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REVIEW

Functionalizable low-fouling coatings for label-free biosensing in complex biological media: advances and applications

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Abstract This review focuses on recent advances in the development of functionalizable antifouling coatings and their applications in label-free optical biosensors. Approaches to the development of antifouling coatings, ranging from self-assembled monolayers and PEG derivatives to ultra-low-fouling polymer brushes, are reviewed. Methods of preparation and characterization of antifouling coatings and the functionalization of antifouling coatings with bioreceptors are reviewed, and the effect of functionalization on the fouling properties of biofunctional coating is discussed. Special attention is given to biofunctional coatings for label-free bioanalysis of blood plasma and serum for medical diagnostics.

Keywords Label-free optical biosensors · Non-fouling coatings · Functionalization · Self-assembled monolayers · Polymer brushes · Blood plasma

Abbreviations

Ab	Antibody
ALCAM	Activated leukocyte cell adhesion molecule
ANACH	Anachelin
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
CTAs	Chain-transfer agents
DOPA	Dopamine
DSC	Disuccinimidyl carbonate
EDC	Ethyl(dimethylaminopropyl) carbodiimide
Fbg	Fibrinogen
FTIR	Fourier-transform infrared spectroscopy
hCG	Human chorionic gonadotropin
HSA	Human serum albumin
IgG	Immunoglobulin G
LOD	Limit of detection
LSPR	Localized surface plasmon resonance
Lys	Lysozyme
NHS	*N*-Hydroxysuccinimide
OEG	Oligo(ethylene glycol)
OWLS	Optical-waveguide light-mode spectroscopy
PBS	Phosphate-buffered saline
pCBAA	Carboxybetaine acrylamide
pCBMA	Carboxybetaine methacrylate
PEG	Poly(ethylene glycol)
pHEMA	Poly(2-hydroxyethyl methacrylate)
pHOEGMA	Poly(oligo(ethylene glycol) methacrylate)
pHPM	Poly(3-hydroxypropyl methacrylate)
pHPMA	Poly(*N*-(2-hydroxypropyl) methacrylamide)
PLL	Poly(L-lysine)
pMeOEGMA	Poly(oligo(ethylene glycol) methyl ether methacrylate)
pMPC	Poly(methacryloyloxyethyl phosphorylcholine
pSBMA	Poly(sulfobetaine methacrylate)
RAFT	Reversible addition-fragmentation transfer polymerization
SAMs	Self-assembled monolayers
SET-LRP	Electron-transfer living radical polymerization

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SI-ATRP	Surface-initiated atom-transfer radical polymerization
SPR	Surface plasmon resonance

Introduction

Label-free optical affinity biosensors enable direct real-time observation of interactions among biomolecules and rapid detection of chemical and biological species [1]. These biosensors have become an important research tool in molecular biology and hold vast potential for bioanalytical applications in numerous fields, including medical diagnostics, environmental monitoring, food safety, and security.

In contrast with routinely used label-based bioanalytical methods, for example enzyme-linked immunosorbent assays (ELISAs) or fluorescence biosensors, label-free biosensors are able to detect target analytes directly and therefore do not require the use of multi-step assays or additional reagents (e.g. secondary antibodies specific to the analyte) [2–4]. However, because label-free optical affinity biosensors are based on the measurement of refractive-index changes caused by the binding of analyte molecules to biorecognition elements immobilized on the surface of the sensor, their performance may be compromised by interfering effects producing refractive-index changes unrelated to analyte binding. Potentially strong interferences can be caused by nonspecific adsorption of molecules from a tested sample to the sensor surface; this effect is sometimes referred to as "fouling". These fouling effects may be particularly severe during the analysis of complex real-world samples, e.g. blood plasma or serum.

To suppress nonspecific adsorption to the sensor surface, multiple research groups have pursued the development of coatings that enable the immobilization of biorecognition elements while also providing a low-fouling background [3, 5–11]. Herein, coatings with fouling levels <1000 pg mm^{-2} in single or complex protein solutions are referred as to "antifouling". Surfaces with blood-plasma fouling levels of <100 pg mm^{-2}, of <50 pg mm^{-2}, and below the limit of detection (LOD) of the technique used are classified as "low-fouling", "ultra-low fouling", and "non-fouling", respectively. To preserve the biological activity of the immobilized biorecognition elements, hydrophilic low-fouling surfaces based on poly(ethylene glycol) (PEG) and its derivatives have been a popular choice [12]. Recent advances in polymer chemistry have led to the development of novel zwitterionic and non-ionic polymer brushes, which have been proved to provide excellent resistance to fouling even from the most complex biological fluids, including undiluted blood serum and plasma [6, 8, 11, 13]. There have been several reports of the successful attachment of biorecognition elements to some of these coatings [8, 11]; however, a deterioration of the fouling properties of the coatings was observed after the attachment of biorecognition elements. Therefore, there is a continuing search for functionalizable coatings that can provide a high degree of resistance to nonspecific adsorption from complex real-world samples [14, 15].

This review focuses on recent advances in the development of functionalizable antifouling coatings and their applications in label-free optical biosensors. It covers aspects related to coating materials and architectures, and methods used for the characterization of their fouling properties. Special attention is given to the application of label-free affinity biosensors with advanced functional coatings in medical diagnostics.

In the first part of this review ("Main principles") we introduce several basic terms related to label-free optical biosensors, non-specific adsorption to synthetic antifouling coatings, and functionalization strategies. The next section, "Antifouling coatings", reviews the designs of available antifouling coatings, including their preparation and characterization. This section also includes a summary of current results regarding surface resistance to fouling from single-protein solutions and from blood plasma and serum. The section "Functionalization of low-fouling coatings" provides a detailed focus on functionalized antifouling coatings, and discusses the effects of functionalization on surface resistance to fouling from blood plasma and serum. Finally, the section "Antifouling coatings for biosensing in blood plasma and serum" reviews work on applications in that field.

Main principles

Concept of label-free optical affinity biosensors

Optical affinity biosensors consist of two crucial elements:

1. biorecognition elements (alternatively referred to as bioreceptors): distinctive biomolecules or synthetic molecular structures that can recognize and specifically capture a target analyte; and
2. an optical technique that is able to transduce the binding event into a measureable signal.

The biorecognition elements are typically arranged on the sensor surface in the form of a two-dimensional (2D) or three-dimensional (3D) sensitive layer. When a solution containing analyte molecules is brought into contact with the sensor, analyte molecules in solution bind to the molecular-recognition elements and produce an increase in the refractive index of the sensitive layer. The change in the refractive index Δn_d occurring within a layer of thickness h can be expressed by the de Feijter formula [11]:

$$\Delta n_d = \left(\frac{dn}{dc}\right)_{vol} \Delta c_b = \left(\frac{dn}{dc}\right)_{vol} \frac{\Delta \Gamma}{h},$$

where $(dn/dc)_{vol}$ is the refractive-index increment, Δc_b is the wt/vol concentration of the bound molecules within the sensitive layer of thickness h, and $\Delta\Gamma$ is the corresponding change of surface concentration (mass per surface area). The refractive-index increment $(dn/dc)_{vol}$ ranges from 0.1 to 0.3 $cm^3\ g^{-1}$ for most relevant biomolecules [11]. Proteins and nucleic acids have a fairly consistent refractive-index increment, which typically falls within 8 % of the value of $(dn/dc)_{vol}$=0.18 $cm^3\ g^{-1}$ [16].

Numerous optical methods have been used to measure refractive-index changes induced by analyte binding. These methods include, but are not limited to, interferometry techniques, techniques based on the spectroscopy of modes of optical waveguides, and ring-resonator techniques. Interferometric label-free biosensors are typically realized as integrated optical devices, for example the Mach–Zehnder interferometer [14, 15], the Young interferometer [17, 18], and the Hartman interferometer [18, 19]. The spectroscopy of guided modes of dielectric waveguides (sometimes referred to as optical-waveguide light-mode spectroscopy (OWLS)) has been exploited in both the resonant mirror sensor [20, 21] and the grating coupler sensor [22]. Likewise, the spectroscopy of modes of metal-dielectric waveguides (i.e., surface plasmons) has also been exploited in a multitude of surface-plasmon-resonance biosensors [23]. Ring-resonator biosensors are typically based on the spectroscopy of whispering-gallery modes of resonator structures and have been implemented as microfabricated ring and/or disk resonators on a chip [24, 25] or dielectric microspheres [26, 27]. In most of these optical techniques, the refractive-index changes occurring within the sensing layer are probed by a portion of an optical wave that propagates along the sensing layer. These waves have a field profile that exponentially decays as a function of distance from the sensor surface (across the sensing layer), and are referred to as evanescent waves. In typical optical-based label-free biosensors the penetration depth of the evanescent waves is on the order of hundreds of nanometers. This short penetration depth has implications for the design of the functional coatings. It is generally desired that analyte interaction should occur close to the sensor surface (in the area of a strong evanescent field), because the response of the evanescent wave to refractive-index changes decreases with increasing distance from the sensor surface.

Concept of antifouling coatings for biosensors

The nonspecific fouling of synthetic surfaces from biological media is primarily caused by the nonspecific adsorption of proteins. The large variety of amino-acid residues and conformation flexibility enable proteins to adsorb to nearly any surface via hydrogen bonds and ionic and other polar or hydrophobic interactions. The adsorption of proteins from complex biological media is a dynamic process: in general, high-mobility proteins are first adsorbed to a surface, and are subsequently replaced with lower mobility proteins having a higher affinity to the surface (Vroman effect) [28–31]. Protein fouling is usually low on hydrophilic and electroneutral surfaces because of the reduction in hydrophobic and ionic interactions [5, 32–35].

Protein adsorption on a substrate with a low-fouling coating results from a competition between repulsive forces produced by the coating and adhesion forces induced both by interactions between proteins and the substrate surface and by weak attractive interactions between proteins and the coating [36–40]. There are several characteristics related to the physical structure and properties of the coating (thickness, density, elasticity, etc.) that can prevent proteins from penetrating to the substrate surface and thus from entering a region of long-range electrostatic and van der Waals interactions with the substrate. The chemical composition of the coating will prefer hydrogen bonds and dipolar interactions with water molecules to interactions with amino-acid residues and the peptide backbone of proteins. The presence of a hydration layer consisting of organized water molecules at the coating surface has been revealed to be a necessary condition for its antifouling properties [41, 42].

A common approach to reducing the fouling effects on biosensor coatings is the addition of different surfactants (for example Tween), protein-based additives (including BSA or casein), or non-protein reagents. The mechanisms by which these additives reduce fouling rates are based on minimizing any hydrophobic and/or electrostatic attractions between the complex matrix and the functionalized surfaces. Unfortunately, such blocked surfaces have only limited antifouling properties [34, 43], and no reports have been published revealing low-fouling or non-fouling properties in blood serum or plasma. Furthermore, it has been reported that blocking the surface may lead to a substantial reduction in the biorecognition activity of immobilized bioreceptors [43]. Therefore, biosensor coatings with low-fouling properties in blood plasma or serum without the need of any blocking additives are attractive for biosensing in real-world complex media.

Several research groups have described a variety of low-fouling and potentially functionalizable surface coatings designed to resist fouling from complex samples [5, 6, 8, 44]. Poly(ethylene glycol) (PEG) and its derivatives have been popular low-fouling materials [12]. Self-assembled monolayers (SAMs) of closely-packed amphiphilic molecules are frequently used as antifouling coatings; however, their ability to resist fouling from undiluted media is limited [8, 34, 43, 45]. Advances in nanotechnology and polymer synthesis have resulted in the design of novel classes of non-fouling material, including brushes of hydrophilic electro-neutral polymers [12, 13, 45, 46]. Some of these low-fouling coatings have also been reported as functionalized biosensor coatings, enabling the attachment of bioreceptors [11, 16].

