Polymer Brushes Showing Non-Fouling in Blood Plasma Challenge the Currently Accepted Design of Protein Resistant Surfaces\textsuperscript{a}


Ultra-low-fouling poly[N-(2-hydroxypropyl) methacrylamide] (poly(HPMA)) brushes have been synthesized for the first time. Similar to the so far only ultra-low-fouling surface, poly(carboxybetaine acrylamide), the level of blood plasma fouling was below the detection limit of surface plasmon resonance (SPR, 0.03 ng \cdot cm\textsuperscript{-2}) despite being a hydrogen bond donor and displaying a moderate wettability, thus challenging the currently accepted views for the design of antifouling properties. The antifouling properties were preserved even after two years of storage. To demonstrate the potential of poly-(HPMA) brushes for the preparation of bioactive ultra-low fouling surfaces a label-free SPR immunosensor for detection of \textit{G Streptococcus} was prepared.

Introduction

Protein fouling in complex biological fluids, in particular, blood, plasma, and serum, is an adverse event that can impair the properties or functions of various biotechnological and biomedical devices.\textsuperscript{[1–3]} Some examples include stopping flow through separation columns and porous membranes,\textsuperscript{[4]} non-specific response of affinity biosensors,\textsuperscript{[2,3,5]} reduced circulation time of nanocarriers in the blood stream due to colloidal instability\textsuperscript{[6]} or opsoniza-

\textsuperscript{a} Supporting information for this article is available from the Wiley Online Library or from the author.
tion,7–11 bacteria attachment on contact lenses12 and synthetic grafts,13–15 or disabling of cardiovascular devices by thrombus formation.13,14 Ultra-low-fouling properties are particularly essential for biosensors designed to detect analytes in real-time in complex biological media, such as blood serum or plasma.16 Contemporary surface modifications with antifouling self-assembled monolayers (SAMs),17 grafted polymer layers, and polymer brushes reduce considerably or suppress the adsorption from single protein solutions. Authors have often claimed that they have obtained perfectly antifouling,16 super-low fouling,17 ultra-low fouling,18 and even non-fouling19 surfaces because they had not observed any adsorption from solutions of the main plasma proteins—human serum albumin (HSA) and fibrinogen (Fbg). However, a reduction or even total prevention of the adsorption of the main blood plasma proteins (HSA and Fbg) and immunoglobulin G (IgG) is not evidence that the surface is resistant to blood plasma.3,6,20 Much fewer works showed a reduction in the fouling from blood plasma.13,21 Grafted carboxymethyl dextran or poly(ethylene glycol) have been used but only a minor decrease in plasma fouling was reached. Currently, the design of antifouling surfaces is based on general principles that were established from experiments conducted on SAMs and thin (functional) polymer films. Surface wettability, the presence of hydrogen bond acceptors, the lack of hydrogen bond donors, and a neutral electric charge. Although these brushes totally suppressed the fouling from single protein solutions,17 they show unprecedented low fouling from undiluted blood plasma.10

Remarkably, poly(HPMA) brushes do not follow the behavior of surfaces because they had not observed any adsorption from solutions of the main plasma proteins—human serum albumin (HSA) and fibrinogen (Fbg). However, a reduction or even total prevention of the adsorption of the main blood plasma proteins (HSA and Fbg) and immunoglobulin G (IgG) is not evidence that the surface is resistant to blood plasma.3,6,20 Much fewer works showed a reduction in the fouling from blood plasma.13,21 Grafted carboxymethyl dextran or poly(ethylene glycol) have been used but only a minor decrease in plasma fouling was reached. Currently, the design of antifouling surfaces is based on general principles that were established from experiments conducted on SAMs and thin (functional) polymer films. Surface wettability, the presence of hydrogen bond acceptors, the lack of hydrogen bond donors, and a neutral electric charge. Although these brushes totally suppressed the fouling from single protein solutions,17 they show unprecedented low fouling from undiluted blood plasma.10

Experimental Section

Materials

All chemicals and solvents were purchased from Sigma–Aldrich, Acros, or Serva at the highest available purity and were used as received. The monomers N-(2-hydroxypropyl) methacrylamide
(HPMA) and (3-acryloylaminopropyl)(2-carboxyethyl)dimethylammonium (carboxybetaine acrylamide, CBAA) were synthesized according to literature procedures (see the Supporting Information).^{44,45}

**Preparation of SAMs**

Gold-coated substrates (SPR chips) were rinsed with ethanol and water, dried with nitrogen, and cleaned with UV–ozone cleaner (Jelight) for 15 min. The substrates were immediately immersed in a $1 \times 10^{-3}$ M solution of $\text{HS(CH}_2\text{)}_{11}\text{EG}_6$ (EG = ethyleneglycol) or $\text{HS(CH}_2\text{)}_{11}\text{EG}_3$ in ethanol at 40°C for 10 min and then kept in the dark at room temperature for 1 d. The preparation of the initiator SAM was analogous but the chips were placed in a $1 \times 10^{-3}$ M solution of $\omega$-mercaptoundecyl bromoisobutyrate at room temperature.

