Protein-Resistant Polymer Coatings Based on Surface-Adsorbed Poly(aminoethyl methacrylate)/ Poly(ethylene glycol) Copolymers

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We report on the protein-resistant properties of glass substrates coated with novel copolymers of 2-aminoethyl methacrylate hydrochloride and poly(ethylene glycol) methyl ether methacrylate (AEM-PEG). In comparison to currently available protein-blocking polymer systems, such as poly-L-lysine-poly(ethylene glycol), silane-based poly(ethylene glycol), and poly(ethylene glycol) brushes prepared by surface-initiated polymerization, the proposed AEM-PEG offers the combined advantages of low cost, simplicity of use, and applicability in aqueous solutions. We demonstrate the capability of AEM-PEG to block the surface binding of globular proteins (tubulin), their assemblies (microtubules), and functional motor proteins (kinesin-1). Moreover, we demonstrate the applicability of AEM-PEG for surface patterning of proteins in microfluidic devices.

Introduction

Reducing the nonspecific adsorption of proteins on surfaces is important for biomedical, bioanalytical, and bionanotechnological applications.[1−6] Currently, poly(ethylene glycol) (PEG) based polymers with different architectures are widely used for this purpose. The methods to immobilize PEG on different substrates include the grafting or adsorption of functionalized PEGulating agents and surface-initiated polymerization.[7−12] Among these methods, the ones that can be easily utilized in an aqueous environment for lab-on-chip systems deserve particular interest. For example, graft copolymers of poly-L-lysine (PLL) with PEG side chains (PLL-PEG) were shown to adsorb from biological buffer solutions onto a variety of substrate materials including glass and metal oxides.[13,14] Thereby, the positively charged PLL backbone sticks to the negatively charged substrates (providing stability for the adsorbed layer) and the PEG side chains stretch away from the surface (forming the protein-resistant layer). However, despite the widely demonstrated applicability of PLL-PEG for surface blocking, for generating of protein patterns and gradients, as well as for cell adhesion studies,[15,16] PLL-PEG is rather expensive.

Here we report on the synthesis and the investigation of the protein-repellent properties of a low-cost PLL-PEG analog. In particular, we prepared random copolymers of 2-aminoethyl methacrylate and poly(ethylene glycol) methyl ether methacrylate (AEM-PEG, Figure 1). AEM-PEG comprises a positively charged backbone and PEG side chains, thus, mimicking the structure of PLL-PEG. We tested the protein-resistant properties of glass substrates modified by AEM-PEG and compared our results to surface blockings based on PLL-PEG as well as silane-based poly(ethylene glycol) (silane-PEG).[17]
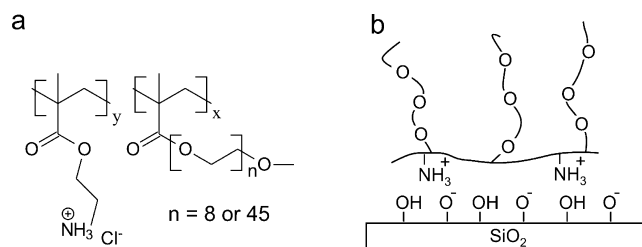

Figure 1. Characteristics of poly(ethylene glycol) methyl ether methacrylate and 2-aminoethyl methacrylate hydrochloride (AEM-PEG). (a) Chemical structure of AEM-PEG. The number of monomer units are denoted by *x* and *y* and the length of the PEG side chains is given by *n*. (b) Schematic representation of AEM-PEG adsorption onto negatively charged surfaces.

Experimental Section

Chemicals. 2-Aminoethyl methacrylate hydrochloride (AEM, Aldrich), 2,2′′-azobis(2-methylpropionitrile) (AIBN, Fluka), *N,N*-dimethylformamide (DMF, Fluka), PLL(20)-g(3.2)-PEG(5) (Surface Solutions, batch SZ24-3, 20.6.2005), and 2-[methoxy(polyethyleneoxy)propyl] trimethoxysilane (ABCR) were used as received. The poly(ethylene glycol) methyl ether methacrylate, M_n = 475 (PEG475, Aldrich), and poly(ethylene glycol) methyl ether methacrylate 50% water solution, M_n = 2080 (PEG2K, Aldrich), were purified by passing through an Al_2O_3 column.