Several approaches have been used to assess the antifouling properties of these surfaces. Single-protein solutions of human serum albumin (HSA), immunoglobulin G (IgG), fibrinogen (Fbg), and lysozyme (Lys) have been often used for these assessments [13, 47]. HSA, Fbg, and IgG are the main plasma proteins and are present in plasma at the highest concentrations. Fbg is a large (340 kD) blood-plasma protein that adsorbs strongly to hydrophobic surfaces and is a model "sticky" serum protein, and Lys is a small protein that is positively charged under physiological conditions (14 kD, pI ~12) and is often used to model the electrostatic adsorption of proteins to surfaces [48]. However, it has been revealed that resistance to fouling from single-protein solutions does not reflect the surface resistance to fouling from complex media. High blood-plasma fouling has been observed on some surfaces that are completely resistant to adsorption from single-protein solutions of the main plasma proteins. [9, 32, 34]. Therefore, surface exposure to complex media (blood plasma or serum) is required to complete an assessment of the surface antifouling properties. A variety of methods have been used to characterize the relevant surface physico-chemical properties (homogeneity, thickness, and wettability). These methods include spectral ellipsometry, contact-angle measurements, atomic-force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), neutron reflectometry, and infrared reflection spectroscopy.

Immobilization of biorecognition elements

Biorecognition elements

To date, the most widely used bioreceptors have been antibodies (Ab). In particular, IgG is capable of binding respective antigens with high affinity and selectivity [49–52]. IgG is a Y-shape protein having a molecular weight of approximately 150 kD. Each tip of the two Y branches contains an antigen-binding site, a paratope, which precisely fits an epitope. An epitope is a structure on the surface of a specific antigen that enables an antibody to bind to the antigen with a high affinity and selectivity. The stem of the Y is a constant region that does not take part in antigen binding. For biosensing applications, an antibody should be immobilized in a suitable orientation, so that the binding sites are accessible for binding the epitope of the target antigen [53, 54]. Larger antigens, including big biological macromolecules, viruses, or bacteria, usually present several epitopes that can act as points of interaction for specific antibodies. Monoclonal antibodies (mAb or moAb) bind only one epitope. Conversely, polyclonal antibodies (pAbs) are a collection of mAbs, each of which identifies a different epitope of the same antigen. An antibody can be enzymatically cleaved into two F_{ab} fragments, containing identical sites for antigen binding, and a passive F_c fragment. Single-chain variable fragments (scFv) can be obtained by another type of IgG cleavage [55].

Antigens or their epitope structures may be also used as bioreceptors. Typically, they are used in biosensors that measure specific IgG antibody levels in blood serum or plasma, which can indicate an individual's immune status with respect to a particular pathogen.

A variety of proteins and polypeptides having a strong binding affinity to specific analytes can be designed and prepared using recombinant methods and phage or yeast displays. Examples include recombinant monoclonal antibodies, engineered antibody fragments, antibody mimetics (e.g. affibodies) [56, 57], recombinant antigens and epitope structures, and peptide aptamers [58].

In addition to nucleic-acid-derived biorecognition elements (widely used for DNA, RNA, or mRNA capturing), DNA and RNA aptamers can be used for the detection of protein analytes [10, 59]. The aptamers are made up of single strands of nucleic acids that can form a 3-dimensional structure capable of specifically binding an analyte via a combination of van der Waals forces, hydrogen bonding, and electrostatic interactions.

Attachment of biorecognition elements

A variety of immobilization techniques have been described for optical label-free biosensors to immobilize a biorecognition element onto a sensor surface. These techniques are based on one or on a combination of three different strategies: (i) physical adsorption, (ii) covalent binding, and (iii) specific non-covalent binding. For more details see Refs. [15, 49, 50, 59–63].

The most widely used type of immobilization to antifouling coatings is covalent binding of bioreceptors to activated surface functional groups. The stability of covalent bonds makes the immobilization durable in biological media, and biosensor regeneration is possible via the release of captured analytes under specific conditions, for example low pH or high ionic strength. Most of these immobilization strategies use the binding of amine groups on the bioreceptor, either to active esters of *N*-hydroxysuccinimide created by the activation of carboxy-functional surfaces or to active carbamates [43, 64, 65] created by the activation of hydroxy-functional surfaces. Less frequent is the use of a reaction of bioreceptor amine groups with epoxide or aldehyde-functional surfaces. A high abundance of amine groups available at different sites of a protein bioreceptor can lead to a decreased availability of its binding center to the target analyte, primarily because of the bioreceptor immobilization via multipoint attachment and/or as a result of unfavorable orientation. Binding via amino-acid residues in the vicinity of the analyte binding center can also result in loss of the bioreceptor activity.

Thiol groups of cysteine residues located in the constant F_{ab} domain of antibody fragments or at selected sites of recombinant proteins and polypeptides can be used for covalent binding of the bioreceptors to maleimide-functionalized surfaces without a decrease in their analyte-binding activity [66, 67]. The immobilization of an antibody via covalent binding of an oxidized oligosaccharide located in the F_c region of an antibody to hydrazine or hydroxylamine-functionalized surfaces enables oriented antibody immobilization without decreasing the antigen-binding activity of the F_{ab} regions.

The non-covalent immobilization of a bioreceptor is based on a biospecific, high-affinity interaction between a tag attached to the bioreceptor and a capturing element immobilized on the surface. Bioreceptors conjugated with biotin are immobilized via binding to one of four biotin-binding centers in the tetrameric glycoprotein streptavidin or neutravidin previously attached to the surface [15]. Recombinant bioreceptors with oligohistidine tags are bound to a surface bearing chelated metal ions [50, 61, 68–70]. Recombinant bioreceptors with oligohistidine tags (His_n-tag, where n is the number of histidine residues) are bound to a surface functionalized with a chelating moiety, for example nitrilotriacetic acid complexed with a metal ion, usually Ni (NTA). Because of the reversibility of the coordinate bond, regeneration of the functionalized surface or re-use of the surface by repeated release and attachment of bioreceptors can be achieved. Possible approaches include addition of a competitive ligand (histidine, imidazole), reprotonation of the His-tag, or addition of chelating agent, for example the hexadentate ethylenediaminetetraacetic acid. A decomposition of the coordinate bonds at low pH makes it impossible to release captured analytes by the usual decrease in pH without detaching the immobilized histidine-tagged bioreceptors from the surface.

Bioreceptors conjugated with oligo(or poly)-nucleotides can be immobilized via hybridization with their complementary oligo(or poly)-nucleotides attached to a surface. Some antibodies can be immobilized in an orientated fashion without damage of the F_{ab} binding regions, via a biospecific interaction of their F_c region with protein A or protein G anchored to a surface [71–73]. Immobilization driven by oligonucleotide hybridization can be particularly attractive for the preparation of multi-spot biosensor microarrays. A designed pattern of different oligonucleotides can be spotted and bound to the sensor surface. An array of different bioreceptors conjugated with specific complementary oligonucleotides can subsequently be created via oligonucleotide hybridization.

A maximum level of biorecognition activity for a functionalized surface is one of the critical requirements in biosensor development. In general, a randomly oriented covalent immobilization results in multipoint coupling of a protein bioreceptor, which can either change its structure near the binding center or fix it at an orientation unfavorable for analyte binding. Furthermore, because the carboxy, amino, or epoxy-bearing residues are involved in protein folding, such multipoint-coupling methods can potentially lead to protein instability or even denaturation. Therefore, approaches enabling site-specific immobilization have attracted much attention [74–77]. These include the approaches based on F_c antibody fragments or protein A or G. Currently available preparation methods for recombinant proteins and synthetic oligopeptides are expected to encourage the design and preparation of new protein bioreceptors having specifically located functionalities, for example histidine tags, oligopeptide tags for the covalent attachment of biotin or oligonucleotides, specific peptide sequences for coiled-coil hybridization [78], engineered antibody fragments, antibody mimetics, recombinant antigens, epitope structures, and peptide aptamers. Similarly, non-natural amino acids with alkyne or azide functionalities can be introduced in a selected site of the recombinant protein structure. These functionalities (commonly nonexisting in proteins) react exclusively with their complementary functionalities on the substrate, which do not react with any reactive groups present in the bioreceptor [74, 75, 77, 79].

Methods based on the physical adsorption of protein bioreceptors, based mainly on ionic and hydrophobic interactions [50], are inherently not applicable to low-fouling surfaces that are designed to be resistant to protein adsorption.

Antifouling coatings

In this section, we introduce the main approaches to the development of antifouling coatings.

Self-assembled monolayers (SAMs)

Self-assembled monolayers are highly ordered monolayers of close-packed amphiphilic molecules (Fig. 1). A typical structure of the molecules is R_1–$(CH_2)_n$–R_2, where R_1 is a hydrophilic moiety at the ω-position, $(CH_2)_n$ is a nonpolar alkane chain, and R_2 is a group on the α-position that is chemically reactive to the substrate. Typically, alkane chains having 11 or more methylene groups (n) are used to promote the assembly of closely-packed amphiphilic molecules on a surface. The chemisorption of the molecules via R_2 and ordering (via hydrophobic interactions and dispersion forces among their alkane chains) result in the formation of a SAM capped with a close-packed layer of the R_1 hydrophilic moieties. Thiol (–SH) groups have been mostly used as R_2 for the preparation of SAMs on gold or silver; silane (e.g., $–SiCl_3$) is used as R_2 on glass and silicone; and silane or phosphate is used as R_2 on a variety of metal-oxide surfaces. A dense hydrophobic interlayer of organized alkane chains and a layer of hydrophilic moieties on top of the SAM decrease fouling rates by preventing the direct contact of proteins with the substrate surface and by decreasing the free interfacial energy between the surface and aqueous media,

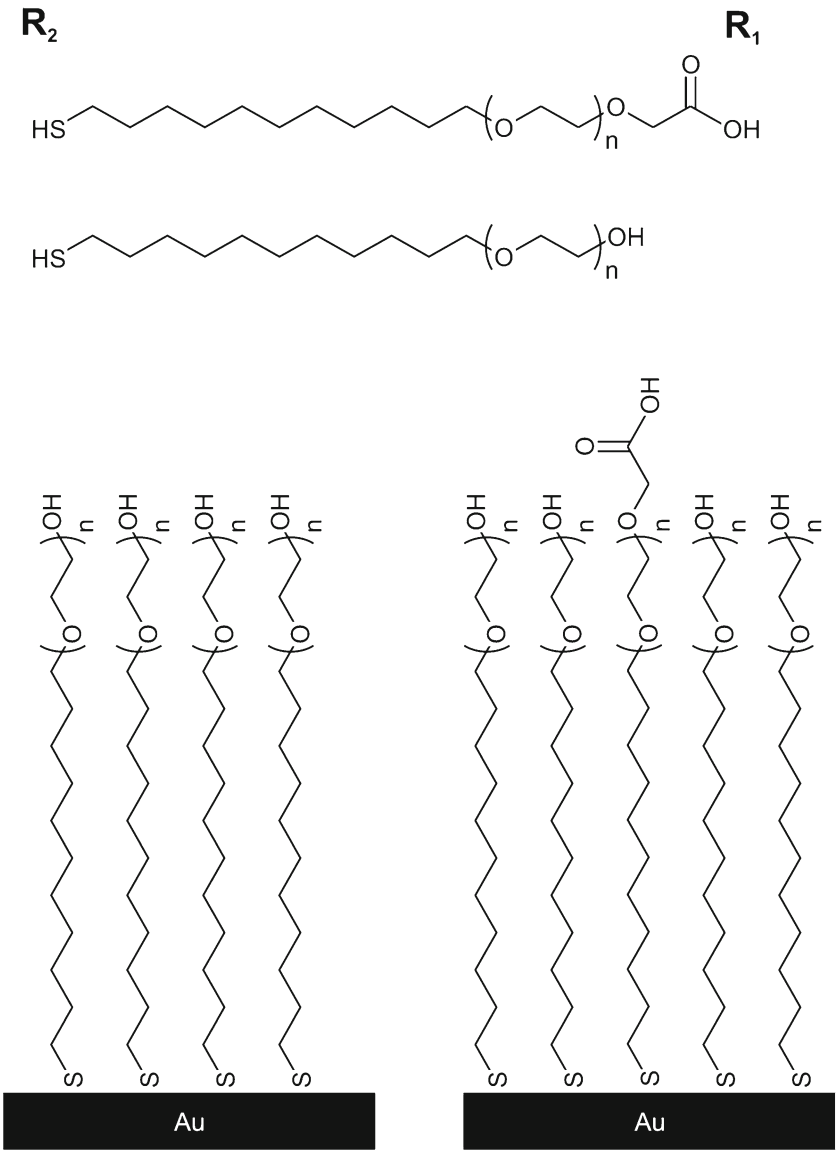


Fig. 1 Self-assembled monolayers (SAMs) of alkanethiols terminated with oligo(ethylene glycol) (OEG) or mixed OEG–OH and OEG–COOH

respectively. Close-packed oligo(ethylene glycol) (OEG) moieties containing only a few ethylene glycol units on OEG-terminated SAMs have been able to prevent adsorption from single-protein solutions [80, 81].