**Polymerization of HPMA and CBAA**

Ethanol (10 mL) was degassed using three freeze–pump–thaw cycles and transferred to a Schlenk tube containing CuBr$_2$ (19.1 mg, 133 μmol), CuBr (5.9 mg, 26.5 μmol), and Me$_2$Cyclam (40.9 mg, 160 μmol). The blue solution of the catalyst was added to the monomer HPMA (953 mg, 6.7 mmol) or CBAA (1 500 mg, 6.7 mmol). Finally, the polymerization solution was transferred to the reactor containing the substrate coated with a SAM of $\omega$-mercaptoundecyl bromoisobutyrate and the reaction was allowed to proceed for 2 h at 30°C. The substrates coated with poly(HPMA) or poly(CBAA) brushes were washed with ethanol and water and stored in phosphate buffered saline (PBS).

**Polymerization of Monomethoxy-Terminated Oligo(ethylene glycol)methacrylate (MeOEGMA)**

To a degassed solution of CuBr$_2$ (8.1 mg, 36.4 μmol), 2,2‘-dipyridyl (145 mg, 930 μmol), and MeOEGMA (5.7 g, 19 mmol) in 10 mL of water, CuCl (37 mg, 375 μmol) was added. The polymerization mixture was transferred under Ar protection to the reactor containing the initiator-coated substrate and the reaction was allowed to proceed for 90 min at 30°C to obtain 30 nm thick polymer brushes.

**Polymerization of 2-Hydroxypropyl Methacrylate (HPM)**

The novel poly(HPM) brushes were synthesized for the first time using a modified procedure described elsewhere. To a degassed solution of CuBr$_2$ (8.1 mg, 36.4 μmol), 2,2‘-dipyridyl (145 mg, 930 μmol), and HPM (6.4 g, 44.3 mmol) in 10 mL of water/ethanol (1 : 1), CuCl (37 mg, 375 μmol) was added. The polymerization mixture was transferred under Ar to the reactor containing the initiator-coated substrate and the polymerization was allowed to proceed for 90 min at 30°C to obtain 30 nm thick poly(HPM) brushes.

**Biofunctionalization of Poly(HPMA) Brushes**

Hydroxy groups in poly(HPMA) brushes with the same thickness as those used for fouling studies (19 nm) were activated by incubating them overnight in a solution of $N,N$-disuccinimidyl carbonate (DSC, 0.1 M) and 4-dimethylaminopyridine (DMAP, 0.1 M) in anhydrous $N,N$-dimethylformamide (DMF) at room temperature for 24 h. The activation was carried out under inert atmosphere. The success of the activation was confirmed by FT-IR spectroscopy. Freshly activated chips were rinsed with DMF and water. Antibody was immobilized by spreading a 150 μL drop of a 50 μg · mL$^{-1}$ solution of rabbit antibody against peptidoglycan-polysaccharide antigen unique to group G Streptococcus in PBS, pH 7.4. The samples were placed in a glass chamber saturated with humidity for 12 h at 4°C. The samples were rinsed with PBS and kept in PBS for 24 h before use. The binding of the peptidoglycan-polysaccharide antigen at concentrations of 600 and 6 000 ng · mL$^{-1}$ in PBS and the subsequent binding of secondary mouse antibody against the captured antigen was monitored in real-time using a Biacore 300 SPR apparatus.

**Results and Discussion**

Poly(HPMA), poly(CBAA), poly(MeOEGMA), and poly(HPM) brushes were grown from gold surfaces modified with a SAM of $\omega$-mercaptoundecyl bromoisobutyrate by SI-ATRP. The chemical structure of the brushes was verified by FT-IR GASF (Figure 1, Supporting Information). A dry thickness of 30 nm was selected as a minimum to reach optimal antifouling properties for poly(MeOEGMA) brushes. For poly(CBAA) and poly(HPM), a thickness of 18 and 30 nm, respectively, was selected following literature reports. The adsorption from solutions of the main plasma proteins Fbg (1 mg · mL$^{-1}$), HSA (5 mg · mL$^{-1}$), IgG (8 mg · mL$^{-1}$), and lysozyme (Lys, 1 mg · mL$^{-1}$) in PBS as well as from undiluted human blood plasma was measured by SPR spectroscopy (Figure 2 and Supporting Information). All the brushes were able to suppress adsorption from the single protein solutions, but only poly(CBAA) and poly(HPMA) were able to fully suppress the fouling from blood plasma below the SPR detection limit of 0.03 ng · cm$^{-2}$ as depicted in Figure 2 and in Table 2 of the Supporting Information. The observed plasma deposits, 22.5 ng · cm$^{-2}$ on poly(MeOEGMA), 40.5 ng · cm$^{-2}$ on poly(HPM), and 71 ng · cm$^{-2}$ on OEG6 would be too high for many applications, particularly for label-free biosensing. The results were...
qualitatively confirmed by FT-IR GASR, by which no plasma deposits were detected on poly(CBAA) and poly(HPMA) (Supporting Information) after 15 min incubation. It is worth noting that for biosensing applications, resistance to a 15 min contact with blood plasma is a sufficient proof of fouling resistance, however, other applications require longer contact times with blood plasma. Therefore, the resistance to longer incubation in undiluted blood plasma was evaluated. No fouling was detected even after 2 h contact with undiluted human blood plasma.

