydrolysis of C3 in plasma with a lack of inhibition by one of the regulatory proteins such as factor H. C3 generally does not bind directly to a surface, but to previously adsorbed proteins, especially albumin, fibrinogen, or IgG. A direct covalent attachment of C3b to incompatible surfaces is considered to be important for its further bioactivity. The covalent bonding can occur through amide or ester bonds, therefore nucleophilic amino groups on material surfaces may be especially complement activating. The quantification of proteins of the complement system, e.g. C3b, by SPR or QCM is one option to evaluate the influence of a biomaterial on the complement activation. The formation and anchoring of C3b leads to the release of anaphylatoxins like component 3a (C3a) and component 5a (C5a). Regardless of the pathway, a more effective measure of complement activation is therefore the concentration of C5a in plasma or serum after incubation with a biomaterial.[147,148] A major point is the catalytic production of C5a, which provides an amplified readout that is detectable by an enzyme-linked immunosorbent assay (ELISA). In the search for biomaterials exhibiting low complement activation, the preferential adsorption of regulatory molecules, specifically factor H, is of interest. Factor H adsorption as a regulator of the alternative pathway may reduce the immunological response to biomaterials.[149]

Bead-Based Affinity Chromatography of Complex Protein Samples to Identify Polymer Binding Partners

The separation of biochemical mixtures by affinity chromatography is achieved by the specific interaction between a stationary phase and the biomolecules. The stationary phase consists of polymer-coated beads, which specifically adsorb proteins from complex protein mixtures, e.g., human serum or plasma. It is a feasible and straightforward approach with regard to subsequent protein identification as well as biocompatibility and nanotoxicology testing.

As a general rule, the binding of human serum to unfunctionalized beads should be compared to polymer-functionalized beads that discriminate additional, intrinsic polymer-specific binding proteins. To achieve this goal, the column packing of beads further optimizes the subsequent handling, e.g. protein loading, washing, and the specific elution of interacting binding partners as well as the regeneration of the functional support. Upon application of 1D and 2D polyacrylamide gel electrophoresis (PAGE), protein eluents from stripped polymer-functionalized beads can be separated and the individual protein spots can be analyzed by mass spectrometry and sequencing procedures.

It is believed that the plasma proteome consists of approximately 3700 proteins.[150,151] Keeping in mind that the protein corona formation on nanoparticles is primarily determined by high-abundant proteins like albumin, immunoglobins, fibronectin, and apolipoproteins, it is not surprising that with similar approaches but on different surfaces similar protein patterns were identified as binding proteins.[152] Therefore, proteins with a higher binding affinity are often masked due to their lower abundance in complex biological protein solutions. Furthermore, the identification of real binding partners is often questionable, because the indirect binding by bridging proteins is a common phenomenon. To evaluate direct interactions with the polymer surface, additional techniques like SPR, ITC, or microscale thermophoresis (MST) are required to verify protein binding on individual purified samples. Since the protein corona formation is a dynamic event, depletion of high-abundant proteins from plasma or serum by affinity chromatography offers the perspective to detect additional relevant binding partners.

2.5. Theoretical Tools to Test Mechanistic Hypotheses

For a surface to be bioinert or bioactive, protein adsorption has to be prevented or tuned. This is somehow the central dogma, on which theoretical models for the investigation of the effect of coatings on biomaterials rely. As described in the introduction, water-mediated hydrophobic and hydration forces were thought to be the major players determining protein adsorption, a view that is summarized in the concept of "Berg's law".[21] According to this empirical rule, protein adsorption on hydrophilic surfaces does not occur, because proteins are not able to displace the water molecules, which are in contact with the surface, whereas the opposite should be true for hydrophobic substrates. Although this view was consistent with many early experiments, subsequent studies of various charged hydrophilic gels[64,95,96,103,153] clearly showed that protein adsorption can also take place on these surfaces. This calls for theoretical models, which are able to account for these findings. In this regard, it is now well-recognized that the physicochemical processes behind protein adsorption are governed by a complex interplay of the conformational degrees of freedom of the (responsive) polymer and various polar and nonpolar interaction mechanisms.[20] Nonpolar interactions include van der Waals forces as well as solvent-mediated phenomena like hydrophobic attraction[22] or hydration repulsion,[23] which are considered in Berg's law. Polar interactions, which can be manipulated by a change of salt concentration and pH, include nonspecific electrostatic and ionic screening effects as well as more local, specific nonbonded interactions, e.g., salt bridges or hydrogen bonds. A quantitative description of these highly interconnected and collective mechanisms requires multiscale coarse-graining modeling approaches for both the solvated polymers[24–26] and the proteins[27,28] to access all relevant time and length scales, and at the same time preserving chemical specificity.

In this regard, the steadily increasing power of computers enables to study protein interactions with surfaces on a microscopic level using particle-based computer simulations,[154] such as explicit water molecular dynamics (MD) computer simulations[155] or more coarse-grained, solvent-free Langevin simulations.[156] The early work in that area focused on size and "excluded-volume" effects in competitive protein adsorption upon simple model surfaces and elucidated the competition between effective protein–protein, protein–surface interactions, and protein size ratios in binding.[157,158] More recent coarse-grained simulations provided insights on shape effects and kinetics.[159] On the microscale, explicit water simulations on purely hydrophilic, charged solid surfaces revealed that in such a system the mechanism driving protein adsorption is the electrostatic attraction between parts of the protein and the surface.[160] More recently, protein adsorption on realistically modeled polyurethane polymer surfaces showed that the interplay between the hydrophobic residues, the surface roughness, and the hydrophilicity of the polymer surface determined the overall protein adsorption affinity.[161] MD studies of lysozyme adsorption onto a crystalline polyethylene surface gave molecular-level insight into the fine balance between the orientation-dependent protein–surface interaction and the (de)hydration of the reactants during adsorption.[162] Both these detailed simulations show that protein heterogeneity plays a key role for balancing the specific interaction mechanisms and in general not a single interaction feature can be assigned to protein–surface adsorption. While these kinds of simulations provide valuable molecular insight, the time and length scales, which are accessible with these methods, are still too small to gain a complete understanding of the adsorption process.[163]

On a rather mesoscopic scale (with less specific resolution of protein or surface atoms or hydration effects), complementary approaches have been introduced on the basis of lattice models,[164] single-chain mean field theory,[165,166] density functional theory (DFT),[167–171] and self-consistent mean field theory,[172–176] where mean field approximations for the electrostatics and the excluded-volume packing of the proteins can be included. In a series of pioneering works, Szleifer and co-workers emphasized the effects of excluded-volume interactions between the various components of the system as well as nonspecific protein–surface and polymer–surface interactions.[168,169,171] These studies gave a firmer theoretical basis to the empirical rule that an important molecular mechanism, which prevents protein adsorption on polymer-grafted surfaces, is the competition between proteins and the repeating units in the polymer for surface adsorption sites and proteins for surface adsorption sites. Besides, the theory was able to provide further insight into how polymer parameters such as molecular mass, grafting density, or hydrophobicity determine specific trends observed in protein adsorption. For example, it was possible to qualitatively and quantitatively explain why long polymers can be as effective as short ones in preventing protein adsorption even at a much lower grafting density. This was attributed to the deformability of the polymer backbone, which upon compression by the adsorbed proteins allows its repeating units to accumulate close to the surface creating a barrier for adsorption, a mechanism that works better the stronger the hydrophobic surface–monomer attraction is. A similar mechanism was described by Halperin and Kröger, who showed that polymer brush swelling and collapse may induce three different stable adsorption regimes for proteins: direct binding at the substrate onto which the polymer is bound, embedded in the brush, or on the brush surface.[172,173] These models are important for clarifying the role of polymer architecture on protein adsorption tuned by polymer–surface interactions. Notably, they did not contain any electrostatic interaction between polymer chains and proteins. However, most polymer coatings and proteins are charged and create mesoscale electrostatic potentials and fields. In this regard, mean field electrostatic extension of the DFT approaches to include competitive adsorptions on bare surfaces indicates that multiple layers of proteins occur and that their structures are controlled by the salt concentration.[167] Moreover, field-theoretical models combining classical polymer brush theories,[177,178] charge regulation, and protein packing, indeed demonstrate how the electrostatic potentials self-regulate during protein binding and couple to the polymer conformations for polyelectrolyte brushes.[166,174–176] Recently, it was also demonstrated on a more conceptual level, how electrostatic cooperativity can be separated from intrinsic binding effects by fitting advanced binding models to experimental binding isotherms for lysozyme and core–shell microgels.[96] All these findings together support the relevance of electrostatic mechanisms in protein adsorption to polymeric surfaces. The details of rather local electrostatic correlation effects such as salt bridge binding or counterion release mechanisms[91] are still awaiting full experimental and theoretical confirmation (see Figure 5).