Chapman et al. pioneered research on protein adsorption onto ω-functional SAMs. A survey of the resistance of more than fifty SAMs terminating in different groups [48] to fouling from Fbg and Lys solutions led to a description of physical-chemical properties that should be fulfilled to achieve protein resistance of the coating. These properties included the surface being hydrophilic, electro-neutral, having hydrogen-bond acceptors, and lacking hydrogen-bond donors [48, 82].

Oligo(ethylene glycol)-terminated SAMs

Oligo(ethylene glycol)-terminated SAMs (OEG_n SAM, where *n* denotes the number of ethylene glycol units) have been the most widely used antifouling coatings. OEG SAMs have been used in several important theoretical and experimental studies as a model surface to investigate the mechanism of surface protein-resistance [83–91]. High internal and terminal hydrophilicity and a high lateral packing density have been recognized as factors that determine the protein-fouling resistance of OEG_n SAMs. [85].

Although OEG_n SAMs can effectively reduce or prevent adsorption from single-protein solutions (blood-plasma related) in PBS, there have been several reports of a lack of resistance to fouling from undiluted blood plasma and other complex media [8, 9, 45, 92]. The fouling from blood plasma decreased with increasing number of ethylene glycol units, *n* (Table 1). Mixtures of R_2–$(CH_2)_{11}$–OEG_n and R_2–$(CH_2)_{11}$–OEG_n–F have been used for the preparation of SAMs to which bioreceptors can be attached via the functional groups F (where F includes COOH, NH_3, and biotin) [93–96].

Table 1 Adsorption from single-protein solutions and fouling of blood plasma onto SAM surfaces

Surface	Fouling (pg mm^{-2})					Technique	Ref.
	HSA	Fbg	Lys	IgG	Blood plasma		
OEG_2	105	300	150	180	1500–2500	SPR, ellipsometry	[9, 10, 45, 92]
OEG_4	–	100	–	–	900	SPR, ellipsometry	[45]
OEG_6	0	36	17	59	500	SPR, ellipsometry,	[8, 9, 43, 45, 92]
TMA:SO (1:1)	–	–	–	–	2500	SPR	[46]

Zwitterionic SAMs

Zwitterionic surfaces contain positively and negatively charged moieties in balanced amounts, thus creating surfaces with a net neutral charge. Whereas OEG_n surfaces are hydrated via hydrogen bonding, zwitterionic surfaces can bind water molecules even more strongly via hydrogen bonds and ionic solvation [32, 46, 97]. Holmlin et al. prepared mixed SAMs from a 1:1 combination of alkanethiols terminating in positively charged tri(methyl)ammonium (TMA) and alkanethiols terminating in a negatively charged sulfonate group (SO) [46]. The mixed TMA–SO SAMs reduced the adsorption from Fbg and Lys solutions to less than 1 % of a protein monolayer. The SAMs prepared from only one of these charged components had higher fouling levels, corresponding to a near-saturated protein monolayer [46]. These results emphasized the important effect of electro-neutrality in preventing protein adsorption.

SAMs composed of alkanethiols terminating in highly wettable sulfobetaine (SB) or phosphorycholine (PC) zwitterion groups have also been revealed to be capable of reducing adsorption from single-protein solutions [44, 46, 97, 98]. The results suggest that SB SAMs are substantially more effective in resisting Fbg and Lys adsorption than PC SAMs [44, 46, 98].

Other SAMs

Surfaces resistant to adsorption from single-protein solutions have been prepared from SAMs terminating in a variety of hydrophilic groups. These groups include tri(propylene sulfoxide) (TPS) [99], Mannitol [100], gulitolor mannonamide [101], hyperbranched or dendritic polyglycerol [102], and oligopeptides [103]. None of these SAMs were tested for fouling from blood plasma or serum.

Summary

Table 1 summarizes the published data on protein adsorption from single-protein solutions of IgG, HSA, Fbg, and Lys, and fouling from undiluted blood plasma on SAMs (including the methods of characterization). SAMs capped with densely packed helical OEG_n seem to provide a more effective resistance to both protein adsorption and fouling from blood plasma than SAMs capped with other hydrophilic moieties. Unfortunately, none of the reported SAMs had low-fouling properties when exposed to undiluted blood plasma or serum.

The great advantage of SAMs lies in the ease of fabrication and the availability of alkanethiols. For example, $HS(CH_2)_{11}EG_n$ with n=2–6 can be relatively easily synthesized or purchased from a variety of sources. In summary, SAMs are suitable for use in biosensor and microarray applications involving buffer solutions or diluted complex media (<~15 %) [15, 104–106]. The lowest fouling from blood plasma on SAMs was obtained for $(CH_2)_{11}EG_6$ SAMs (Table 1). The thickness of these SAMs is only 2.5 nm [83]. It is worth mentioning that such a low thickness of SAMs is attractive for biosensors with a highly localized evanescent field, e.g. sensors based on localized surface plasmon resonance (LSPR) [107, 108].

PEG-based coatings

For many years, surface coatings based on nontoxic, nonimmunogenic, and uncharged ethylene glycol oligomers (OEG) and polymers (PEG) have been used to reduce protein fouling on a variety of biomedical surfaces.

Also, linear PEG chains have been grafted to surfaces to create antifouling coatings. The high solubility and strong interaction of PEG chains with water does not enable reliable grafting of PEG homopolymers themselves by physical adsorption. In a few reports, PEG containing structural motifs capable of electrostatic or hydrophobic physisorption have been immobilized on different substrates; however, only poor stability and poor resistance to fouling were observed [109–112].

The electrostatic adsorption of comb polymers, composed of a polyelectrolyte backbone of poly(*L*-lysine) (PLL) and pendant PEG chains (PLL–*g*–PEG) on negatively charged surfaces, has not been revealed to be stable at higher ionic strength [12, 113]. Improved stability was achieved when physisorption was combined with covalent binding of amino groups on the PLL backbone to aldehyde groups present on a previously deposited plasma polymer [12]. A triblock copolymer of PEG with poly(propylene oxide) (PPO) in the form PEG–PPO–PEG [114] or with poly(butadiene) (PB) in the form PEG–PB–PEG [115] has been adsorbed via

hydrophobic blocks of PPO or PB onto different hydrophobic surfaces and subsequently chemically grafted by γ-irradiation. Zoulalian et al. grafted a terpolymer of PEG–poly(alkyl phosphonate)–PEG (PEG–PAP–PEG) to TiO_2 and Nb_2O_5 surfaces via the PAP block, which obtained a reduction in fouling from blood plasma as observed by ellipsometry, XPS, and OWLS [116, 117]. Even if the PAP block provided a multivalent linkage to TiO_2 and Nb_2O_5 surfaces, a significant loss of the polymer was observed after only 3 h in different aqueous solutions [116], limiting its use for real applications. Only fouling from single-protein solutions has been tested on the other previously mentioned physisorbed polymers. Stable antifouling coatings composed of linear PEG chains have been prepared by the chemical binding of PEG with a functional terminal group to reactive groups present on a substrate surface (Fig. 2) [118]. Reactive ω-trichloro silane-PEG (PEG-$OSiCl_3$) [119] and ω-mercapto-PEG (PEG-SH) [40, 120–123] have been grafted via covalent bonds to silicon and gold surfaces, respectively. Similarly, PEG conjugated with a catecholic chelating group, for example dopamine (PEG–DOPA) [124–126] or anachelin (PEG–ANACH) [127], has been tethered onto different metal oxides by complexation via coordinate bonds [85, 88, 128]. Activated carboxyl groups on a self-assembling monolayer of mixed alkanethiols terminating in either OEG or carboxylic groups have been used for the covalent attachment of ω-amino-PEG (M_w=3400 g mol^{-1}) [9]. The resulting PEG–SAMs coatings decreased blood-plasma fouling.

Several works have reported a decrease in protein adsorption with both increasing grafting density (σ=number of polymer chains per nm^2) and increasing length of the PEG chains grafted to a surface [40, 121, 123, 129–132]. Layers with increasing grafting density pass from a single grafted chain (mushroom) regime to a brush regime with deformed polymer coils. A polymer brush is formed when the average distance between polymer-chain anchoring points S ($S^{-2}=\sigma$) approaches the size of the polymer in solution, which is characterized by the double Flory radius R_F of the polymer random coil ($S=2R_F$), (Fig. 2a) [133]. At higher grafting densities the polymer coils become deformed (Fig. 2b) [36, 133, 134].

High grafting densities have been achieved when PEG-SH, PEG–DOPA, and PEG–ANACH were grafted from a solvent of poor thermodynamic quality nearing a "cloud point" condition. Under such conditions, the PEG coil dimensions are reduced and thus minimize the excluded volume interactions between adsorbing polymer chains [121, 122, 135]. When the coatings are used in aqueous solutions, a high osmotic pressure can cleave the coordinate bonds between PEG–DOPA or PEG–ANACH and the substrate. More than three dopamine groups per PEG chain were required to ensure the coating stability [125].

Brash et al. performed extensive studies on the PEGylation of gold using R–PEG-SH of different molecular weights and terminal groups [40, 120–122, 136]. The surfaces were exposed to blood plasma spiked with radiolabelled fibrinogen, where fibrinogen adsorption from plasma decreased with increasing grafting density [121, 122]. The lowest adsorption of fibrinogen was observed at the highest grafting densities, reached when PEG-SH was immobilized from a solvent of poor thermodynamic quality near "cloud point" conditions [40, 121].

Pop-Georgievski et al. coated gold and several other substrates with ultrathin layers of poly(dopamine) and grafted ω-amino-PEG from a melt to these layers, obtaining a grafting density much higher than that obtained by grafting the polymer from solutions (even on non-reactive substrates) [118]. The poly(dopamine) interlayer adhered strongly to a variety of inert substrates and contained multiple functional groups available for grafting [118, 126]. The melt grafting of PEG ensured no excluded volume interactions, maximum grafting density, and substantially higher thickness. The achieved level of blood-plasma fouling (20 pg mm^{-2}) is the lowest reported for PEG grafted to any surface to date [118]. However, a decrease in the PEG thickness, with loss of resistance to plasma fouling, was observed after only one day's storage in PBS, most probably brought about by the washing out of non-covalently bound entangled polymer chains [118].

PEG-like surfaces have also been prepared by plasma polymerization and/or deposition techniques [137–139]. These coatings have been revealed to reduce the adsorption of BSA, IgG, and Fbg [138, 139].

PEG coatings that have resistance to fouling from blood plasma or serum are summarized in Table 2. In summary, only the PEG grafted from melt to a poly(dopamine) layer had ultra-low-fouling properties; however, this surface lost its resistance within one day of storage in PBS.

Polymer brushes

Polymer brushes are ultra-thin polymeric films consisting of closely-packed polymer chains tethered to a surface by one end (Figs. 2 and 3) [6, 133, 134]. If the grafting density is very high, the polymer chains adopt an unusual conformation, wherein the individual polymer coils overlap [38, 134]. Under these conditions, the polymer molecules are strongly stretched away from the surface and achieve a unique molecular conformation different from the random coil [6, 134].