Importantly, while the resistance of poly(CBAA) to protein adsorption corresponds well with the accepted ideas for the design of surfaces resistant to blood plasma fouling, i.e., high wettability expressed by low advancing and receding water contact angles, $\theta_{\text{adv}} = 23^\circ$ and $\theta_{\text{rec}} = 8^\circ$, and to the lack of hydrogen bond donors, poly(HPMA) brushes are out of line with these criteria. Remarkably, the novel brushes based on poly(HPMA) totally suppressed plasma fouling despite being hydrogen bond donors and displaying a moderate wettability, measured by water contact angles, $\theta_{\text{adv}} = 40^\circ$ and $\theta_{\text{rec}} = 21^\circ$, which were closer to those of poly(MeOEGMA) ($\theta_{\text{adv}} = 55^\circ$ and $\theta_{\text{rec}} = 22^\circ$) or poly(HPM) ($\theta_{\text{adv}} = 50^\circ$ and $\theta_{\text{rec}} = 36^\circ$). The hydration of the poly(HPMA) brushes in water, characterized by a swelling of 176% of their dry thickness, was even lower than that of poly(MeOEGMA) brushes (216%). The excellent non-fouling properties of poly(HPMA) brushes together with our previous results showing no direct relationships between the plasma fouling on various surfaces and their wettability or ability to suppress adsorption of the main plasma proteins,[3] suggest that the principles of blood plasma fouling remain not well understood.

After incubation in blood plasma, SPR chips coated with poly(HPMA) brushes were stored in PBS. No fouling on the chips was detected when the used chips were stored in PBS buffer and re-incubated in blood plasma after three months. A plasma deposit of only 17 ng·cm$^{-2}$ was observed when these chips were incubated for a third time after two years storage in PBS. The stability of the poly(HPMA) antifouling properties easily out-performs that of the so far only ultra-low-fouling material, poly(CBAA) brushes, on which 125 ng·cm$^{-2}$ deposit was observed when repeating the incubation in plasma after two years of storage in PBS (Supporting Information).

Most of the real applications of non-fouling surfaces require covalent attachment of bioactive molecules, e.g., antibodies, antigens, aptamers, and cell interacting molecules,[5,20] to perform specific functions without the interference of fouling from the surrounding biological medium.[14] To demonstrate the potential of poly(HPMA) brushes for these applications a label-free SPR immuno-sensor for detection of $G$ Streptococcus was prepared. A
rabbit antibody against a peptidoglycan-polysaccharide antigen unique to group G Streptococcus (Ab) was covalently attached to poly(HPMA) brushes activated with DSC and DMAP using a procedure described elsewhere. Figure 3A shows the real-time binding of the peptidoglycan-polysaccharide antigen at concentrations of 600 and 6,000 ng·mL⁻¹ in PBS and the subsequent binding of secondary mouse antibody against the captured antigen observed by SPR spectroscopy utilizing a Biacore 3000 apparatus. Concentrations as low as 600 ng·mL⁻¹ could be detected (Figure 3A) while no increase in blood plasma fouling was observed after the immobilization of the antibody (Figure 3B) demonstrating the versatility of these brushes for the preparation of ultra-low-fouling bioactive surfaces.

Conclusion

In conclusion, a new class of ultra-low-fouling polymer brushes based on the biocompatible poly[N-(2-hydroxypropyl) methacrylamide] was prepared for the first time. Together with poly(CBAA), these novel protein resistant brushes are the only surface modifications capable of preventing the fouling from blood plasma below the detection levels of label-free monitoring platforms based on SPR. Importantly, the new poly(HPMA) brushes are based on a hydrogen bond donor and are moderately hydrophilic, which is in contrast to the currently accepted views for the design of protein resistant surfaces. Thus, these brushes open a new paradigm for the field of non-fouling surfaces based on new materials. The covalent functionalization of poly(HPMA) brushes with antibodies demonstrated their potential application for the preparation of ultra-low-fouling surfaces with specific biological activities.

Acknowledgements: This research was supported by the Academy of Sciences of the Czech Republic under Contract No KAN200670701, by the Grant Agency of the Academy of Sciences of the Czech Republic (No. IAAX 00500803), and by grant SVV-2011-263.

Received: March 25, 2011; Revised: April 14, 2011; Published online: June 3, 2011; DOI: 10.1002/marc.201100189

Keywords: biosensors; blood plasma; HPMA; polymer brushes; surfaces