Intimately linked to the thermodynamic description of protein adsorption, the modeling of kinetics also constitutes an important area of research. The rates of protein adsorption and desorption are governed by the local protein mobility in solution and in the polymer matrix. Mobility magnitudes partially originate from local protein–polymer and protein–protein interactions, whereas long-ranged hydrodynamic effects also play a role. Protein diffusivities in polymer networks, for instance, are generally described in terms of the reduction of free network volume, an enhanced hydrodynamic solute drag in a compressible continuum, and increases of the diffusive path length due to obstructions.[179,180] Because of the heterogeneous distribution of interactions and the mobility among different proteins, the composition of the adsorbed protein layer in multicomponent systems exhibits a nonmonotonic behavior in time, the Vroman effect.[15,181] A correct description of this effect and its dependence on the system's parameters is probably the most important challenge

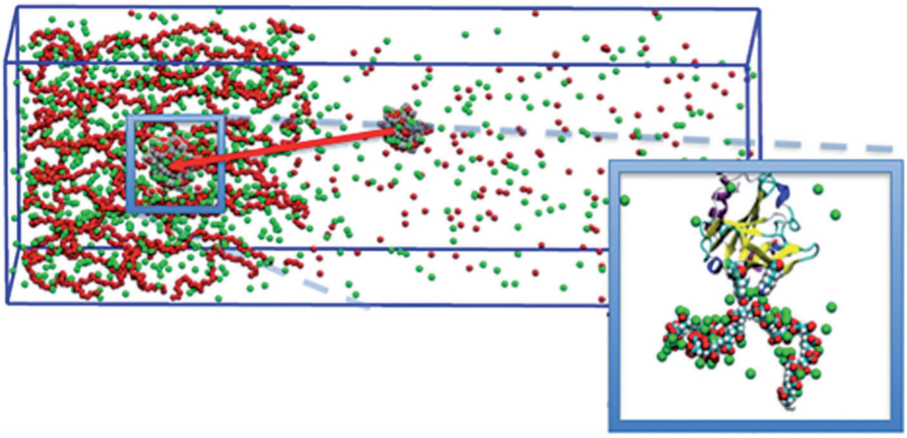


Figure 5. Illustration of salt bridges, a typical ionic correlation effect driving adsorption on a charged polymer brush. Red and green beads represent the negative and positive charges, respectively. In the inset, two "salt bridges" between the polymer and a protein embedded in the polymer matrix are shown. In this model, the protein adsorption is thermodynamically favored even when both polymer and protein bear the same net charge. The latter adopts different forms depending on the type of salt present in the solution. Apart from the aforementioned salt bridges, Manning condensation and counterion release effects can typically provide thermodynamic forces, which are strong enough to overcome monopole–monopole repulsion.[91]

in developing models of protein adsorption kinetics. Typically, the Vroman effect is explained as the outcome of a competition between the initially faster adsorption of small proteins and the higher thermodynamic stability of bigger (and hence slower) proteins replacing the smaller ones at longer time scales. However, this simplified picture does not take many important facts into account. Diffusion, for example, is not the only mechanism through which proteins move; convective fluxes arising from free-energy gradients are also important. Moreover, some of the thermodynamic forces driving protein motion are not size-dependent. As a result, bigger proteins are not always slower or more stable than smaller ones. In order to describe these scenarios to obtain a more rigorous picture of protein adsorption kinetics, models for adsorption on both bare and polymer-coated surfaces have been studied using a wealth of different methods, ranging from particle-based molecular simulations[159,163] to more effective coarse-grained models based on continuum theories[167,168,170] or master equations.[95,182] Particle-based molecular simulations have been used not only to understand single protein dynamics,[163] but also to study the collective effects due to the proteins' shape[159] and to clarify the role of size-induced effects.[157,158] For example, it was shown how shape can lead to different effective interactions on bare surfaces, thus inducing nonmonotonic competitive adsorption kinetics,[159] which is a possible manifestation of the Vroman effect. Although it is useful to study the influence of such molecular aspects like the proteins' shape and chemical structure, particle-based simulations cannot access the relevant time scale, ranging from milliseconds to hours or days,[64,95,103,168,182,183] which is necessary to understand the Vroman effect in real systems.

For this purpose, the two major current approaches have been master equations that describe Langmuir kinetics and ab initio models based on a so-called "generalized diffusion approach",[168] i.e., "dynamic DFT" (DDFT).[184,185] Langmuir kinetic models can be built once the equilibrium binding constant for the single-component protein adsorption is available for all species in solution and, at least for simple systems, they have been shown to reliably rationalize the observed kinetics.[95,182] For example, they allow to clarify how long-lived steady states can appear in systems with multiple protein types that last for hours before true equilibrium is reached,[182] thus explaining the apparently contradicting experimental results. The molecular mechanism behind these long-lived states is the following: whereas the adsorption is dominated by the diffusion timescale, which is dictated by protein size and solvent viscosity, desorption is controlled by the need to detach a strongly bound protein from a surface. This protein might not be the thermodynamically more stable one, yet initially adsorbs faster on the surface purely due to its higher diffusion coefficient (one possible manifestation of the Vroman effect). The timescale for protein desorption can be roughly calculated from the knowledge of the equilibrium adsorption constant and the diffusion-controlled Debye rate,[64] and turns out to be of hours for typical proteins on bare surfaces. A drawback of Langmuir kinetic models is that all effects of the various driving forces in the system are simply gathered in one number, which is the equilibrium binding constant for the different species. As such, they are not particularly useful to quantitatively predict how specific design choices, for example the polymer grafting density, will affect the observed kinetics. Also, it is important to recognize that the assumption of Langmuir kinetics is not always valid,[186] especially when size effects are important. Moreover, since the dynamic change of the various interspecies interactions driving adsorption is only partially taken into account, Langmuir kinetic models cannot properly explain the many possible cooperative binding mechanisms.

In order to overcome these problems, important steps in the direction of building a well-justified theoretical framework for sorption kinetics have already been taken in the realm of DDFT approaches by Szleifer and co-workers, in which equilibrium free-energy functionals are used to generate chemical gradients in diffusion equations.[167–171] Using such approaches it was found, for instance, that polymers which are not attracted to the surface can greatly increase the timescales required for proteins to adsorb. Instead, polymers more strongly attracted to the grafting surface, although less effective in slowing down adsorption kinetics, are better at reducing the final, equilibrium concentration of adsorbed proteins. The role of parameters such as the polymer's length and grafting density have also been analyzed[168,169] as well as that of protein charge and salt concentration of the medium[167] in a multiprotein mixture adsorbing on bare surfaces. Although computationally more expensive than Langmuir's kinetic models (see above), DDFT-based models are still able to access the relevant timescales typically involved in competitive protein adsorption,[168] thus providing a way to compare real systems of interest. An obvious advantage of such approaches is that, being based on molecular parameters such as the polymer grafting density and length,[168,169] they directly relate to experimentally controllable quantities, and thus provide useful design rules for the material scientist. Much remains to be studied within the DDFT framework. In particular, the kinetics become quite complex in charged systems due to the coupling of protein motion to the local and time-dependent electrostatic fields close to the surface.[167] These effects, especially the inclusion of a charged polymeric coating, have just started to be investigated. Given the wealth of interactions governing the binding processes, a very rich behavior can be expected in future model studies on competitive adsorption kinetics of proteins to polymer-coated surfaces.

3. Bioinert and Biospecific Surfaces—Recent Developments and Applications

3.1. Bioinert and Protein-Resistant Coatings

Several polymers that have been employed to prepare protein-resistant coatings (see also introduction) were systematically reviewed in recent literature.[40,187,188] Less attention has been paid to the 3D architecture of these coatings than to their chemical structure. However, the anchoring sites, steric effects, and topology of the polymers are highly important to obtain effectively covered substrates and perfectly prevent protein adsorption. Thus, different strategies for dense bioinert coatings are discussed in the following part.

Monolayer Coatings

Because of their highly controllable structure and related simple coating approaches, monolayer coatings are the most intensively studied and applied strategy (Figure 6). Effective methods for their preparation include self-assembly, non-covalent physisorption, and covalent chemisorption.