Polymer brushes can be prepared by either a "grafting-to" or a "grafting-from" approach. The "grafting-to" approach (Fig. 2) consists of the attachment of polymer chains to a surface via physisorption [32, 113], chemisorption [125, 140], or covalent bonding [40, 120–123]. The final grafted-to brush structure is characterized by the grafting density σ, which is the number of polymer chains per nm^2 [133, 134]. The protein adsorption typically decreases with both increasing grafting density and increases in the length of the attached

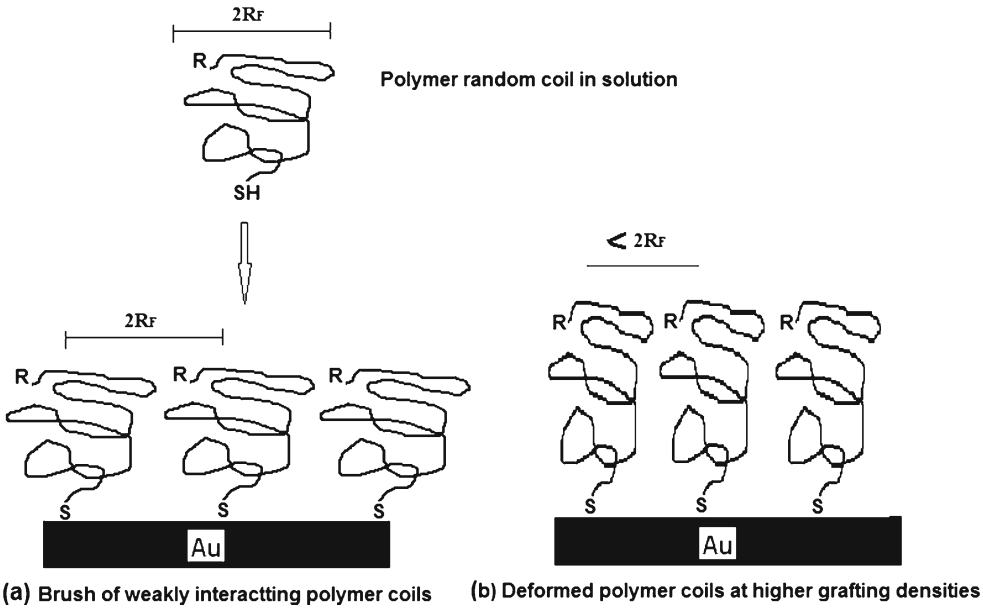


Fig. 2 Grafting SH–PEG chains from solution to gold surface. Polymer chains are chemisorbed via the SH-terminal group, where *R* can be the OH-terminal of PEG or a functional group for the attachment of bioreceptors

grafted-to polymer chains [40, 121, 123, 129–131, 141]. Although this method is experimentally straightforward, only limited grafting densities have been achieved [133, 134]. To date, the grafting of polymers from solutions to a variety of substrates has resulted in coatings with blood-plasma fouling higher than 100 pg mm^{-2}. The "grafting-from" approach (Fig. 3) uses controlled surface polymerizations for the preparation of very dense brushes by growing each chain at virtually the same rate. Methods of preparation include anionic and cationic polymerization [142, 143], nitroxide-mediated polymerization (NMP) [144], ring-opening polymerization [145], reversible addition-fragmentation transfer polymerization (RAFT) [146], single-electron-transfer living radical polymerization (SET-LRP) [147], and atom-transfer radical polymerization (ATRP) [148]. Polymer chains grow at the same rate from initiators or chain-transfer agents (CTAs) attached to a surface after their exposure to a monomer containing polymerization solution [6, 134]. Initiators, CTAs, or pre-formed polymers are attached via a variety of chemical reactions specific to the substrate surface [13, 149–154]. Chemically inert substrates require either specific modification [6, 151, 155] or a coating having an ultrathin layer with reactive groups, for example autopolymerized poly(dopamine) [118, 156] or RF-magnetron-sputtered amino-rich nylon [10].

The surface-initiated ATRP method (SI-ATRP) is the most widely used technique for the preparation of brushes (Fig. 3) [134, 157]. Surfaces coated with a designed pattern of initiator SAM brushes can be prepared by depositing an initiator SAM using microcontact printing (μCP) or dip-pen nanolithography.

Low-fouling, ultra-low-fouling, and non-fouling brushes with very high grafting densities of nearly stretched polymer

Table 2 Adsorption from single-protein solutions and fouling of blood plasma onto PEG surfaces. Detection technique and thickness of PEG layers are specified

Surface	Fouling (pg mm^{-2})						Thickness (nm)	Technique	Ref.
	IgG	HSA	Fbg	Lys	Blood serum	Blood plasma			
PPL–*g*–PEG	–	–	–	–	200–2930	–	–	OWLS	[113]
PEG–PAP–PEG	–	–	–	–	–	300	–	XPS, OWLS, ellipsometry	[116, 117]
PEG–$DOPA_3$	–	–	–	–	10–60	–	–	OWLS	[122, 125]
PEG–ANACH	–	–	–	–	150	–	2	Ellipsometry	[127]
PEG from melt-on polydopamine	–	0	0	–	–	20	11	SPR	[118]
PEG from melt-on polydopamine (1 day storage)	–	–	–	–	–	900	6.5	SPR	[118]
PEG–SAM	75	0	30	120	–	900	–	SPR	[9]

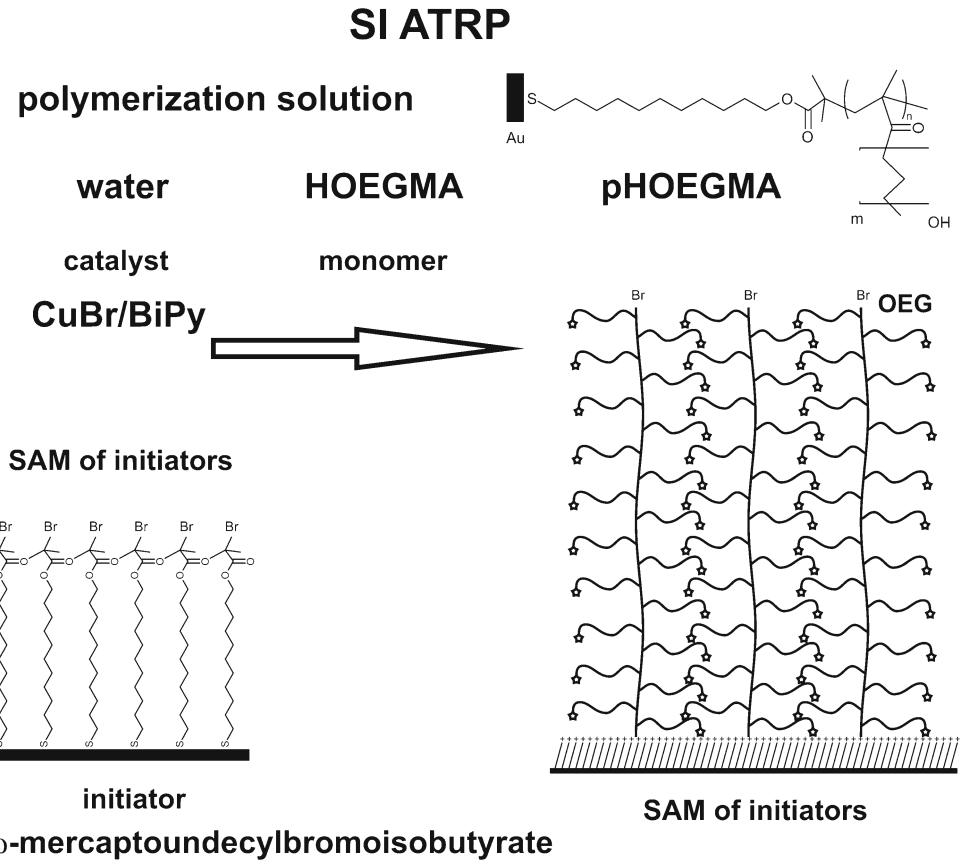


Fig. 3 Polymer brushes grafted from a surface by surface-initiated atom-transfer radical polymerization (SI-ATRP)

chains have been prepared by the "grafting-from" SI-ATRP approach (Fig. 3). Polymer chains grow from a substrate surface with covalently bound ATRP initiators after exposure to a polymerization solution containing monomers and a catalyst system dissolved either in water or in a water–polar solvent mixture. If SI-ATRP polymerization is living and well controlled, the length of the "grafted-from" polymer chain increases linearly with the polymerization time. This makes it possible to adjust the brush thickness at a nanometer scale or to prepare brushes with a multiblock architecture [10, 156, 158, 159]. Unlike "grafted-to" brushes, the grafting density and molecular mass of polymer chains in "grafted-from" polymer brushes cannot be quantified.

Brushes of polymers with oligo(ethylene glycol) side chains

Ma et al. revealed that brushes of oligo(ethylene glycol)methacrylate monomers (HOEGMA) prepared by SI-ATRP can be used as low-fouling surfaces [160, 161]. These cylindrical brushes consisted of pHOEGMA chains with a methacrylate backbone and OEG side chains. The dense low-fouling brushes of pHOEGMA have been grown from gold [65, 68, 94, 156, 160, 161], silica and/or silicon [151, 162, 163], TiO_2 [164], polymeric nanocapsules [154], and a variety of polymer surfaces [10, 155].

Huck et al. obtained a well-controlled SI-ATRP of pHOEGMA and pMeOEGMA brushes by optimizing the polymerization system. [162]. The application of well-controlled living SI-ATRP made it possible to adjust the brush thickness at a nanometer scale of up to 180 nm by simply changing the polymerization time; in this manner they established a minimum thickness that ensured the best protection against blood-plasma fouling [8, 10, 92, 159]. Rodriguez-Emmenegger et al. assessed the fouling from blood plasma and different body fluids on both pHOEGMA and pMeOEGMA brushes prepared under the optimized conditions [92], obtaining an approximately 90 % decrease in plasma fouling with a brush thickness of 30 nm [8, 10, 92, 159, 165].

Kizhakkedathu et al. grafted PEG-based *N*-substituted acrylamide from polystyrene particles and oligo(ethylene glycol) methyl ether acrylamide (pMeOEGA) from latex particles. The fouling from undiluted blood plasma on pMeOEGA (measured using SDS-PAGE) was as high as 370 pg mm^{-2} [166, 167].

Poly(zwitterionic) brushes

Poly(zwitterions) are macromolecules containing identical numbers of anionic and cationic species on the same monomer units, which can be completely dissociated and thus will maintain an electro-neutral state [167, 168].

Polymer brushes of methacryloyloxyethyl phosphorylcholine (MPC) have been prepared by SI-ATRP polymerization [169–171]. The adsorption of BSA, Lys, Fbg, IgG, von Willebrand factor, and Hageman factor in PBS and fouling from blood plasma [9] have been evaluated. Even if there was only minor adsorption from solutions of the main plasma proteins on the pMPC brushes, huge fouling (3450 pg mm^{-2} measured by SPR) was observed when the MPC brushes were exposed to blood plasma [9]. It should be noted that, being one of the most hydrophilic surfaces (water-contact angle of θ_{adv}=18° and θ_{rec}=7° [9, 171]), the pMPC had fouling levels from blood plasma comparable with that seen on bare gold.

Highly hydrophilic surfaces have been prepared by coating substrates with polymer coatings based on *N*-(3-sulfopropyl)-*N*-(methacryloxyethyl)-*N*,*N*-dimethylammonium sulfobetaine methacrylate (SBMA) [172–175]. There was no adsorption from solutions of the main plasma proteins on pSBMA brushes prepared by SI-ATRP [9, 176, 177]; however, fouling from blood plasma ranged from 75 to 2400 pg mm^{-2} and was dependent on the thickness of the brush [9, 178]. The lack of functional groups for covalent coupling to bioreceptors and the high coating thickness required to reduce the plasma fouling (60 nm in dry state) substantially limits the use of pSBMA for biosensors.