Fabrication of Silane-PEG Substrates. Piranha-cleaned glass substrates were incubated in 2.3 mg/mL toluene solution of 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane for 18 h. The substrates were rinsed several times in toluene, ethanol, and pure water.

Synthesis of AEM-PEG Copolymers. Poly(ethylene glycol) methyl ether methacrylate (1 g) with different molecular weights, a variable amount of 2-aminoethyl methacrylate hydrochloride, and 2 mg of AIBN were dissolved in 5 mL of water/DMF 1:1 mixture. Polymerization was performed under a nitrogen atmosphere for 24 h. The resulting polymer solution was diluted to a concentration of 1 mg/mL and used for further experiments. Obtained polymers were defined as AEMα-PEGβ, where α is the molecular weight of PEG side chains (475 stands for 475 g/mol, 2080 stands for 2080 g/mol), and β is the mass of AEM in milligrams used for the synthesis of polymers For example, sample

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PEG2K-AEM6.5 was prepared using 1 g of poly(ethylene glycol) methyl ether methacrylate (M_n = 2080 g/mol) and 6.5 mg of 2-aminoethyl methacrylate hydrochloride. Degree of polymerization was estimated by SEC using dimethylacetamide as eluting solvent. For determination of composition we applied NMR (CDCl₃). We found that NMR spectra of copolymers as prepared (no purification after the synthesis) and after dialysis against water for 7 days are quantitatively similar. In fact, the ratio between peaks corresponding the PEG side chains ($\delta \approx 3.65$) and AEM groups ($\delta \approx 3.65$ and $\delta \approx 3.4$) did not change after dialysis.

Polymer Adsorption Experiments. Glass coverslips or silica wafers cleaned in piranha solution (H_2SO_4 and H_2O_2 2:1) at 70 °C for 40 min and were used as substrates. Narrow channels (3 × 18 × 0.1 mm) were fabricated between the cleaned glass coverslips and formed the flow cells for further experiments. The channel was filled with PEG-AEM copolymers solution (1 mg/mL in pure water). After about 10 min the channel was rinsed with BRB80 (80 mM PIPES/KOH pH = 6.9, 1 mM EGTA, 1 mM $MgCl_2$) buffer.

Adsorption of Microtubules and Tubulin. Taxol-stabilized, rhodamine-labeled microtubules (∼30 nM tubulin, 1 mM ATP, 1 mM $MgCl_2$, 10 μM Taxol, and oxygen scavenger mix; all in BRB80 buffer) were prepared as described elsewhere.[2] Unpolymerized tubulin was not removed. The substrates were exposed to the microtubule/tubulin solutions for 5 min. Afterward, not-adsorbed microtubules and tubulin were removed by rinsing with BRB80 (containing 10 μM Taxol).

Microtubule Motility Experiments. Motility experiments were performed in flow channels with adsorbed polymers. A casein-containing solution (0.5 mg/mL in BRB80) was perfused into the flow cell and allowed to adsorb to the surfaces for 5 min. Next, 50 μL of a motor solution containing 2 μg/mL wild-type kinesin-1 in BRB80 (full length drosophila conventional kinesin expressed in bacteria and purified as described in ref 18) was perfused into the flow cell and allowed to adsorb for 5 min. Thereafter, a motility solution containing rhodamine-labeled taxol-stabilized microtubules[2,19] was applied.

Ellipsometry. The thickness of the polymer layers was measured at $\lambda = 633$ nm and an angle of incidence of 70° (in dry state) and of 68° (in specially designed cell for in situ measurements) with a null-ellipsometer (Multiscope, Optrel Berlin) as described elsewhere.[20,21] The density of PEG-side chains in the polymer layer adsorbed on the glass (Γ_{PEG}) and the distance between individual PEG chains on the surface (D) were calculated using eqs 1 and 2, respectively:

$$\Gamma_{PEG}(mg/m^2) = H \cdot \rho \cdot (1 - \varphi_{AEM}^{mass}) \qquad (1)$$

$$D(nm^{-1}) = \sqrt{\frac{M_{PEG}}{\Gamma_{PEG} \cdot N_A}} \qquad (2)$$

where H is the dry thickness of the adsorbed polymer layer, ρ is the bulk mass density of polymer, φ_{AEM}^{mass} is the mass fraction of AEM, M_{PEG} is the molecular weight of PEG, N_A is Avogadro's number.