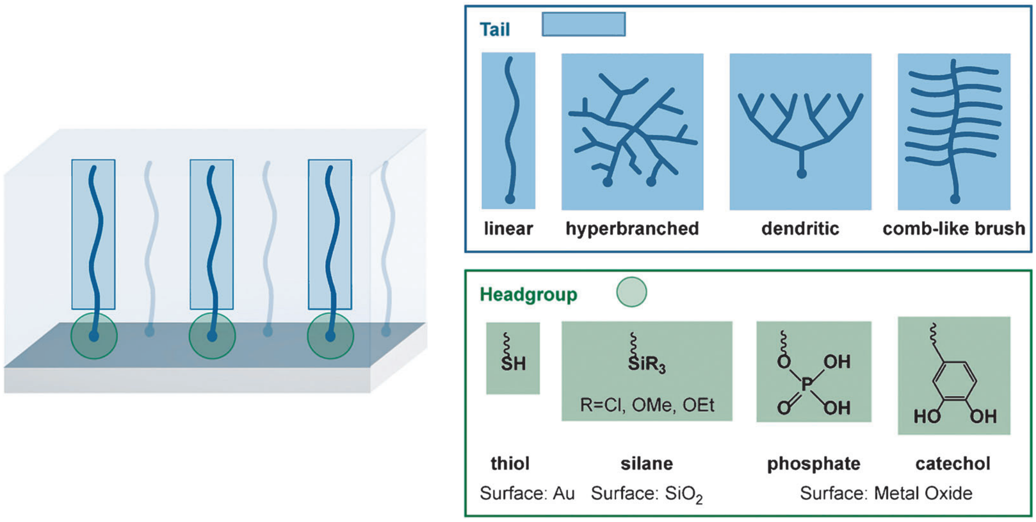


Figure 6. Scheme of a typical monolayer. Polymers for monolayer coatings typically contain head anchor groups and functional tail chains. Commonly used anchors are, e.g., thiol, silane, phosphate, and catechol groups, depending on the respective surface. Tail chains can be linear, hyperbranched, dendritic, and comb-like brush architectures of hydrophilic polymers.

Self-Assembled Monolayers (SAMs)

Self-assembled monolayers (SAMs) are ordered molecular assemblies formed by the spontaneous adsorption of organic molecules on solid surfaces.[189] This self-assembly provides a convenient, flexible, and simple way to modify metal, metal oxide, and semiconductor surfaces.[190]

Whitesides and co-workers used ω-functionalized alkanethiolate SAMs on gold to study the interaction of proteins with organic surfaces. The hexa(ethylene glycol)-terminated SAMs showed efficient protein resistance.[33] Grunze et al. then studied oligo(ethylene glycol) (OEG) SAMs on both gold and silver surfaces in detail and investigated the influence of the polymer chain conformation on the protein resistance.[191] OEG chains on a gold surface adopt a helical conformation, which repels protein adsorption. In contrast, OEG chains on silver surfaces adopt a *trans*-conformation, which leads to the adsorption of proteins. Computer simulations by the Monte Carlo method and neutron reflectivity measurements suggest that due to the substantial conformational disorder of helical SAMs, water molecules can penetrate these coatings more easily than coatings consisting of *trans*-orientated SAMs.[55,192,193] Moreover, the protein resistance was dramatically decreased on the hydrophobic-group-terminated OEG SAMs as well as on the oligo(propylene glycol) SAMs, which have a more hydrophobic interior. It is suggested that both, internal and terminal hydrophilicity, favor the penetration of water molecules into the interior of the SAMs, which is a necessary prerequisite for protein resistance.[194] More recently, even a thermoswitchable linear polyglycerol-copolymer SAM has been introduced for cell and protein adhesion.[195]

Whitesides and co-workers also identified the protein resistance of several hundred kinds of SAMs with different functional groups on gold. A number of new functional groups were identified as protein-resistant. More importantly, the mechanism of action of protein-resistant surfaces was studied by correlating the property with their molecular structure. Consequently, the empirical guidelines for designing a protein-resistant surface became generally known as the “Whitesides rules” (see also introduction).[29,30]

Self-assembly, one of the most effective methods to prepare monolayer coatings with high density and surface coverage, requires an accurate molecular structure to reach a thermodynamic equilibrium. This molecule must contain an anchor group for reversible binding on the surface, e.g., based on thiol–gold chemistry, and an ordered tail chain, which usually consists of linear alkyl moieties. A planar crystalline surface is required in order to achieve homogeneous coatings.[190] Alternatively, less ordered but diverse monolayer coatings that can be obtained by noncovalent physisorption or covalent chemisorption are considerable alternatives for broader applications.

Physisorption (Non-Covalent Adsorption)

The physisorption extends the application of bioinert coatings to diversified surfaces, especially to chemically inert polymeric surfaces. Typical physisorption methods include the Langmuir–Blodgett deposition, hydrophobic adsorption, and electrostatic attraction. Block copolymer brushes can be physisorbed through one block onto the surface.[196]

Langmuir–Blodgett monolayers of a mixture of dipalmitoylphosphatidylcholine and 10–30 mol % of cholesterol suppress protein adsorption due to the packed phosphocholine groups.[197] However, because of the complex instrumentation and limitations of substrate size and shape, Langmuir–Blodgett deposition has not been widely used. Instead, amphiphilic block copolymers can be physisorbed on hydrophobic surfaces by immersing substrates in a dilute aqueous block copolymer solution, e.g., of PEG-PPO [poly(propylene oxide)]. These copolymers were able to reduce the non-specific protein adsorption on a hydrophobic surface to a level of less than 3 $\mathrm{ng\,cm^{-2}}$ under optimal conditions.[198] In a very recent study, protein-resistant amphiphilic copolymers of poly[(2-methacryoyloxyethyl phosphorylcholine)-*b*-(*n*-butyl methacrylate)] (polyMPC-BMA) were cross-linked by trimethoxysilylpropyl methacrylate (TSMA) side chains to enhance the stability. This cross-linked coating was stable even in ethanol and SDS aqueous solutions. In contrast, the same copolymer without cross-linking could be easily removed by washing under these conditions.[199]

Poly(L-lysine)-*b*-poly(ethylene glycol) (PLL-PEG) block copolymers were reported to adhere to anionically charged surfaces through the positively charged PLL block and create a steric barrier through the PEG block.[200] In later studies, PLL-PEG and PLL-dextran comb-like polymers with PLL backbones and PEG or dextran side chains were developed. They successfully reduced protein adsorption on different negatively charged metal oxide surfaces like TiO_2, $Si_{0.4}Ti_{0.6}O_2$, and Nb_2O_5.[196,201] Additionally, long-chain *n*-alkanoic acid monolayers were examined on metal oxide surfaces.[202]

Chemisorption (Covalent Adsorption)

Covalent bonds tether polymer coatings much stronger to a surface than physisorption; therefore covalent immobilization is necessary for many applications. Besides thiol chemistry on gold and silver surfaces, many anchor groups can form monolayer coatings. Typically, silanes are commonly used on silicon oxide and other hydroxylated surfaces[203] and phosphate groups can anchor monolayers on many metal oxide surfaces.[204] In recent years, catechol was recognized as a suitable anchor on noble metal and metal oxide surfaces.[205] These anchors have already been widely used or have potentially valuable application for protein-resistant surface coatings. Furthermore, different specific coupling reactions can be employed according to the functional groups on the surfaces.

Besides the variety of anchor groups, different architectures with tails can also be employed for monolayer coatings using chemisorption. Remarkable examples are hyperbranched and dendritic polymers exhibiting a highly branched 3D architecture.[206] These structures can cover larger surface areas than linear molecules of equal molecular weight. Because a dendritic architecture exposes multiple functional groups on the surface, it is an excellent platform for further multiple functionalizations.[34] The multivalent anchoring and the tree-like structure of the hyperbranched and dendritic polymers have particular advantages in coating rough surfaces. Dendritic polyethylenimine,[207] aliphatic polyester dendrons,[208] and carbazole dendrons[209] have been good shields on gold surfaces. Therefore, these chemically active layers have been converted into inert forms by OEG/PEGylation of the multiple amine or hydroxy groups exposed on the surface. Such PEGylated dendritic monolayers successfully enhanced the protein resistance of the original material.

Oligo-/polyglycerol (OG/PG) and PEG dendrons can be directly used to prepare bioinert coatings without further surface passivation steps. Hyperbranched PG monolayer coatings exhibit structural features comprising aliphatic polyether chains, hydrophilic surface groups, and a highly branched architecture.[50,210] As a result, they have shown a similar or even better protein resistance than PEG monolayer coatings. Furthermore, PG has a higher thermal and oxidative stability than PEG.[50] The monolayer coatings of second-generation PEG dendrons on TiO_2 surface were equally protein-resistant to the linear PEG analogue with the same molecular weight.[211] The analysis of the hydration properties suggested that the dendritic monolayers were relatively stiff with low hydration and low dissipation capacity. Linear monolayers, on the other hand, were viscoelastic, strongly dissipating, and highly hydrated coatings.[211] Overall, hyperbranched and dendritic monolayers with their confined and multifunctional architecture are considered to be attractive for surface bioengineering.