Zhang et al. were the first to introduce poly(carboxybetaine methacrylate) brushes (pCBMA), revealing their resistance to fouling from Fbg, human choriogonadotropin (hCG), and Lys. They also revealed the covalent attachment of bioreceptors to pCBMA [13]. This work was followed by the synthesis of carboxybetaine acrylamides (pCBAA) with a methylene (pCBAA-1), ethylene (pCBAA-2), propylene (pCBAA-3), and pentylene (pCBAA-5) spacer between the charged groups, and carboxybetaine methacrylate with an ethylene spacer (CBMA-2): these brushes were also tested for Fbg adsorption as a function of ionic strength and pH [179]. A total resistance to Fbg adsorption was achieved for the pCBAA-2. Later, the fouling from undiluted blood plasma on pCBAA-2 was found to be below the detection limit of the SPR sensor used (<3 pg mm^{-2}) [8, 34, 92, 152]. Although pCBMA-2 has a resistance to plasma fouling [9] similar to that of pCBAA-2, the latter has a higher hydrolytic stability [178, 180]. A non-fouling di-block brush was prepared by growing pCBAA-2 from living terminal groups of a pMeOEGMA brush [8, 10, 92, 159]. The main disadvantage of both pCBMA and pCBAA brush preparation is a lack of thickness control in their SI-ATRP [8, 10]. Therefore, the brush thickness cannot be tuned by polymerization time, but only by empirical changes to the polymerization system.

Other types of polymerization have been used to gain control over the polymerization process, including nitroxide-mediated polymerization [181], reversible addition-fragmentation transfer [180], and single-electron-transfer living radical polymerization (SET-LRP) [182]; however, no reports on the resistance of these new coatings to blood plasma have been presented. Poly(CBMA-2) brushes have also been prepared by the "grafting to" approach. pCBMA-2 chains bearing dopamine at one end (DOPA-pCBMA) were grafted via the DOPA terminus either to an SiO_2 film deposited on a gold layer of a SPR sensor or to silicon microcantilevers on a suspended microchannel resonator [140, 152]. Both studies obtained low fouling from undiluted blood plasma (51 pg mm^{-2}).

Poly(betaine) brushes do not usually have long-term stability in aqueous solutions. Several studies have reported a reduction in the thickness (approximately 20 %) of pCBAA-2 brushes after only 15 days' storage in PBS, most probably caused by the detachment of chains [183], which results in a deterioration of their antifouling properties [8, 183]. Similarly, the thickness of pSBMA brushes was reduced from 31 to 21 nm after four days' storage at 40 °C in water [176].

More recent attempts include the use of poly(ampholyte) brushes composed of alternating positively and negatively charged monomers having an overall neutral charge [178, 184–186]. McCormick et al. revealed a high alternating tendency of the oppositely charged monomers [186], copolymerizing as ion pairs [187, 188]. Bernard et al. revealed that polymer brushes of (2-(methacryloyloxy)ethyl) trimethylammonium chloride (pTMA) and 3-sulfopropyl methacrylate potassium salt (pSA) could be prepared by ATRP [185] using the concept proposed by McCormick. Only minimal fouling from Fbg or Lys [184, 188, 189] was observed on these surfaces, whereas higher levels of fouling (76.5 pg mm^{-2}) [184] from 20 % blood plasma were found using a SPR sensor. Even using much thicker brushes, the resistance of poly(TMA-*co*-SA) was comparable with that of OEG_n SAMs and substantially worse than that of pCBAA-2.

Hydroxy-contained brushes

Hydrophilic polymer brushes of non-ionic, hydroxy-functional monomers based on 2-hydroxyethyl methacrylate (HEMA) [43, 92, 156, 158, 183, 190], 3-hydroxypropyl methacrylate (HPM) [8, 191], and *N*-(2-hydroxypropy) methacrylamide (HPMA) [8, 92, 158, 183, 192] have been prepared via SI-ATRP, and their protein resistance has been evaluated by SPR [8, 92, 152, 183, 190, 191]. All of these brushes completely prevented fouling from single-protein solutions.

The fouling from undiluted blood plasma on pHPM was 410 pg mm^{-2} [8], or 530 pg mm^{-2} if a mixture of HPM isomers under poorly controlled conditions was used [183]. Zhao et al. prepared pHEMA coatings that had a minimum plasma fouling of approximately 30 pg mm^{-2} at a thickness of 30 nm [183].

Fouling from undiluted blood plasma below the SPR detection limit has been achieved on 18 nm thick pHPMA brushes grafted from gold SPR chips by SI-ATRP [8]. No fouling was observed, even when the pHPMA surface was repeatedly exposed to blood plasma after three and 24 months of storage in PBS: the fouling observed in the same experiment on non-fouling pCBAA-2 brushes increased to 1250 pg mm^{-2} after 24 months. The fouling of pHPMA brushes was in the range 0–13 pg mm^{-2} for plasma from different individual donors, and 0–8 pg mm^{-2} for pooled plasma from different commercial sources [192]. Fouling rates were below the limit of SPR detection for a variety of biological fluids, including cerebrospinal fluid, urine, saliva, fetal bovine and calf sera, eggs, and milk [92].

In comparison with any other known antifouling surface, the total resistance to undiluted blood plasma and the long-term stability seem to be the most important properties of pHPMA brushes. A disadvantage of pHPMA-brush preparation is the lack of control of its SI-ATRP, which precludes controlling their thickness by polymerization time or using their living terminal groups for immobilization of bioreceptors. The thickness is adjusted by empirically changing the polymerization system. The not fully reversible activation of hydroxyl side groups (see section "Activation and deactivation of functional groups of low-fouling coatings") also limits the biosensor applications of pHPMA brushes.

Other brushes

Polymer brushes of first and second generation dendritic acrylate monomers (linear oligoglycerol methacrylate and glycerol methacrylate) have been grown from gold, and fouling from single-protein solution and blood plasma has been assessed [193]. A more crowded architecture of the brush (for example that of dendritic brushes) resulted in fouling levels from blood plasma (240 pg mm^{-2}) that were lower than those for linear or bottle brushes (510–690 pg mm^{-2}), indicating the importance of the architecture of the brush [193].

Different peptoids [194], poly(β-peptoid)s [183], and polyoxazolines [195] have been grafted to surfaces that have obtained minimum adsorption of proteins from single-protein solutions and low fouling from undiluted blood plasma (40 to 97 pg mm^{-2}) for short contact times.

Saccharide polymer brushes [196] and brushes of *N*-substituted acrylamide containing different carbohydrates [197] have also been used to achieve reduced protein fouling from single-protein solutions; however, poor results were obtained when the surfaces were exposed to blood plasma.

The published data on the fouling resistance of polymer brushes are summarized in Table 3. It can be seen from the table that the hydroxyl-functional pHPMA and carboxy-functional pCBAA and pCBMA have no detectable fouling from undiluted blood plasma and therefore can be regarded as non-fouling coatings. High blood-plasma fouling is observed on some surfaces that are completely resistant to adsorption from single-protein solutions of HSA, Fbg, and IgG, which are present in plasma at the highest concentrations. However, there is no adsorption of the main plasma proteins from PBS on "ultra-low-fouling" surfaces. It can be assumed that total resistance to the adsorption of these proteins from single-protein solutions is a prerequisite for achieving "ultra-low fouling" surface properties for blood plasma, but does not necessarily mean these properties will be achieved.

Summary

Results regarding the ability of SAMs to resist fouling from undiluted blood plasma and/or serum have revealed that, despite a variety of designs having low-fouling properties, the SAMs provide only a limited resistance to blood-plasma fouling. Currently, the most effective resistance has been reached with closely packed OEG_6 SAMs (fouling of 500 pg mm^{-2}) [8, 92]. The present state of the art does not indicate any trend of significant improvements in the low-fouling properties of SAMs-based coatings. Because of the well-established procedures and the high reproducibility of SAMs preparation, SAM surfaces can be used as an antifouling standard to compare the properties of other surfaces.

A much more effective resistance to plasma fouling has been achieved by surfaces coated with polymer brushes composed of close-packed polymer chains end-tethered to the surface. High grafting densities can be obtained by grafting linear PEG chains to surfaces under conditions that minimize the excluded volume interactions between the immobilized polymer chains, for example grafting from a solvent of poor thermodynamic quality near "cloud point" conditions or from a melt. Plasma-fouling levels as low as 20 pg mm^{-2} have been achieved using the latter technique [118]. The grafting of peptide-like polymers to a surface seems to provide a reasonable resistance to serum and plasma fouling [183].

The highest resistance to fouling from blood plasma has been achieved for polymer brushes grafted from a surface by SI-ATRP. Both pCBAA-2 and pHPMA brushes have non-fouling properties; however, the thickness of these brushes can only be adjusted with a limited reproducibility by changing polymerization conditions. This is the result of a limited control of their SI-ATRP.

Results have confirmed that fouling from single-protein solutions does not reflect resistance of a coating to fouling from blood plasma or serum [8–10, 32, 34]. A high blood-plasma fouling has been observed on selected coatings, even though the surfaces were totally resistant to adsorption from model solutions of albumin, Fbg, or IgG, which are present in plasma at high concentrations [8–10, 92].

There remain many challenges to the precise lab-to-lab evaluation of synthetic surfaces for their antifouling properties

Table 3 Adsorption from single-protein solutions and fouling of blood plasma onto polymer-brush surfaces. The detection technique and thickness of brush layers are specified

Surface	Fouling (pg mm^{-2})					Thickness (nm)	Detection technique	Ref.
	IgG	HSA	Fbg	Lys	Plasma			
pHOEGMA M_n 526	–	–	0	–	300	25	SPR	[161]
pHOEGMA M_n 526	0	0	0	0	163	30	SPR	[8, 92]
pMeOEGMA M_n 300	0	0	0	0	189–230	30	SPR	[8, 10, 92, 159]
pMeOEGMA M_n 350	–	–	–	–	370	–	SDS-PAGE	[166]
pMPC	–	–	70–100	–	–	–	Radiolabelled	[170, 198]
pMPC	135	0	75	0	3450	–	SPR	[9]
pSBMA	150	0	0	0	2400	20	SPR	[9, 176, 177]
pSBMA improved polymerization	–	–	–	–	75–200	15–90	SPR	[178]
Dendritic glycerol acrylate	–	40	70	–	240	17	SPR	[193]
poly(β-peptoid)s	–	2	0	4	97	–	SPR	[183]
pHPM	0	0	0	0	410	30	SPR	[8]
pHPM mix isomer	–	–	–	–	500	30	–	[183]
pHEMA	–	0	0	0	30–35	12–30	SPR	[183]
pHEMA	–	–	–	–	278	18	SPR	[43]
DOPA-pCBMA	–	–	0	0	63	–	SPR	[152]
pCBMA-2	0	0	0	0	0	15–20	SPR	[8, 32, 92, 181]
TMA-co-SA	25	0	30	30	76	40	SPR	[184, 186]
pHPMA	0	0	0	0	0	18	SPR	[8, 92, 158]

B.P., human blood plasma. A value of zero means below the limit of detection

on the basis of the measurement of plasma fouling on the surface. There is no universal blood-plasma "standard" to which the fouling can be measured and related in different experiments, or which can be used as a reference in plasma-screening measurements. The fouling is significantly different if plasma samples from different donors or pooled plasma from different sources is used [43, 192]. Therefore, plasma-fouling tests performed in different laboratories obtain rather different values resulting from the different blood-plasma samples. Table 4 summarizes fouling-level results for plasma samples collected from five different donors and pooled plasma from three different suppliers on both OEG-terminated SAMs and five antifouling brushes [192]. It was observed that even pooled plasma from a single supplier might provide different results if different batches are used.

Only experiments performed using the same batch of plasma or serum can be correctly quantitatively compared. In consequence, when developing a particular biosensor, the antifouling properties of a surface should be always evaluated in the medium relevant to applications of that specific biosensor [192].

Because the sensor sensitivity decreases with increasing physical thickness of the coating, special attention should be given to calibration of the raw sensor responses to an actual thickness and refractive index of the coating in the tested medium. However, only the thickness of dry coatings has usually been assessed, and the surface swelling in aqueous solutions or even in tested complex medium has not been considered; most results therefore have not been correctly calibrated to the actual optical thicknesses of the coatings in tested media [178]. The effect of decreasing sensor sensitivity with increasing optical thickness of the coating is typically combined with a decreased fouling with increased thickness of the coating. Therefore, the minimum wet thickness to ensure antifouling properties is recommended for use in bioanalytical experiments.