Fluorescence Microscopy. Fluorescence images were obtained using an Axiovert 200 M inverted microscope with a 20× objective (Zeiss, Oberkochen, Germany) equipped with a FluoArc lamp. For data acquisition a standard TRITC filterset (excitation: HQ 535/50; dichroic: Q 565 LP; emission: HQ 610/75, Chroma Technology) in conjunction with a Micromax 512 BFT camera (Photometrics, Tucson, AZ) and a MetaMorph imaging system (Universal Imaging, Downingtown, PA) were used.

Electrokinetic Measurements. The streaming potential measurements were carried out with the Electrokinetic Analyzer (EKA) by Anton Paar GmbH, Graz, Austria, using a special rectangular cell (developed and constructed at the Leibniz Institute of Polymer Research, Dresden, Germany) for small flat pieces. Details of the measuring technique and the used device are reported elsewhere.[22,23] The zeta potential was calculated according to Smoluchowski.[24]

Results and Discussions

Adsorption of Polymers. Two series of AEM-PEG copolymers with different lengths of the PEG side chains (Figure 1) were synthesized using free radical polymerization. The obtained copolymers were denominated as AEMα-PEGβ, where α is the mass of AEM in milligrams per gram of PEG monomer used for synthesis and β is the molecular weight of the PEG (in g/mol). The degree of polymerization varied between 600−1000 monomer units. While the obtained polymers were all water soluble, increasing the fraction of AEM resulted in gelation of the polymer water solutions.

We studied the adsorption of AEM-PEG copolymers on negatively charged glass surfaces (IEP$_{GLASS}$ = 2; Figure 2). It was found that the thickness of the adsorbed polymer layers as function of the composition exhibited a peak-like character (Figure 2a and b).[25,26] The incorporation of small amounts of positively charged AEM groups in the polymer chains first resulted in a sharp increase of the polymer adsorption. This behavior indicates that a minimum number of positively charged groups per chain are required for sustained polymer adsorption to the surface. On the other hand, at larger AEM amounts the layer thickness decreased due to the lower number of PEG side chains per polymer molecule. Importantly, this decrease was not linear. We believe that this nonlinearity results from internal electrostatic repulsions of AEM groups (leading to an increased footprint of the polymer coils on the surface) and from repulsions between the charged polymer coils themselves.

We calculated the distances between individual (AEM-bound) PEG chains on surface and compared these values to the gyration radii of PEG chains of similar molecular weight (Figure 2d). The distance was found to be minimal for the polymers AEM3.2-PEG475 and AEM3.2-PEG2080. It is important to note that these minimal distances were almost equal to the gyration radii of free PEG chains with similar molecular weight[27] in aqueous environment. This indicates that, although the (AEM-bound) PEG chains are not in the brush regime, they completely shield the glass substrate.

Zeta potential measurements proved the substrate shielding by the adsorbed polymers. While the glass substrate was strongly negatively charged ($\zeta = -60$ mV at pH = 5), the adsorbed AEM-PEG polymers reduced the negative charge. The most pronounced reduction in surface charge was observed for AEM3.2-PEG2080 and AEM230-PEG475 (Figure 3). In these cases, the glass substrates were almost uncharged. Considering the low thickness of the adsorbed AEM230-PEG475 layer (see Figure 2b), we can assume that the main reason for the charge reduction is the large amount of AEM in the polymer chains. On the other hand, the adsorbed AEM3.2-PEG2080 layer was significantly thicker and the reduction in surface charge was most likely caused by the formation of a relatively dense PEG layer (see Figure 2b and d).

Combining the results on polymer adsorption, we predict that AEMα-PEG2080 copolymers with α in the range from 1.6 to 6.5 will most efficiently prevent the surface adsorption of proteins. These polymers form the thickest and the densest PEG layers, while at the same time, they completely reduce the negative surface charge.