Unlike metal and semiconductor surfaces, only a few common polymer surfaces contain active functional groups for chemical conjugation. Therefore, chemical pretreatment or irradiation methods are required to activate the surfaces for further functionalization. Typical activation methods, such as chemical reagents, ozone oxidation, plasma treatment as well as electron beam and UV irradiation, have been well described in a recent review[212] and will thus not be repeated here. We will only include a few new and efficient methods.

Poly(ether imide) porous membranes are easily functionalized by chemical reactions, because the repeating imide unit can be reacted with nitrogen nucleophiles, which results in the formation of amide bonds between the bulk polymer and the attached structure. Functionalization can be achieved, whereas the main polymer chain is kept intact. The membrane surface was functionalized by grafting PEGs,[213] linear polyglycerols,[51] heparin, polyethylenimine,[214] and tris(hydroxymethyl)aminomethane.[215] PEG and linear polyglycerols did not entirely prevent protein adsorption, and interestingly they had only a minor effect on thrombocyte adhesion and activation. Detailed analyses suggested that a structure–function relationship established, e.g., for PEG, on ideally flat systems cannot be easily transferred to rougher polymer membrane surfaces. Flat inorganic surfaces might therefore not be the perfect model for real-life applications.[213]

Polydimethylsiloxane (PDMS) and polytetrafluoroethylene (PTFE) are two important materials that are typically chemically inert. Semetey et al. reported a hydrosilylation click reaction for the conjugation of vinyl groups from modified methylcellulose to the free hydrosilane groups on the surface of commercial silicone elastomers in one step in water. This integrated strategy produced surfaces that were highly resistant to protein adsorption.[216] In another recent report, the hydration capacity and protein resistance of PTFE were improved by surface-initiated poly(sulfobetaine methacrylate) (polySBMA) through plasma treatment.[217]

Although covalently grafted coatings were successfully introduced to many types of material surfaces, including PTFE, most of them could not reduce protein adsorption as efficiently as self-assembled monolayers on gold due to the low surface activity and grafting density.

Alternatively, the "grafting from" approach was reported to have the theoretical advantage of achieving thicker and denser monolayer coatings.[205] A high-density comb-like polymer brush coating has been developed with this approach. The photoinitiator benzophenone (BP) was employed to activate both organic (polyethersulfone and polycarbonate-urethane) and inorganic (ceramics) surfaces.[218,219] The excited BP moieties abstract a hydrogen atom from the polymeric or ceramic surfaces. Starting from these free radicals on the surface, the polymerization was initiated with monomers exhibiting short bioinert side chains. Because the footprint of the "bottle brush" is significantly larger than that of the initiator,[220] a very high surface packing ratio can be achieved. Comb-like polymer brushes of poly(hydroxyethyl methacrylate) (polyHEMA) and poly-[oligo(ethylene glycol) methyl methacrylate] (polyOEGMA) dramatically decreased protein adsorption on the surfaces.[218]

Polymer brush coatings with a highly controlled thickness were achieved with in situ atom transfer radical polymerization (ATRP) on surfaces. The ATRP initiators with anchor moieties were immobilized on different substrates.[221,222] A well-designed polymer brush of polyOEGMA could decrease the nonspecifically adsorbed single-protein amount to below 1 ng cm^{-2}.[220] A dendritic polymer brush with PG dendron side chains also effectively decreased the protein adsorption from human blood plasma.[223]

Zwitterionic molecules, which perfectly combine the strong interaction with water and the rule of absence of net charge, are also excellent candidates for protein-resistant materials (Figure 7).[35,224] In recent years, several zwitterionic polymer brushes, including polysulfobetaine,[225] polycarboxybetaine,[226] polyphosphobetaine,[227] and mixed charge polymers,[228] were synthesized by a "grafting from" approach. However, the complicated synthesis of phosphobetaine monomers, e.g., 2-methacryloyloxyethyl phosphorylcholine (MPC) limited the application of polyphosphobetaines.[35] Poly(sulfobetaine methacrylate) (polySBMA) and poly(carboxybetaine methacrylate) (polyCBMA) were the most widely used polysulfobetaine and polycarboxybetaine, respectively. The polyCBMA coatings show improved resistance to protein adsorption compared to the polySBMA coatings, because the distance between the charged groups in CBMA was shorter than in SBMA. The shorter distance resulted in a stronger hydration layer on the surface. In addition, both polyCBMA and polySBMA coatings adsorbed less proteins than polyOEGMA, which indicated that zwitterionic polymers are viable alternatives to PEG-based materials.[226] Mixed-charge polymer coatings, wherein the positively and negatively charged moieties are derived from separate monomers, also resist protein adsorption at an equally low level as the polymers mentioned above.[228] Because of their high protein resistance and stability, zwitterionic polymers are considered to be a new benchmark for protein-resistant materials as well as a new platform for biospecific surfaces[229] besides PEG. However, they are sensitive to extreme pH and ionic strength, which may limit their efficiency under those conditions.

The stability of tethered monolayer coatings is a vital factor in practical applications since the interaction between some of the anchors and substrates is usually relatively weak. Monolayer coatings with multiple anchors were developed to overcome this problem. The relatively poor stability of thiol-tethered monolayers is limited by the finite strength of the metal–sulfur bond and by the susceptibility of some simple thiolate systems toward decomposition reactions.[190] Thioctic acid, a linker molecule bearing a carboxylic acid linked to an 1,2-dithiolane ring, can replace alkanethiolates to form more stable coatings.[230] The other attractive alternative is poly-(propylene sulfide) (PPS), a polymer comprising sulfide repeating units. Multivalent anchors are generally considered to be more stable.[231] The multiple sulfides of PPS strongly chemisorbed to gold and showed a much better resistance to oxidative conditions than alkanethiolates. The poly(propylene sulfide-*block*-ethylene glycol) (PPS-PEG) block copolymer could passivate gold surfaces against protein adsorption. These coatings were proven to be insensitive to sulfur oxidation under ambient conditions for at least 41 days.[232] The other example is catechol on a TiO_2 surface. Catechol forms a charge–transfer complex with the metal oxide surface.[233] Three catechol units in the anchor group are required for an essentially irreversible binding and confluence of the formed monolayers. The coatings with the oligocatecholic linear anchor with dendritic PEG tails were stable for at least 12 days in physiological buffer.[211] Dendritic polyethylenimine can be stably immobilized on a self-assembled monolayer that presents carboxylic anhydride groups.[207] Due to its large footprint and the flexible hyperbranched structure, this dendritic polymer can compensate the defects of self-assembled monolayers with low grafting density. Equipped with short PEG groups, the polyethylenimine surfaces become highly protein-resistant.

Monolayer coating architectures promise high effectiveness and simplicity. However, a dense coating has only been achieved on a few types of substrates, due to the steric effect and the defects of the coatings. Furthermore, to obtain a strong covalent surface binding a harsh treatment is required, which may compromise the mechanical properties of

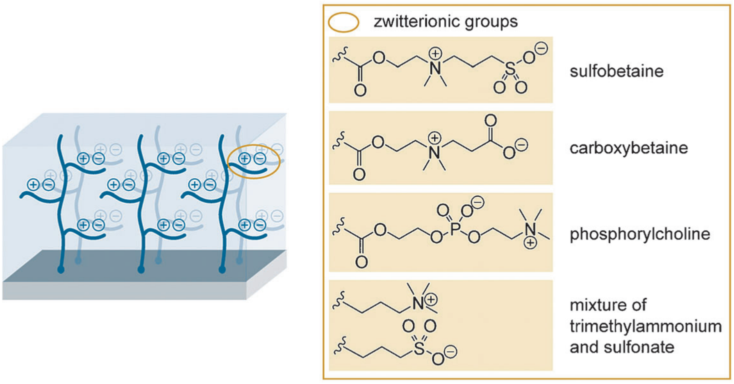


Figure 7. Molecular structures of sulfobetaine, carboxybetaine, phosphorylcholine, and a mixture of trimethylammonium and sulfonate groups (pseudobetaines).

the underlying substrates. Long-term stable monolayer coatings on a chemically inert surface still remain a challenge.

Multilayer Coatings

Surface gelation[234] and spin coating[235] are usually employed to prepare thick and dense layers on various substrates, but the thickness is not always controllable. Some multilayer-based technologies, however, offer a high level of thickness control for these coatings (Figure 8). Multilayer coatings can shield the defects in each single layer to reach a high surface coverage. More importantly, the intracoating interactions can be enhanced, e.g., by hydrophobic forces, hydrogen bonding, electrostatic interaction, and covalent cross-linking, during multilayer formation. These interactions enhance the stability and thereby enable the immobilization of such coatings on surfaces even with only weak anchors. In other words, many multilayer coatings can be formed on inert surfaces under mild conditions.