Table 4 The fouling from plasma samples collected from five different donors and pooled plasma from three different suppliers measured in one experiment on five antifouling polymer brushes [192]

Brush	A	B	C	1	2	3	4	5
	Pooled plasma			Individual donors				
pHEMA	1677	105	40	898	203	119	1950	68
pHOEGMA	194	145	315	144	627	40	220	237
pMeOEGMA	137	97	233	203	101	102	119	288
pCBAA	113	16	81	51	42	0	119	20
pHPMA	8	0	0	0	0	8	8	13

Values are pg mm^{-2}

A, plasma mixed from 10 donors; B, pooled plasma from Sigma–Aldrich; C, pooled plasma from Biochemed, USA. A value of zero means below the limit of SPR detection (3 pg mm^{-2})

In addition, it is important to mention that there are surfaces that have low-fouling properties when exposed to undiluted blood plasma; however, the same surfaces have also had fouling rates below the limit of SPR detection when exposed to other biological fluids, including saliva, urine, and cerebrospinal fluid [92].

Discussion of approaches to design novel low-fouling coatings

Existing theories are not able to fully explain blood-plasma fouling on surfaces that are totally resistant to the adsorption of the main plasma proteins from model solutions (Table 3). Studies based on neutron reflectometry [40, 122, 199] and theoretical simulations [85, 123] indicate that resistance to protein adsorption correlates with the interfacial structure of water molecules arranged with an angular distribution of dipole moments bound around repeating ethylene glycol units in PEG and OEG-based coatings. Similarly, the strong hydration of the pCBAA zwitterionic moieties (having specifically arranged water molecules [200–203]) will probably contribute to the resistance of pCBAA to plasma fouling. However, the relationship between blood-plasma fouling and the chemical composition of low-fouling polymer brushes has yet to be revealed [8, 92, 192]. For example, the two surfaces totally resistant to fouling from selected undiluted blood-plasma samples, i.e. highly wettable zwitterionic pCBAA and medium-wettable pHPMA brushes, have quite different chemical structures [8]. Some studies have pointed out that most of the known surfaces resistant to single-protein solutions were decorated with kosmotrope moieties [172, 204], which could prevent the approach of a protein by a long-range interaction with the protein water shell [205]. Other studies have reported on the effect of architecture, configurational entropy, and elasticity of polymer chains in a brush [6, 36–38, 206–208]. The search for the interfacial water structures associated with some structural motifs and gradients in density and mobility of polymer chains throughout the polymer brush in relation to the fouling resistance continues to be a challenge for current research on antifouling surfaces.

A different strategy based on analysis of blood deposits on antifouling surfaces may contribute to a better understanding of plasma fouling in relation to the design of new antifouling coatings [165]. Only seven different proteins were present in plasma deposits on pHOEGMA or pMeOEGMA brushes grafted from a gold SPR surface. HSA and Fbg were both present in the deposits, despite the fact that no adsorption of these proteins from single-protein solutions in PBS was observed. Their adsorption from plasma was probably mediated by other proteins [165]. The study of plasma fouling on pHOEGMA, pHEMA, and pSBMA brushes grafted from silica particles suggested that apolipoprotein B100, which was the most concentrated protein in the deposit, was adsorbed as a part of low-density-lipoprotein (LDL) particles via interaction of LDL phospholipids with the brushes [209]. It would be useful to perform such analysis of the deposits from blood-plasma samples on other ultra-low fouling and non-fouling coatings, for example pHPMA and pCBAA, which are fully resistant to blood-plasma fouling. Such complex studies may provide new insights into factors governing the fouling of proteins from blood plasma onto synthetic surfaces, including internal wettability, mobility, flexibility, and elasticity of polymer chains. The results may contribute to the design of novel non-fouling, functionalizable coatings. In addition, alternative polymerization methods, for example SET-LRP [182], might provide better control over grafting non-fouling pCBAA and pHPMA brushes from sensor surfaces.

Functionalization of low-fouling coatings

A variety of strategies have been reported for the immobilization of bioreceptors to sensor coatings [3, 15, 50, 62, 63, 210]. Most of them can also be applied to the immobilization of bioreceptors to antifouling coatings (see also "Immobilization of biorecognition elements" section). The critical challenge for functionalization of antifouling coatings is the preservation of the low-fouling capabilities of the surface after functionalization with bioreceptors [15, 43, 50, 59, 211]. Furthermore, the functionalization procedures should be compatible with the diverse set of bioreceptors used and preserve their binding activity.

Activation and deactivation of functional groups of low-fouling coatings

The covalent attachment of bioreceptors to activated functional groups of antifouling coatings has been the most widely used functionalization approach [5, 8, 34, 43, 190, 212]. The carboxyl groups on the top of mixed low-fouling SAMs or in zwitterionic side chains of pCBAA and/or pCBMA were transformed to reactive *N*-hydroxysuccinimidyl esters [13, 43]. Similarly, the hydroxyl groups terminating the side chains of pHOEGMA, pHEMA, or pHPMA were transformed to reactive carbamates [8, 43, 213]. These reactive intermediates were then used for the covalent attachment of bioreceptors containing amine groups.

Large molecules, including antibodies and many other proteins, that cannot penetrate inside a polymer brush can be bound only via reactive intermediates on top of the brush, and several side groups along the polymer chains will remain activated. Therefore, an effective deactivation of the residual functional groups is required to minimize fouling to functionalized coatings [34].

Because the chemical inertness of sensor coatings is a prerequisite of their antifouling properties, the conditions used for

activation or modification of functional groups present in the coating before the immobilization of bioreceptors and the conditions for deactivation of residual functional groups after the bioreceptor immobilization need to be optimized with respect to physico-chemical properties of each type of antifouling coating [34, 43, 65, 214].

An approach based on transforming the carboxylates in the zwitterionic groups of pCBAA to active succinimide esters by optimized NHS–EDC chemistry has been described (Fig. 4) [34]. The activation resulted in a brush with an excess of positive charges, promoting the attachment of antibodies (negatively charged at pH 8.5) by an electrostatic attraction. The IR reflection spectra indicated that after the attachment of antibodies most residual NHS esters hydrolyzed spontaneously back to the original carboxylates in tens of minutes at pH 7.5–9 (Scheme 1), and the fouling measured by SPR increased to 140 pg mm^{-2} [34, 43].

Hydroxy-functional polymer brushes, for example pHOEGMA, pHEMA, and pHPMA, contain a single functional group per monomeric unit. These polymers have been successfully functionalized using homo or heterobifunctional activating agents [8, 47, 65, 190]. A study by Gautrot et al. of different activating agents for hydroxyl-functional brushes indicated the superior performance of disuccinimidyl carbonate (DSC) as a coupling agent [65, 214]. A schematic of the activation of hydroxyl groups and the successive covalent attachment of primary-amine-containing bioreceptors is shown in Scheme 2. IR reflection spectra indicated that after the attachment of antibodies, several reactive succinimidyl carbonate intermediates remained in pHEMA or pHOEGMA brushes for days. The presence of the bulky intermediates was indicated by increases in both the thickness and refractive index of the pHEMA brush. These changes to the brush structure resulted in an increase in plasma fouling to 2000 pg mm^{-2} [43].

To avoid the activation of numerous hydroxyl side groups along homopolymer chains, di-block brushes of MeOEGMA-*b*-HOEGMA and MeOEGMA-*b*-CBAA were synthesized with the reactive groups only along shorter top blocks. Whereas the fouling on the pHOEGMA brush increased to 590 pg mm^{-2} after immobilization of antibodies via activated hydroxyl groups, the fouling on MeOEGMA-*b*-HOEGMA only increased to 270 pg mm^{-2} after Ab immobilization via activated hydroxyls on short pHOEGMA blocks. Conversely, Ab attachment via activated carboxyl groups of pCBAA blocks in MeOEGMA-*b*-CBAA brushes resulted in an increase in fouling of only 30 pg mm^{-2} [158].

Functionalized low-fouling coatings

Mixed SAMs composed of alkanethiols terminated in low-fouling OEG or reactive carboxylic groups have been revealed, after the activation of carboxyl groups with a variety of succinimide derivatives, to be suitable for the covalent attachment of bioreceptors. Numerous bioreceptors have been attached to activated carboxyl groups on SAMs of mixed ω-carboxy and ω-oligo(ethylene glycol) terminated alkanethiols; these surfaces were then used with SPR biosensors capable of detecting a variety of analytes in blood plasma or serum [15, 105, 215]. The transformation of the terminal hydroxyls on OEG_6 SAMs to reactive succinimidyl carbonates has also been used to attach antibodies in a sensor for *Cronobacter* detection [190].

Several reports have described a successful strategy for the immobilization of bioreceptors to ultra-low-fouling zwitterionic pCBAA-2 brushes (Fig. 5) [34, 216]. Using this method, researchers prepared the first biosensor capable of detecting analytes in undiluted blood plasma without any detectable fouling (via a SPR biosensor) [34]. Jiang's group used modified procedures to functionalize ω-dopamine-pCBAA grafted to a silicon suspended microchannel resonator [140], a SiO_2-coated SPR sensor [152], and catechol-pCBAA grafted onto the gold surface of a SPR sensor [217, 218]. In these methods, the antibodies against the target analyte (ALCAM) were injected in an aqueous solution of NaOH of pH 10 [216, 217]. These conditions may not be suitable for antibody or other protein bioreceptors because of the possible denaturation at high pH. Antibody-functionalized hydroxyl-functional

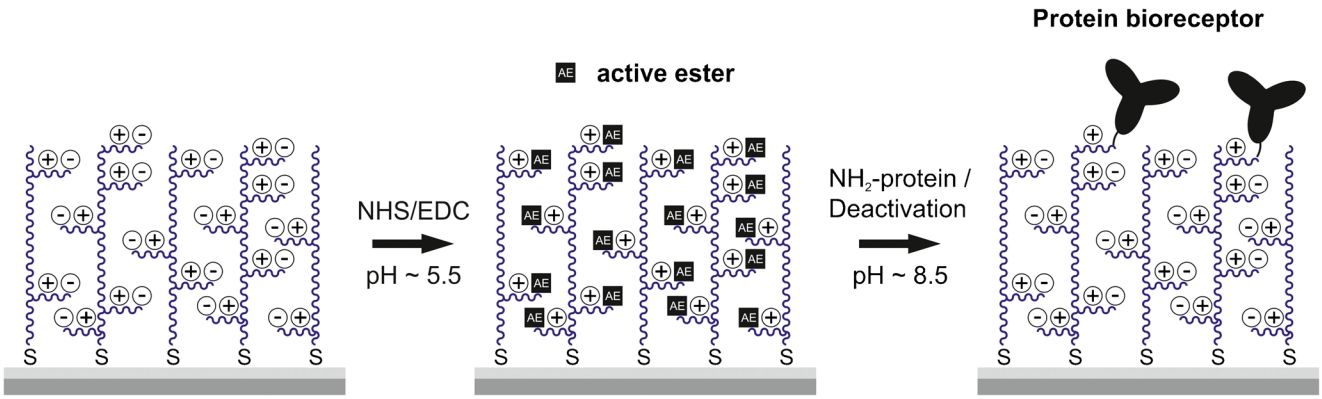
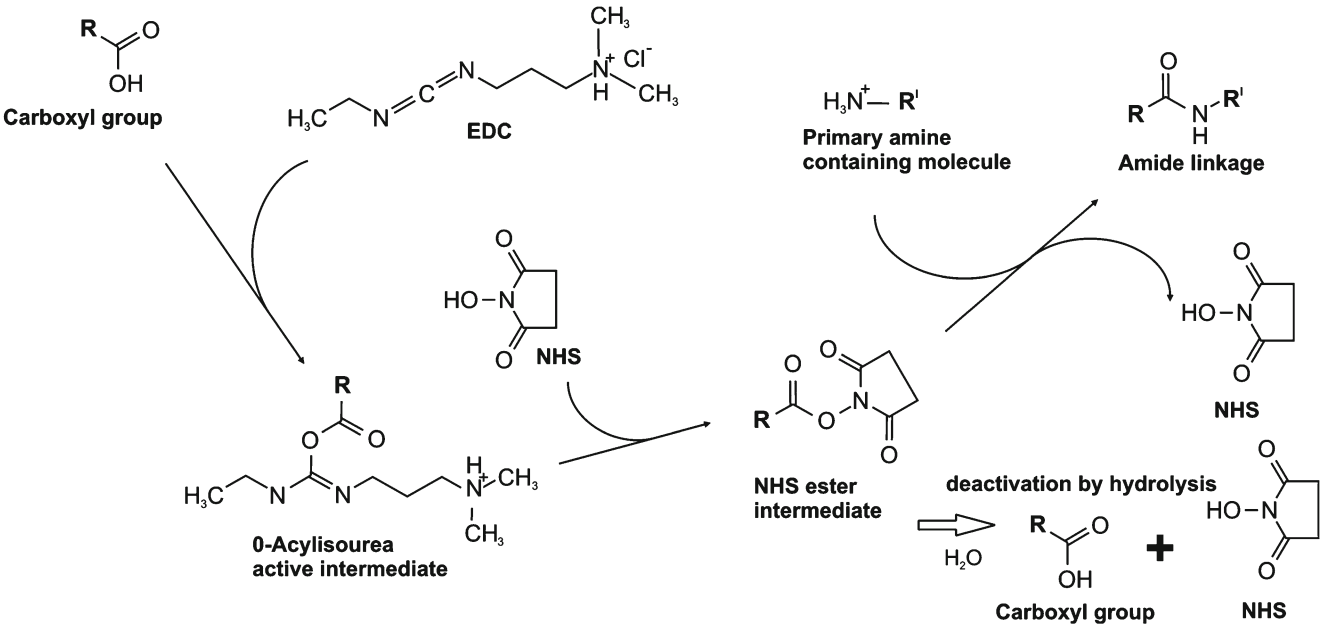