Protein Adsorption. We tested the adsorption of fluorescently labeled proteins to AEM-PEG coated surfaces on the examples of tubulin (globular protein with a size of about 5 nm) and in vitro reconstructed microtubules (protein assemblies with a diameter of about 25 nm and lengths of several tens of μm; Figures 4 and 5). In addition, we investigated the binding
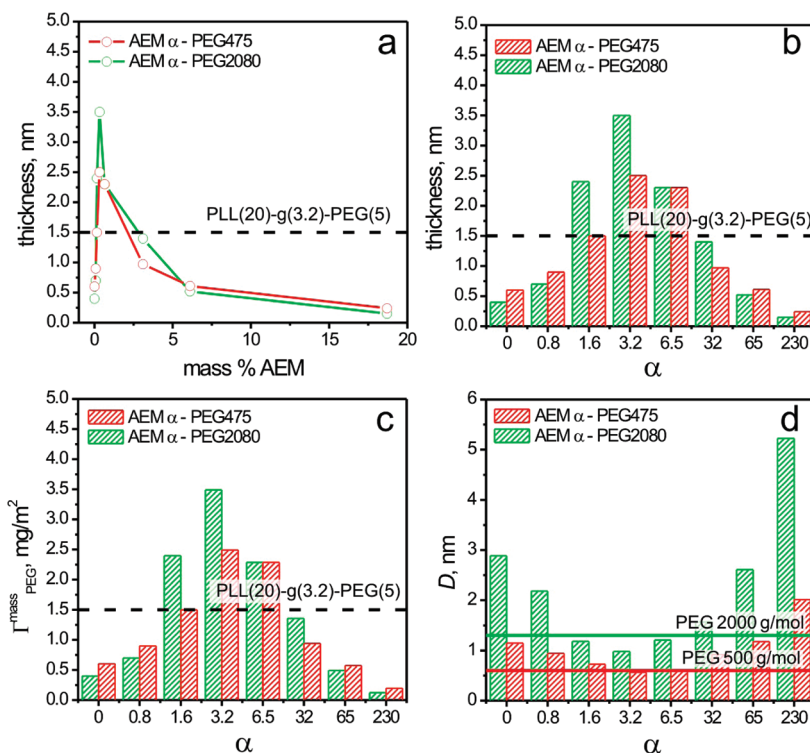
Figure 2. Surface adsorption of AEM-PEG to glass. (a and b) Two different representations of the thickness of adsorbed AEMα-PEG2080 (green) and AEMα-PEG475 (red) copolymers as function of their composition. (c) Calculated grafting density and (d) distance between individual (AEM-bound) PEG chains vs AEM-PEG composition. For comparison, the thickness (dashed lines in a and b) and the grafting density (dashed line in c) of adsorbed PLL(20)-g(3.2)-PEG(5) layers are plotted. Moreover, the gyration radii of PEG with molecular weights of 500 g/mol and 2000 g/mol in water (red and green lines in d) are given for reference.
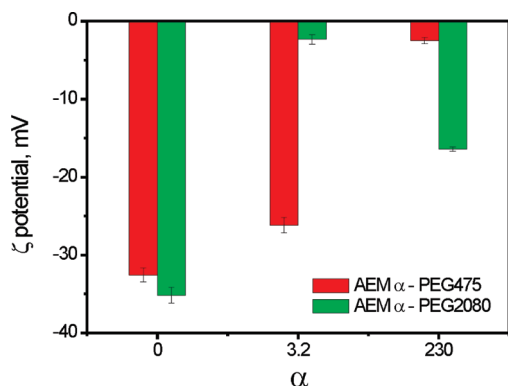

Figure 3. Zeta potential of glass surfaces with adsorbed PEG-AEM copolymers at pH = 5 and 1 mM KCl.

of unlabeled kinesin-1 motor proteins, which the presence on the surface was probed in microtubule gliding assays (Figure 6).

When applying a solution containing rhodamine-labeled microtubules and unpolymerized rhodamine-labeled tubulin to glass surfaces treated with AEM6.5-PEG2080 (Figure 4a) and AEM3.2-PEG2080 (Figure 4b), we observed very low protein binding comparable to a glass surface treated with silane-PEG (Figure 4c). Surprisingly, we found PLL(20)-g(3.2)-PEG(5) to be rather inefficient in surface blocking (Figure 4d), comparable to untreated (piranha-cleaned) surfaces without polymers (Figure 4e) and surfaces treated with AEM-PEG copolymers with high amounts of AEM (images not shown). On those samples, microtubules were not prevented from binding to the surface and a significantly increased fluorescent background signal originating from unpolymerized tubulin was observed. We believe that the sticking of microtubules on the PLL-PEG coated

glass substrate is due to the high density of accessible positively charged lysine groups. On the other hand, as it was reported by Textor et al.[14] and by Vogel et al. in[16] the optimization of the length of PEG side chains and the composition of PLL-PEG allows for very efficient blocking of adsorption of model proteins and kinesin motor proteins.