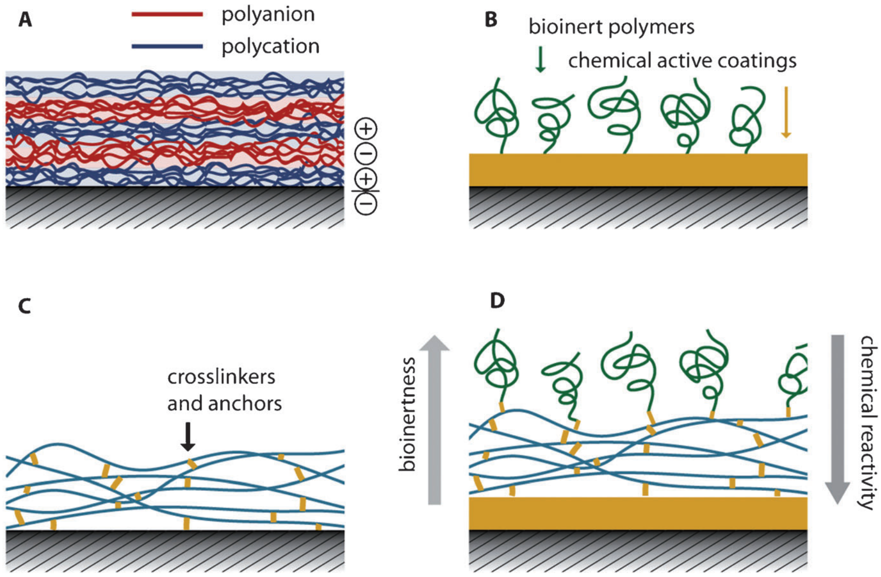


Figure 8. Scheme of a) layer-by-layer (LbL) assembled coating, b) a bioinert coating tethered onto a chemically active layer, c) a chemically homogeneous multilayer coating, and d) a hierarchical polymer multilayer coating, in which the chemical activity gradually decreases and the bioinertness gradually increases from bottom to top.

The polyelectrolyte layer-by-layer (LbL) assembly achieved by the consecutive adsorption of polyanions and polycations from solution is a general approach for the fabrication of multicomponent films on a wide range of surfaces.[236] However, the classic LbL films fail to prevent protein adsorption, because they always display a net charge on their surface, which is in conflict with the rule of absence of net charge.

To overcome this conflict, Textor et al. combined charged LbL coatings with poly(L-lysine)-poly(ethylene glycol) (PLL-PEG) copolymers to shield the charge. As mentioned above, PLL-PEG can be assembled on a negatively charged surface by adsorption of the positively charged PLL block. Thereby, the PEG block is exposed on the surface. Silicon oxide, melamine formaldehyde (MF), and poly(lactic acid) (PLA) surfaces were successfully coated with alternating multilayers of poly(allylamine hydrochloride) (PAH, polycationic) and poly(styrene sulfonate) (PSS, polyanionic). After termination with a monolayer of PLL-PEG, the coatings effectively resisted protein adsorption.[237] Hammond et al. prepared protein-resistant LbL multilayers by adjusting the hydrophobicity of the polycations to neutralize the surface net charge. Cationic *N*-alkylated polyethylenimines with a well-designed alkyl chain length were assembled with anionic poly(acrylic acid)s (PAAs) on silicon surfaces. The hydrophobic alkyl chains limited the classic electrostatic assembly of the hydrophilic PAAs. The amount of adsorbed PAA can be controlled in a way that it is just sufficient to neutralize the surface charge instead of reversing it. In this case, the protein adsorption was minimized, which was attributed to the hydrophilic PAA topmost layer and the absence of net charge on the surface.[238] With the LbL assembly, the thickness of the coatings is well controllable and harsh conditions can be avoided. However, multiple coating steps in solutions with different pH values are necessary to erase the defects in the individual layers. Although many LbL coatings can be assembled on different surfaces, they still lack stability on noncharged surfaces.

In recent years, several material-independent coating methods have been developed that require only one or a few steps. These coatings were chemically or physically cross-linked to enhance their stability. On the other hand, they exposed plenty of functional groups for further modification. Thus, these chemically active coatings could be combined with bioinert coatings to improve protein resistance on a broad range of material surfaces.

The monomer [2.2]paracyclophane-4-methyl 2-bromoisobutyrate, which contains an ATRP initiation moiety, was coated on different surfaces by chemical vapor deposition and polymerization. PolyOEGMAs were subsequently initiated from the coating to generate protein-resistant coatings.[239] Another similar approach was realized by radio frequency magnetron sputtering of nylon 6,6 on the surfaces. Bioinert polymer brushes were then initiated by ATRP initiators that grafted to the amino groups on the surface.[240] Furthermore, hydrophobic surfaces were modified with protein layers. Denatured human serum albumin was adsorbed on the surface and cross-linked by glutaraldehyde to enhance the stability of the layer. Afterwards, a similar post-treatment was carried out with ATRP.[241] These interesting methods extend the ability to modify chemically inert surfaces including Teflon. However, the coatings of [2.2]paracyclophane-4-methyl 2-bromoisobutyrate and nylon 6,6 were only stabilized by hydrophobic interactions, and the cross-linked protein coatings must be degradable in physiological environment. Thus the long-term stability of these coatings in biomedical applications might become a problem. The most widely applied material-independent coating is the mussel-inspired polydopamine, which works on virtually all types of material surfaces, and requires only a simple immersion under mild conditions.[242,243] Many kinds of bioinert polymers were grafted on polydopamine coatings by Michael addition or Schiff base reactions to form protein-resistant secondary layers.[234,244–246]

Starlike bioinert polymers, e.g., the mixture of epoxy- and amino-functionalized star-PEGs, can be stabilized on substrates as chemically homogeneous multilayer coating with a high degree of cross-linking. To enhance the stability, bioinert polymers with the same anchors and cross-linkers could spontaneously form tethered homogeneous multilayer coatings in only one step.[247] Catechols, which can work as both anchor and cross-linker, were grafted to the functionalized sides chains of PEG[248] or the branched chains of dendritic polyglycerol (dPG)[249] to allow multivalent anchoring and cross-linking. It was found that a well-balanced amount of catechols is necessary to obtain coatings with good stability and good protein resistance.[249] Too little catechol groups led to ineffective anchoring of the polymers, whereas too many led to protein adsorption.

These chemically homogenous coatings expose some free cross-linkers on the surface, which may cause adsorption during prolonged contact with proteins. Due to their high chemical activity, the material-independent coatings discussed above interact strongly with proteins. It is difficult to completely shield the chemically active sites by immobilizing bioinert monolayers. In a very recent study by Wei et al., these two kinds of coatings were combined in a hierarchical multilayer architecture to reduce the drawbacks of both types (Figure 8D). A catechol- and amine-rich dPG-based material-independent coating was employed as the foundation layer. A bioinert multilayer that was composed of dPG with an appropriate density of catechol groups was employed to completely shield the activity of the foundation layer. Finally, a monocatechol functionalized dPG formed a flexible final layer by coupling to the exposed free catechol groups on the surface of the second layer to terminate all active groups. In this hierarchical coating, the chemical activity gradually decreases and the bioinertness gradually increases from bottom to top. An architecture with these characteristics can be used to form a material-independent coating with excellent protein resistance properties and long-term stability.[250]

Superamphiphobic and Lubricated Self-Cleaning Coatings

The hydrophilic coatings mentioned above interact with water molecules to prevent protein adsorption. In a different approach, self-cleaning coatings have been designed by decreasing the surface energy to prevent contamination.[251] Self-cleaning coatings have been widely studied, but have been seldom considered for biomedical applications. The main problems originate from their biocompatibility and the effectiveness of these coatings in a physiological environment. However, some exploratory studies regarding the contact of self-cleaning coatings with blood were reported very recently. Typical superhydrophobic surfaces fail to repel blood plasma, because the highly concentrated amphiphilic proteins greatly decrease the surface tension of blood.[252] Vollmer and Butt et al. developed a superamphiphobic coating consisting of a fractal-like network of fluorinated silicon oxide nanospheres (Figure 9A–C). Gas could be trapped between the nanospheres to prevent the liquid, including fresh blood, from contacting the solid surfaces. After 48 h exposure to whole human blood, only a few blood proteins were detected on the coated surfaces.[252]

The geometry of superhydrophobic or superamphiphobic surfaces creates an energy barrier to prevent liquid from penetrating into the pores. A pressure that exceeds a certain critical value will destroy the balance of the three phases of gas, liquid, and solid on the surface.[253] At this point, superamphiphobic surfaces lose their effectiveness in repelling liquids. Vogel and Aizenberg et al. developed slippery fluorinated lubricant-infused porous coatings as omniphobic surfaces (Figure 9D–F). Nanoporous surface structures were designed to lock the fluorinated lubricant on the surface, forming a defect-free, self-healing liquid interface. Thus, a second liquid with the liquid-borne contaminants applied to this surface could be effectively repelled. It was shown that undiluted fetal bovine serum with its blood proteins did stain the dry surface, but could not stain the lubricated surfaces. In addition, the repellent character of this lubricant-infused surface was stable under high pressure, and could bear strong shear forces and mechanical damage.[254] However, the main problem for biomedical application is the potential release of fluorinated molecules.