Fig. 4 A scheme of the activation, protein immobilization, and deactivation of a pCBAA brush of a pCBAA brush, adopted from [34]



Scheme 1 NHS–EDC activation of carboxyl groups, attachment of bioreceptors via a primary amine, and hydrolysis of residual NHD esters back to carboxyl groups

brushes have also been reported using homo or heterobifunctional activating agents [8, 47, 65, 190].

Aldehyde functionalities have been used for coupling of bioreceptors. A micropattern of immobilized streptavidin on low-fouling poly(*N*-[(2,3-dihydroxypropyl)acrylamide]) brushes was prepared by generating spatially distributed aldehyde functionalities with sodium periodate, without deterioration of its low-fouling background for both single-protein solutions and blood plasma [219].

An OWLS sensor with reduced serum fouling was prepared by a step-by-step procedure consisting of grafting a graft-copolymer of poly(*l*-lysine) with PEG and PEG-biotin side chains to a Nb_2O_5 transducer surface, the covalent attachment of neutravidin, and the attachment of biotinylated anti-rabbit IgG. [220]. A sensor prototype based on polymer brushes resistant to Fbg and Lys adsorption and capable of binding biotinylated entities was prepared on both SPR and quartz crystal microbalance (QCM) sensors by the covalent attachment of biotin to pre-activated pHOEGMA brushes followed by the attachment of streptavidin [47]. Other potential ways in which protein arrays can be prepared include spotting biotinylated proteins to avidin-coated surfaces [50, 221] and patterning surfaces with spatially-defined photo-activation of previously deactivated biotin [52, 222] or with photochemically bound biotin [223] to obtain patterns of biotin available for subsequent binding of avidin derivatives.

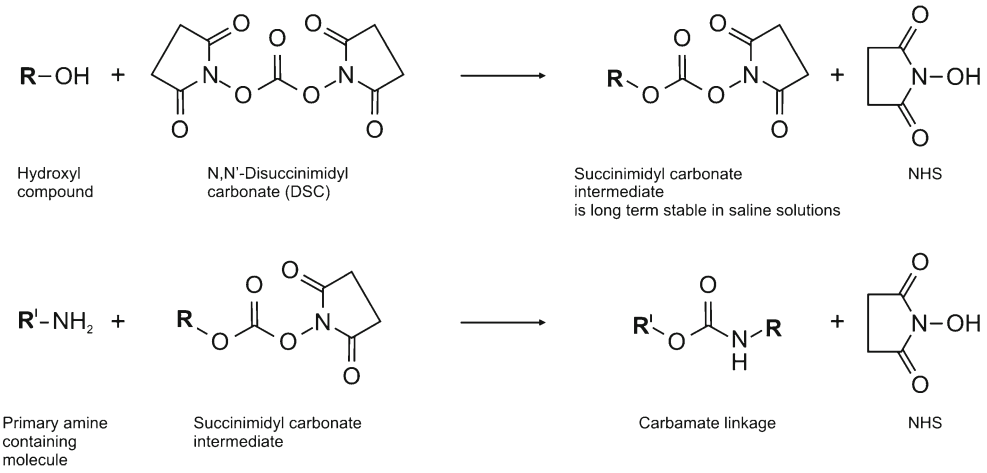
Several groups have immobilized different histidine-tagged proteins and peptides on chelator thioalkane SAMs [69, 224], silanized silicon [225], and patterned PLL–*g*–PEG surfaces [70]. Gautrot et al. immobilized a green fluorescent protein to NTA-functionalized pHEMA and pHOEGMA brushes [68], obtaining an increased loading compared with previous systems [61, 69, 224], with a minimal loss of the antifouling properties of the brushes. The observed higher immobilization level may be attributed to the 3D architecture of the brush used, in contrast with the 2D structure of SAMs.

Boozer et al. prepared a mixed SAM composed of OEG alkanethiols and thiolated single-strand DNA onto which antibodies conjugated with complementary oligonucleotide sequences were hybridized [94]. This functionalization was used for the preparation of a multi-channel SPR biosensor capable of real-time detection of human chorionic gonadotropin [93]. Recently, biotinylated oligonucleotides were attached to streptavidin-functionalized pHOEGMA brushes. The immobilized oligonucleotides were subsequently hybridized with their complementary oligonucleotides conjugated with BSA as a model of oligonucleotide-directed immobilization [10]. Later, the same procedure was used for the immobilization of BSA conjugated with oligonucleotides and antigens on a multichannel SPR biosensor. The biosensor was capable of recognizing four stages of the Epstein–Barr virus by detecting four antibodies in clinical samples of patients' sera [213].

Architecture of functionalized low-fouling coatings

The biorecognition capabilities of functionalized coatings are affected by both the accessibility of functional moieties (e.g. activated functional groups) for bioreceptor immobilization and the accessibility of the immobilized bioreceptors to a target. Three basic architectures of these functionalized coatings have been described:

Scheme 2 The activation of the hydroxyl group by reaction with DSC, and the attachment of bioreceptors via the primary amine



1. a three-dimensional polymer matrix;
2. immobilization on side chains of polymers in a brush; and
3. immobilization strictly at the interface between a coating surface and the tested medium.

The three-dimensional immobilization has typically been used for the immobilization of bioreceptors in the carboxymethyl dextran matrices commonly used in Biacore chips (Fig. 5) [60, 226]. These matrices have a high capacity for protein immobilization and can provide a more homogeneous and natural microenvironment than two-dimensional surfaces, thus preventing the denaturation of proteins [77, 227]; however, a high fouling constrains the use of such coatings for detection in blood serum and plasma.

Brynda's group developed multilayer biorecognition assemblies composed of different antibodies. These multilayers were prepared by alternating the deposition of positively charged Ab below its isoelectric point and dextran sulfate polyanions (DS) at a low pH. After covalent crosslinking of the antibodies, DS anions were expelled from the Ab assembly by increasing the pH above the Ab isoelectric point (Fig. 6). These multilayer biorecognition assemblies were then used with biosensors based on a variety of optical methods, including SPR [228, 229], optical-waveguide light-mode spectroscopy [228, 230, 231], and a Young interferometer [230]. These layers achieved a high loading and activity of bioreceptors and obtained minimum rates of fouling, enabling the direct detection of analytes spiked in blood plasma [230]. For small analytes, for example β_2-microglobulin of m.w. 13 kD, the sensor response increased with increasing number of antibody layers, indicating that analytes penetrated inside the cross-linked assembly. Larger analytes, for example hCG, were captured only by the two upper antibody layers [228].

Most polymer brushes prepared by conventional surface-initiated polymerization are composed of polymers with hydrophilic side chains that can be post-functionalized to immobilize bioreceptors along the chains (Fig. 4). Small activating agents diffuse inside the brush and create active moieties on each side chain along the polymer chains. Usually only those located near the brush surface are involved in the attachment of larger

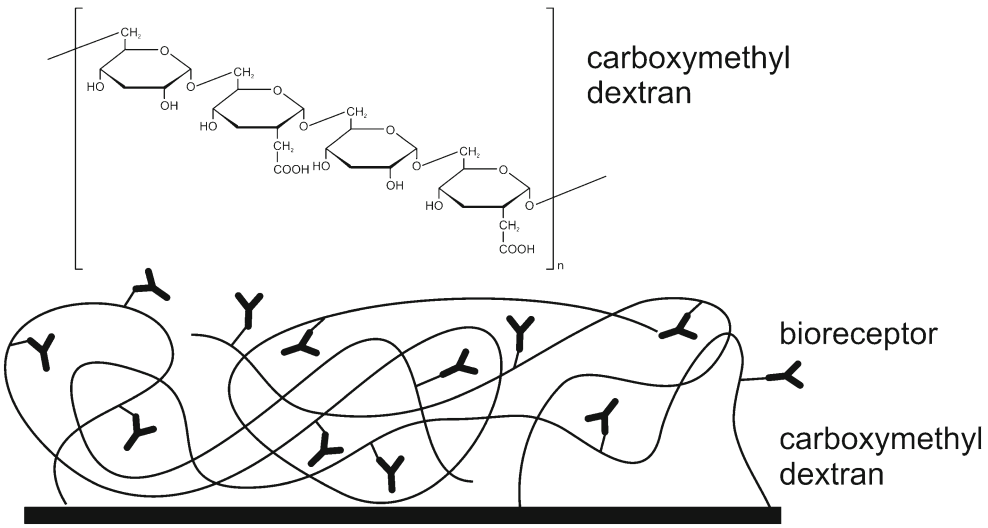


Fig. 5 Carboxymethyl dextran chains are covalently attached to the surface and bioreceptors are immobilized via activated carboxyl groups along the dextran backbone

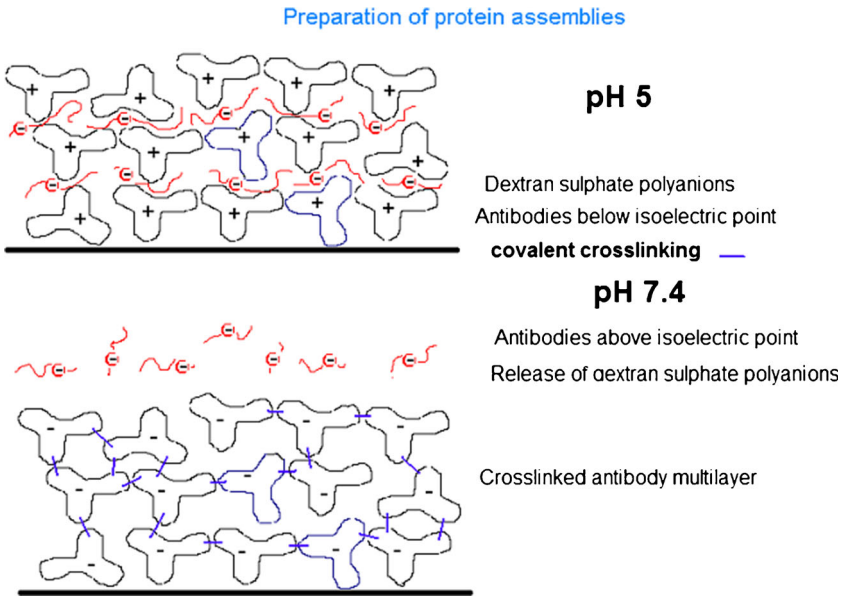


Fig. 6 Multilayer biorecognition assemblies of antibodies prepared by alternating the deposition of positively charged antibody below its isoelectric point and of dextran sulfate polyanions

bioreceptors, for example antibodies, that do not penetrate inside the densely packed polymer brush [43, 65]. For instance, the concentrations of antibodies or streptavidin immobilized on activated pHEMA and pCBAA brushes were comparable with or lower than those immobilized on activated SAMs [43].