There is still a long way to go before in vivo biomedical applications are possible; however, superamphiphobic and lubricated self-cleaning coatings have already opened a new window for protein-resistant materials.

Guidelines for the Design of a Protein-Resistant Surface Coating

To sum up, most coating architectures reviewed above show excellent protein repellent properties, but there are advantages and disadvantages to these systems. To design a protein-resistant coating for a practical application, the cost/efficiency ratio is always the first and most relevant factor. It requires commercially available materials and convenient coating methods. Although, OEG/PEGylation are not the best, they are still the most widely used at present. To achieve a high performance, zwitterionic polymers can be considered as alternative candidates. A one-step SAM could be the best choice on chemically active substrates such as gold. Chemically inert polymer substrates should be activated by irradiation or active foundation coatings. Hierarchical multilayer architecture can increase the protein resistance of the coatings. To enhance the stability, multivalent surface binding and inner-layer cross-linking should be considered. Superamphiphobic and lubricated self-cleaning coatings can now be used for in vitro devices to prevent blood plasma adsorption, and may be used in vivo after further development. Overall, it is

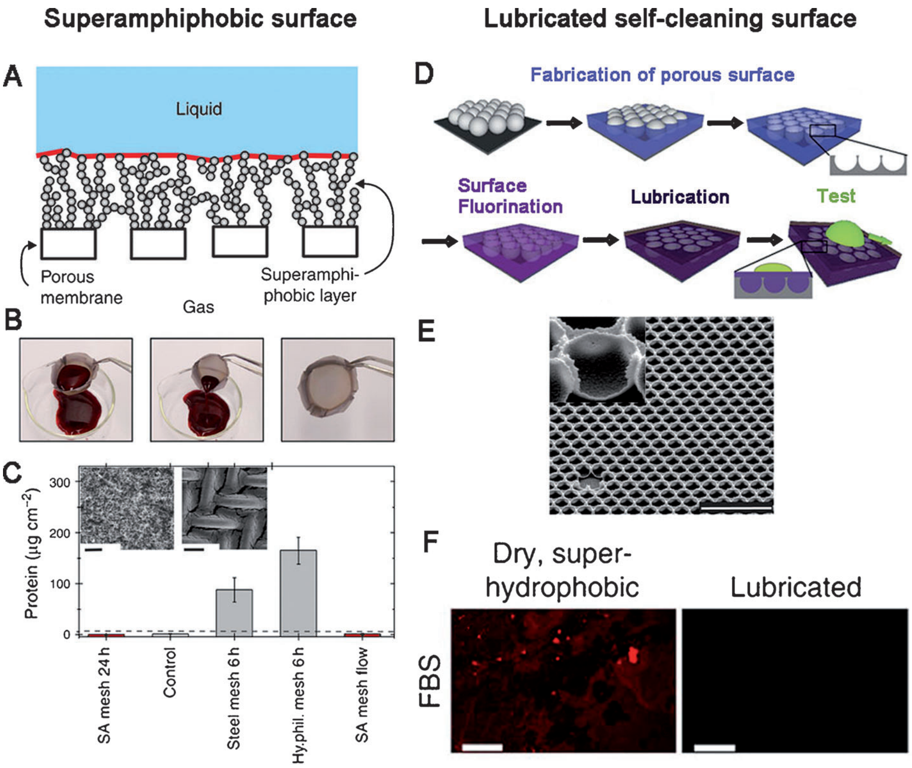


Figure 9. A) Scheme of a superamphiphobic surface coating. A fractal-like network of fluorinated silicon oxide nanospheres was stabilized on the stainless steel meshes. B) Whole human blood was rinsed out of a superamphiphobically coated basket made of a steel mesh. C) A protein adsorption assay on superamphiphobic and controlled surfaces. Inset shows the SEM image of a superamphiphobic membrane after 48 h exposure to whole human blood (left, scale bar 10 μm) and the corresponding metal mesh after 2 h exposure to whole human blood (right, scale bar 50 μm). A–C) were reprinted from Ref. [252] with kind permission of the Nature Publishing Group. D) Construction scheme of lubricated self-cleaning coatings. A perfluorinated lubricant is filled into the fluorinated porous network and forms a stable liquid film held in place by the nanostructures. E) SEM image of the nanoporous network before filling with lubricant. The scale bar is 5 μm. F) Fluorescent images of Sypro Ruby-stained surfaces after incubation with undiluted fetal bovine serum (FBS). D–F) were reprinted from Ref. [254] with kind permission of the Nature Publishing Group.

sensible to consider both the 3D architecture as well as the chemical structure for designing a perfectly protein-resistant surface coating.

Furthermore, it is important to note that despite the outstanding performance of these coatings in the laboratory, only a few coatings are able to prevent adsorption on a very efficient level in real-world media like blood plasma and serum. The underlying principles of the fouling caused by highly complex proteinaceous species are still not well studied. Huck and co-workers identified 98 plasma proteins that foul current "protein-resistant" polymer coatings.[255] It was reported that bioinert coatings can alter the amount of adsorbed proteins but not the composition of the adsorbed layer. The predominant constituent of the adsorbed layer was identified as low density lipoprotein (LDL) particles, which consisted of phospholipids, triglycerides, cholesteryl esters, and apolipoprotein B100 (apoB100). This discovery will deepen the understanding of the principles of interactions between complex proteinaceous and polymer-coated surfaces and lead to the new development of protein-resistant coatings.

3.2. Applications in Biomedicine

Besides the improvement of the in vivo biocompatibility of implanted devices, protein-resistant coatings are also highly relevant in many other biomedical applications such as diagnostics, blood contact materials, packaging, and biopharmaceuticals. Some recent developments in these fields will be introduced in the following section.

In Vitro Diagnostics

Coatings used for solid-phase immunological assays mainly have to fulfill two criteria. They have to reduce the nonspecific adsorption from protein solutions or complex media, like blood plasma, and at the same time allow for the immobilization of a protein or peptide ligand. The simplest approach for the immobilization of proteins is the physisorption based on hydrophobic interactions with the PS substrate, which is mostly used for microtiter plate assays. To account for nonspecific adsorption, the remaining binding sites are subsequently blocked by applying detergents or protein blockers. In the case of protein microarrays, the physisorption to nitrocellulose casted glass surfaces is the generally applied method, because the porous structure of nitrocellulose enables a high ligand density as well as a stabilization of the ligand.[256] Similar to microtiter plate assays, the remaining binding sites are blocked by suitable blocking buffers. The simplicity of these methods, however, may implicate several drawbacks like increased nonspecific adsorption and denaturation of the immobilized ligand.[257,258] Whereas the nonspecific adsorption leads to a higher background noise, the denaturation of immobilized ligand decreases the signal intensity. Additionally, the lack of regenerability of the ligand-coated surface after each measurement cycle presents a problem when determining binding or rate constants by means of time-resolved detection methods like SPR or QCM. A protein-repellent polymeric matrix offering suitable reactive groups for ligand immobilization addresses all of the abovementioned problems.

Since glass, a commonly applied substrate for protein microarrays, and oxidic materials share the same functional groups on the surface (silanol groups), which was shown in optical waveguide lightmode spectroscopy (OWLS) and in silicon microring resonators,[259] all of these surfaces can be functionalized by means of silane chemistry. The simplest way to modify SiO_2 surfaces is to apply standard bifunctional silane coupling agents like 3-aminopropyltriethoxysilane (APTS), 3-isocyanatopropyltriethoxysilane (ICPTES), or (3-glycidyloxypropyl)triethoxysilane (GOPTES). They can be used for the direct modification with a specific biomolecule or as adhesion promoters for the further functionalization with an inert matrix. Apart from lacking an inert matrix, many of the primary amines needed for the first approach may not be accessible due to steric hindrance. Pathak et al. addressed this problem by using 1,1′-carbonyldiimidazole on glass and silicon surfaces as adhesion promoter for the subsequent modification with poly(propylene imine) (PPI) dendrimers of generation 1–5.[260] The authors showed that the accessibility of the amines significantly increased with higher generations. In order to address the problem of nonspecific adsorption, Hucknall et al. used ATRP to graft up to 100 nm thick poly[(oligo(ethylene glycol))-*g*-methacrylate] brushes onto glass surfaces and applied them in protein microarrays with a fluorescence readout.[221] By comparing these coatings with commercially available nitrocellulose membranes, the authors demonstrated that the reduction of the background signal permitted limits of detection (LODs) that were a hundredfold lower. When the polymer was grafted to a gold surface, the nonspecific adsorption from undiluted bovine serum was below the detection limit of a commercial SPR device. Additionally, the authors showed that ligand immobilization could be achieved without a covalent coupling, just by physical entanglement of the grafted polymer chains. A promising alternative to hydrolytically unstable siloxane bonds is the photochemical grafting of terminal alkenes to silicon and silica surfaces.[261,262] This approach is also applicable to carbon-based materials as well as other semiconductor surfaces like germanium, gallium, and nitride.

Three-dimensional polymeric layers generated by "grafting from" approaches, as applied by Hucknall et al., are also of particular interest in evanescent-field-based biosensors as the height can be easily adjusted to fit the decay length of the evanescent field. However, the coating density has to be kept low enough to allow the penetration of the ligand and analyte molecules, respectively. In time-resolved methods, like SPR or QCM, the nonspecific adsorption is generally compensated by running a reference channel in contact with the same analyte solution and by subsequently subtracting the reference signal from the measured signal. However, the accuracy of the data is considerably higher, when nonspecific binding is kept at a minimum.[36] For commercial SPR chips, this is generally achieved by applying a coating of carboxymethyl dextran, a semisynthetic polysaccharide, in which the carboxylic groups can be used for ligand immobilization. Unfortunately, the reference compensation and the protein-resistant coating do not take the nonspecific adsorption into account that is introduced by the ligand. An alternative referencing approach was suggested by Špringer et al.[263] Instead of using an unmodified matrix-coated channel as reference, the detection channel with the immobilized ligand was exposed to an analyte, whose binding sites were blocked with the same ligand.

A substrate-independent approach for the generation of functional surfaces was presented by Rodriguez-Emmenegger et al.[240] The authors demonstrated that nylon 6,6 could be applied as a substrate-independent adhesion layer for the subsequent brush-grafting of oligo(ethyleneglycol)methacrylates and carboxybetaine acrylamides by ATRP.

Bioinert coatings are widely established in molecular diagnostics to improve the signal-to-noise ratio in biosensing, which is essential for lower detection limits of analytes directly obtained from patients. For example, the early detection of cervical cancer, which is the second most common cancer in women, has significantly decreased due to screening programs. It has now been firmly established that an infection with high-risk papillomavirus (HPV) types is the primary cause of almost all cervical cancers. For example, the "papillo check"[264] is based on a biospecific polymer surface coating (Figure 10). This DNA hybridization assay on microarrays can detect PCR-amplified virus fragments in body fluids obtained from patients. The mixture between a bioinert and biospecific polymer matrix significantly enhances the signal-to-noise ratio depending on the functionality of the side chains.

Surfaces in Contact with Body Fluids (Dialysis, Blood Containers, etc.)

Depending on the type of application, different polymers have emerged as standards for providing surfaces with lower

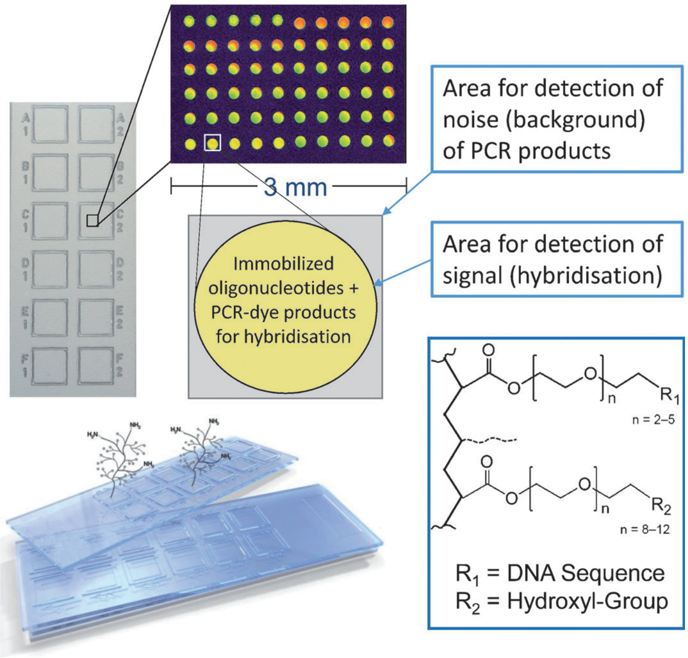


Figure 10. Papillo check for the early detection of the high-risk papillomavirus based on a DNA hybridization assay on microarrays. The biospecific (R_1 = amino-PEG-linked oligonucleotides) and bioinert (R_2 = hydroxy-terminated PEG) groups on the grafted polymer brush can be controlled to obtain high signal-to-noise ratios. With kind permission of PolyAn GmbH, Berlin.[264]

protein adsorption, although the ideal bioinert surface has yet to be found. Important polymers in biomedical applications include polyethersulfone (PES) (dialysis), polyvinylchloride (PVC) as soft plasticized PVC (PVC-P, e.g., in blood bags and tubes), and silicone (shunts, tubes, catheters, implants). Since all of these applications will continue to be of great importance in the future, the drawbacks of the currently applied systems have to be addressed to improve their performance. In dialysis, polymer membranes are used to remove toxins from the blood, e.g., of patients with kidney failure. The performance of the dialyzer is strongly connected to protein adsorption on the membrane, while potential thrombogenic or complement activating properties (see also Section 2.4) have a major impact on the health of patients. Although applied routinely in the clinic, the necessary co-drug treatment with anticoagulants is a cause of concern. One interesting approach is to graft coagulation-inhibiting citric acid onto polyurethanes and blend this functionalized polymer with PES. A much lower protein adsorption and thrombocyte activation was achieved.[265] Surface modification of PES, e.g., with poly(vinyl alcohol), poly(ethylene glycol), or chitosan,[266] is another feasible approach to reduce protein adsorption and improve the long-term membrane performance. In summary, bioinert surfaces of dialyzers have the potential to reduce co-drug treatment, improve patients well-being, and enhance the dialyzer separation performance in clinical applications.

In biomedical applications, PVC is the most widely used biomaterial despite many problems. The major criticism is connected to the inevitable incorporation of plasticizers into PVC,[267] which is necessary for the respective applications. Furthermore, PVC is chemically inert, therefore functionalization of PVC surfaces is of great interest. This is possible with photoreactive groups, e.g., with polymers exhibiting 2-pyridyldithio and benzophenone side chains, which have already been used for the immobilization of CD47.[268] The CD47 functionalization significantly inhibited both the adhesion and activation of platelets and neutrophils. Whereas silicone seems to perform better than PVC-P with regard to hemocompatibility,[269] it does not show a similar good mechanical stability and is more often connected to fatigue. Therefore, to produce devices with suitable bulk and surface properties, it seems to be better to functionalize polymers with silicone or other functional polymers than to increase the bulk mechanical properties of silicone. Figure 11 depicts some typical devices for which bioinert surfaces are urgently needed. The stent is a good example for a commonly used, but not yet ideal device. Because polymer coatings tend to get partially destructed during dilatation, uncoated edges are generated, which can participate in protein adsorption and thrombocyte activation.[270] Surface functionalization of PVC, silicone, or stents might increase the shelf life of blood products, decrease the necessity of drug treatment, and reduce the failure of implants as well as implant-related complications.

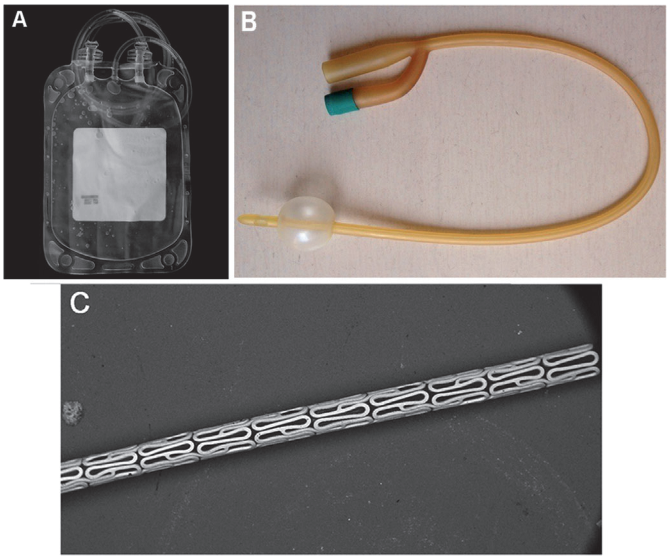


Figure 11. Typical applications of preferably bioinert polymers in the medical field (from left to right): A) blood bag (Reprinted from Ref. [271] with kind permission of Elsevier), B) catheter, and C) polyphosphazene-coated stainless steel stent.