The last type of architecture involves the immobilization of bioreceptors on top of a coating. This approach is commonly used for SAMs and for coatings prepared by grafting heterobifunctional R_1–PEG–R_2 to a substrate surface. PEG is anchored via R_1 and the bioreceptors are subsequently attached via R_2. The living halogen terminal groups of pMeOEGMA and pSBMA brushes grafted from a surface by SI-ATRP were substituted by tris(2-aminoethyl ethanol) or azide and activated by disuccinimidyl carbonate or via click chemistry. Antibodies (anti-Salmonella) were subsequently attached on top of the brushes [232–234]. This immobilization does not modify the properties nor the conformation of the brush, and combined with newer bioorthogonal transformation may result in more efficient strategies for the preparation of biosensors. A prerequisite of the use of this type of immobilization is the control of the living character of the polymerization. The approach is not suitable for immobilizing bioreceptors on non-fouling pCBAA-2 and pHPMA brushes because of lack of control in their SI-ATRP.

Antifouling coatings for biosensing in blood plasma and serum

In medical diagnostics based on disease-biomarker detection it is supposed that trace changes in biomarker concentrations in biological fluids correlate with the risk or progression of a disease, or with the response of the disease to a given treatment. Extensive research on antifouling SAMs, functional PEG derivatives, and polymer brushes has resulted in the development of numerous biosensors capable of the detection of a variety of biomarkers and other analytes in blood plasma or serum [15, 220, 235–237]. These detections are typically performed in a diluted medium spiked with a target analyte and a signal is measured in the detection channel, with interfering effects compensated for by a reference channel.

Because the surface functionalization can change the antifouling properties of the coating, it can be advantageous to use the same immobilization procedure in both the detection and reference channels. Conventional referencing approaches are based on attachment of the bioreceptor and of a similar biomolecule that does not bind the target analyte to, respectively, the detection and reference channel coated with the same antifouling layer, using the same immobilization procedure [15]. However, different functionalities present in the detection and reference channels may mean that antibody cross-reactivity with non-target moieties in a complex biological medium is not fully compensated. In addition, it has been revealed that when screening real clinical samples, the response of a detection channel to a real clinical sample cannot be referenced by subtracting the response of the same surface to plasma or serum collected from healthy individuals [192] (Table 4). Recently, a new approach has been reported that uses a single functionalized surface split into a detection and reference channel [212]. In this approach a complex sample (detection channel) and a complex sample mixed with biomolecules binding to the analyte (reference channel) are introduced to the same surface; in the reference channel the presence of the analyte-binding biomolecules inhibits binding of the analyte to the functionalized surface. The advantages of using such a single-surface referencing approach are (i) only the detection channel captures the analyte, and (ii) levels of nonspecific binding in the detection and reference channels are the same.

Antifouling SAMs have been frequently used for the label-free detection of a variety of analytes spiked in diluted blood plasma or serum [15, 96, 105, 212, 220, 235–237]. Both activated leukocyte cell adhesion molecule (ALCAM) and human chorionic gonadotropin (hCG) spiked into 10 % blood plasma were detected using a SPR imaging sensor with multiple channels with respective immobilized antibodies [215]. Antibodies were immobilized on the sensing areas via the hybridization of antibody–oligonucleotide conjugates to thiolated complementary oligonucleotides microspotted on the sensor surface (DNA-directed immobilization). A low-fouling background was achieved by the covalent immobilization of bovine serum albumin to carboxyl-terminated alkanethiols, filling the areas between the thiolated oligonucleotides and outside the sensing areas. Antifouling peptide-terminated SAMs with immobilized antibodies against matrix metalloproteinase-3 (MMP-3) were used in a SPR biosensor to detect MMP-3 spiked into bovine serum [237]. A response to serum free of MMP-3 was used as a reference.

A SPR biosensor for the detection of ALCAM in undiluted blood plasma was prepared by the covalent attachment of monoclonal antibodies against ALCAM to mixed COOH and OH–OEG SAMs and to pCBAA brushes [34]. The detection and reference surfaces were first exposed to undiluted pooled plasma, after which the steady-state sensor signal was used as a baseline for measuring the response to the same plasma spiked with ALCAM. ALCAM spiked in undiluted plasma was detected with LODs of 100 ng mL^{-1} and 10 ng mL^{-1} on blocked OEG-based SAM and unblocked pCBAA, respectively. The LOD achieved on pCBAA was comparable with the difference between a normal ALCAM concentration of ~84 ng mL^{-1} present in the plasma of healthy individuals and an elevated concentration of approximately 100 ng mL^{-1} present in plasma from patients with pancreatic carcinoma. The fouling from undiluted non-spiked plasma on a reference surface coated with anti-Salmonella attached to pCBAA was below the limit of detection of the SPR sensor used (3 pg mm^{-2}). Such results confirm the hypothesis that surface capability to resist fouling from blood plasma can substantially affect the resulting biosensor characteristics. However, more comparative studies would be beneficial to assess the effects of low-fouling surfaces on detection of analytes in blood plasma and serum.

In a later study, another sensor for the detection of ALCAM was prepared by immobilizing anti-ALCAM to DOPA-pCBMA grafted to a SiO_2-coated SPR sensor [152]. Spiked ALCAM concentrations of approximately 64 ng mL^{-1} were detected in normal blood plasma using anti-Salmonella-coated surface as a reference. The nonspecific fouling of the reference surface was approximately 63 pg mm^{-2} [152]. Anti-ALCAM was attached to DOPA-pCBMA grafted to the silicon surface of a suspended microchannel resonator [140]. In this study the authors used a reference surface functionalized with whole goat IgG. ALCAM concentrations in the range 10–1000 ng mL^{-1} were detected in ALCAM-spiked undiluted foetal bovine serum, a medium with substantially less fouling than that from human blood plasma [140]. After immobilization of IgG on the DOPA-pCBMA brush, the fouling from fetal serum increased to 300 pg mm^{-2} [140]. The cytotoxic drug methotrexate was spiked into undiluted combined human blood plasma and detected by a Young interferometer with a detection waveguide branch coated by a cross-linked multilayer of anti-methotrexate antibodies, where the reference branch was coated with a multilayer of antibodies against horseradish peroxidase [230]. The fouling of non-spiked plasma on the detection surface was approximately 150 pg mm^{-2}.

In the work of Brault et al., solutions of three different antibodies were spotted on the pCBAA surface to create a protein array with a low-fouling background [238]. The respective antigens were detected in antigen-spiked undiluted human blood plasma. The fouling from undiluted human plasma on the antibody-immobilized pCBAA spots and the background was found to be approximately 90 and 60 pg mm^{-2}, respectively. The use of a SPR biosensor to diagnose cancer biomarkers in real clinical sera taken from patients was published by Ladd et al. [236]. They detected levels of antibodies against carcinoembryonic antigen (anti-CEA) in 1 % serum diluted with a BSA solution, using a SPR biosensor based on CEA covalently attached onto a mixed SAM of OH–OEG and COOH–OEG. Serum samples from five healthy individuals were used to establish a threshold value for differentiating a cancerous serum sample from a negative sample. The plasma fouling on the SAM, which might be high and very different in samples taken from different individuals [192], was not taken into consideration. The response to a positive serum was only attributed to the specific capture of anti-CEA by the immobilized CEA, because no adsorption from the BSA solution (1 mg mL^{-1}) was observed.

A SPR biosensor capable of recognizing different stages of Epstein–Barr virosis in clinical serum samples by the simultaneous detection of three antibodies specific for the Epstein–Barr virus (EBV) infection was reported by Riedel et al. [213]. Specific EBV-antigen–BSA-oligonucleotide conjugates were immobilized onto a detection surface coated with a low-fouling pHOEGMA brush via hybridization with complementary oligonucleotides attached to the brush. The same coating onto which the conjugates were not hybridized was used as a reference surface. Even if the responses to negative sera from several healthy individuals were different, there was no significant difference in the response of the detection and reference surfaces to a serum sample from the same healthy individual. Thus, a difference between the responses of the detection and reference surface to a patient serum sample was believed to reflect the presence of the targeted antibody.

In summary, although numerous examples of antifouling sensing techniques have been introduced, very few have

achieved the direct detection of analytes in either complex (>50 %) or undiluted blood plasma and serum. Typically, in detection experiments the analytes were spiked in blood serum or plasma in a selected concentration range and the respective sensor characteristics, including LOD, sensor operating range, and level of fouling to a functionalized surface from measuring biological medium, were determined [34, 230, 235, 237]. There are only a few reports that deal with the screening of clinical samples for a limited number of naturally present target analytes [213, 236].

Conclusions

The development of functionalizable antifouling coatings for label-free optical biosensors faces numerous complex challenges. There is no simple relationship between the fouling properties of antifouling coatings and their physico-chemical properties, chemical composition, and architecture that would provide guidance for the design of antifouling coatings. The responses of antifouling coatings to real-world complex samples have large variations depending on sample composition (for example, significantly different sensor responses have been observed when a functional coating was exposed to plasma samples from different donors or even pooled plasma from different sources). There are no standardized characterization procedures for assessing the fouling properties of antifouling coatings, and differences in the methods used by different research groups make it difficult to draw a fair comparison of biofunctional coatings and provide feedback for their design.

Despite these difficulties, many coatings have been developed and evaluated in a variety of biosensing applications. SAMs of alkanethiolates terminated with oligo(ethyleneglycol) (OEG) have been revealed to provide efficient protection against fouling from single-protein solutions, but rather poor protection against fouling from blood plasma or serum (above 500 pg mm^{-2}). Levels of fouling below 50 pg mm^{-2} have been achieved with pHOEGMA and pHEMA brushes grafted from a surface by SI-ATRP. Levels of nonspecific adsorption from blood plasma below 5 pg mm^{-2} have been reached only with the pCBAA and pHPMA brushes. However, even these levels of resistance to fouling from blood plasma or serum do not reach the sensitivity of the best label-free optical techniques, which are able to detect binding events that result in a surface coverage as low as 0.1 pg mm^{-2}.

The antifouling properties of the coatings are often impaired when they are functionalized with bioreceptors. The most widely used approach to immobilization of bioreceptors to antifouling surfaces is covalent attachment. Before the covalent attachment of bioreceptors, it is necessary to activate or modify chemically functionalizable groups present in the coating. Carboxyl groups on the top of mixed SAMs and in zwitterionic side chains of pCBAA, and hydroxyl groups terminating the side chains of pHOEGMA, pHEMA, and pHPMA, are activated and used to anchor molecules that contain amine groups. Whereas carboxyl groups regenerate quickly after their activation, a substantial portion of the activated hydroxyl groups are converted to intermediates that irreversibly impair the antifouling properties of the polymer brushes. In addition, the covalent binding of protein bioreceptors via amine groups available at different sites of the protein can lead to a decrease in their binding activity as a result of multipoint attachment, unfavorable orientation, or conformation changes near the binding site.

Development of new functional ultra-low-fouling coatings for label-free optical biosensors will undoubtedly benefit from advances in different areas of material research, especially in methods for preparation and characterization of biofunctional materials. New biofunctional coatings based on polymer brushes with well-defined structure will be prepared by adaptation of controlled polymerization techniques; alternatives to SI-ATRP, including RAFT and SET-LRP. Advances in methods for the characterization of low and ultra-low-fouling polymer brushes and identification of protein composition of plasma deposits will contribute to elucidation of the process of deposit formation on biofunctional coatings and help reveal structural and physicochemical properties necessary to achieve resistance to plasma or serum.

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