Primary Packaging and Handling of Biopharmaceuticals

The development of recombinant therapeutic proteins has considerably increased over the last two decades. However, the manufacturing, shipping, and storing of protein-based drugs is challenging because of the likelihood of proteins to denature or aggregate. Although lyophilized proteins show an improved stability toward thermal denaturation, liquid formulations are easier to process, require less manipulation, and are therefore easier to administer.[272] However, in liquid formulations, proteins are more prone to chemical as well as physical stress factors, which cause the proteins to denature, aggregate, or adsorb to the container surface during the commonly targeted 2 year shelf life.[273] This may result in a loss of therapeutic activity and even cause immunogenic[274,275] and allergic reactions.[276,277]

By far the most used primary containers for small-scale applications are Type I borosilicate glass vials. Despite its relative inertness and hydrophilic nature, glass surfaces may still adsorb large amounts of protein.[57,278]

Frieß and co-workers have shown that the adsorption of IgG1 and IgG from human serum (h-IgG) to type I glass vials is mainly driven by electrostatic interactions and is therefore highly dependent on the pH and ionic strength of the formulation.[279] They showed that an increased charge difference between surface and protein correlates with an increased protein adsorption (Figure 12).

In order to preserve the beneficial properties of glass as a primary packaging material, which are the impermeability to gases and moisture, as well as to reduce the nonspecific adsorption, glass vials can be modified with bioinert coatings. Based on the abovementioned results, glass coatings containing polyglycerol (PG) were investigated with respect to the nonspecific IgG1 adsorption.[59] In this case, a significant reduction of adsorbed IgG1 was observed. In contrast to the

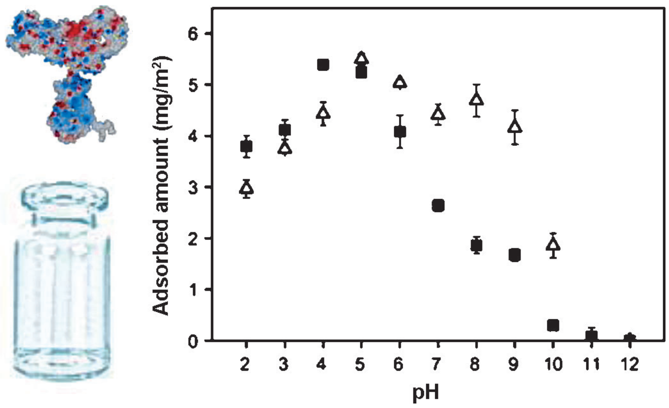


Figure 12. Adsorption profiles of monoclonal IgG1 (■) and pooled human IgG (△) in borosilicate glass vials depending on the pH value. Reprinted from Ref. [279] with kind permission of Elsevier.

uncoated glass vials, the pH and ionic strength of the formulation did not affect the amount of adsorbed IgG. Additionally, the coatings remained unaffected by industrially relevant sterilization methods. Long-term stability tests were performed for up to 3 months and showed a high protein resistance for the PG-modified surfaces.

An alternative to glass are containers made of cyclic olefin polymers (COP) and cyclic olefin copolymers (COC), respectively. COP/COC containers are transparent, which allows for a good visual inspection, and significantly more lightweight as well as break-resistance than glass. Quadry et al. investigated the adsorption of low-concentration formulations (10 μg mL^{-1}) of two proteins (75, 20 kDa),[280] to COP and glass vials after 1 day of storage. For both protein formulations, the amount of adsorbed protein was considerably reduced in the case of the COP vials. In contrast, Mathes et al. found no significant decrease in single-protein adsorption experiments on COC vials compared to glass vials with IgG at concentrations of 1 mg mL^{-1}.[279] Indeed, they were able to show that the amount of adsorbed IgG1 over a range of ionic strengths and pH values even increased after 1 day of storage.

It has to be noted that issues associated with protein adsorption to container surfaces are generally overcome by the addition of excipients, like human serum albumin (HSA), sugars, or nonionic surfactants.[281,283] These excipients not only decrease the adsorption to the primary container, but additionally increase the solubility and bulk stability, and reduce the adsorption of the protein during the manufacturing process, especially when it is filtered and therefore exposed to large surface areas.[284] However, since HSA is obtained from pooled plasma, its use may implicate problems related to batch variations or blood-borne pathogens,[283] whereas nonionic surfactants can increase oxidation and aggregation of the protein.[285] The use of sugars may implicate problems caused by the hyperosmolality of the formulation.

Polymeric coatings can considerably reduce the protein adsorption and the loss of protein might be negligible in the case of high-dose formulations,[279,286] in which it is typically below 0.1 %. However, when labile proteins and low-dose formulations get into contact with container walls during reconstitution or when formulations are diluted for the administration, the loss of protein due to adsorption is commonly quite significant. For example, McLeod et al. reported the adsorption-induced loss of factor VIII during storage in PVC containers.[287] Another example for an increased protein loss for highly diluted protein solutions is given by Quadry et al., who found a significant loss of up to 16 % for protein concentrations of 10 μg mL^{-1} after storage for 24 h in glass vials.[280] Page et al. tested the adsorption from liquid formulations of rhIL-11 to glass vials at concentrations of 1 μg mL^{-1} and found a 40 % loss of activity after storage for 3 h at room temperature.[288] This loss of activity was mainly caused by the adsorption to the glass surface.

Furthermore, when proteins are handled in diagnostics, low-concentration protein solutions are usually exposed to a variety of surfaces.[289] The accompanying protein loss can have a major impact on the outcome of a diagnostic assay. For example, Dixit et al. investigated the activity loss of human fetuin A (HFA) in BSA-treated and untreated PP tubes, respectively, at concentrations of 10 ng mL^{-1} with three different methods.[290] They were able to show that the HFA activity loss due to adsorption and conformational changes after 12 h of storage was above 60 % in untreated PP tubes, whereas the loss was below 40 % in BSA-treated tubes. Therefore, the problem of protein analyte loss in diagnostic assays is an urgent problem that needs to be addressed in the future.

Besides the biomedical applications described above, bioinert coatings were also employed in the field of marine biofouling prevention, bacteria and biofilm inhibition, and infection prevention.[193,291,292] Bioinert materials repel the protein-induced adhesion of organism. The combination of bioinert materials with other specific functional materials such as hydrophobic components (e.g., fluorinated polymers), active moieties (e.g., quaternary ammonium salts) enhances the properties and decreases the cytotoxicity of the applied coatings. However, there is still a lack of long-term stable bioinert coatings in these areas.

4. Conclusions and Future Perspectives

Despite considerable scientific activities, protein interactions between polymeric biomaterials and body fluids or tissues are still poorly understood. Although there are many individual publications, only a few systematic studies provided some first general conclusions. Water-mediated hydrophobic and hydration forces as well as electrostatic interactions are regarded as the major factors determining protein adsorption. On the basis of systematic experiments on self-assembled monolayers (SAMs), the so-called "Whitesides rules" were developed to guide the design of protein-resistant surface coatings. These rules have indeed resulted in many excellent protein-repellent monolayers, although they still have some limitations. Due to the fast development of analytical techniques, these empirical rules can be further improved. Also, theoretical modeling has considerably improved in the last decade. In particular, there seems to be a general agreement on the main forces responsible for protein adsorption. The understanding of dynamics and of competitive adsorption, however, is far less developed. There is also a general lack of systematic studies that combine theory and experiment.

Future research should be focused on the following points:

- More quantitative studies are needed that compare theoretical models to experiments conducted on well-understood surface layers. The results of such studies may then be used to develop further empirical guidelines and to extend the "Whitesides rules".
- The competitive adsorption of proteins to well-defined surfaces should be studied systematically. Such a study must include the dependence on time to gain the much-needed quantitative understanding of the long-term protein resistance of surfaces in complex biological environment.
- The conformational changes of proteins upon adsorption on surfaces as well as the effect of these changes on the biocompatibility should be studied systematically.
- New bioinert coatings that effectively prevent the adsorption of proteins from highly complex protein mixtures (e.g. blood plasma) should be developed based on the evolving theoretical understanding.
- Since most of the coatings are enzymatically degradable during long-term implantation or poorly anchored on the implant surfaces, the cells may integrate with the implanted surfaces to replace the coatings by their extracellular matrix (ECM) components. A long-term human lifetime in vivo viability has not yet been achieved by any protein-resistant coating. There should be studies with regard to this issue.
- Several biomedical applications have been established but the optimal combination of bioinert and biospecific surfaces remains a great challenge for the future.

All these suggested investigations should also provide a better understanding of the most challenging systems such as porous membranes, blood containers, tubings, and many other biomedical devices.

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