Varying the polymer composition, we found that, for all AEM amounts, copolymers with longer PEG chains ($\beta = 2080$) more efficiently suppressed protein adsorption than shorter PEG chains ($\beta = 475$; Figure 5). For compositions with α < 32, independent of the actual AEM amount, no binding of microtubules was observed and the amount of bound tubulin was low.

To further demonstrate the efficiency of surface blocking, we adsorbed kinesin-1 motor proteins[28] on different PEG-modified surfaces and assayed the gliding motility of microtubules.[29] We found that microtubules (i) were gliding (with normal speed of 800 nm/s) on untreated glass surfaces without polymers, (ii) got stuck immotile on PLL-PEG-coated glass surfaces, and (iii) did not bind at all to glass surfaces coated with AEM6.5-PEG2080, AEM3.2-PEG2080, and silane-PEG coated glasses.

We also tested the protein-repellent properties of the polymer layer after treatment in different environment. For this we applied PBS, pH = 7.4, 100 μm, BRB 80, pH = 6.9, buffers as well as organic solvents (ethanol and acetone). We found the protein-repellent properties of the polymer layer are kept after exposure to these media.

Surface Patterning. Finally, we tested the applicability AEM-PEG copolymers for surface structuring in microfluidic devices. In a flow cell formed between two glass coverslips, we filled half of the channel with a AEM6.5-PEG2080 solution. After incubation for 10 min, the solution was removed and replaced by a motility solution containing kinesin-1 motor
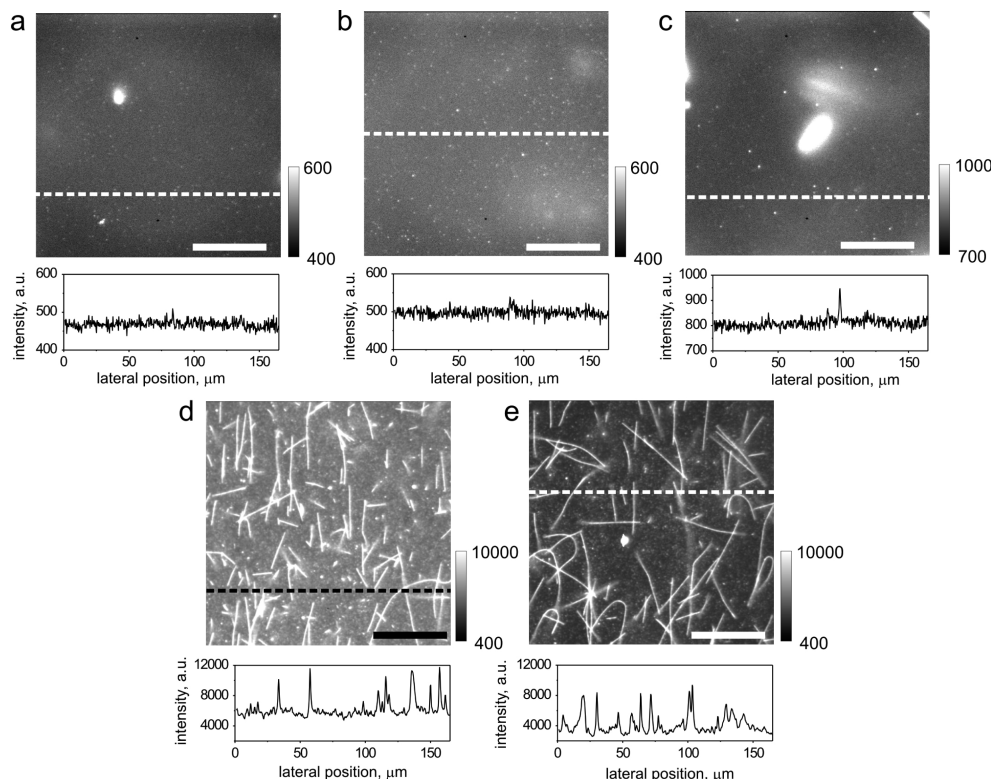
Figure 4. Adsorption of fluorescent tubulin and microtubules on differently treated glass surfaces: (a) AEM6.5-PEG2080, (b) AEM3.2-PEG2080, (c) silane-PEG, (d) PLL(20)-g(3.2)-PEG(5), and (e) piranha-cleaned glass without polymers. The surfaces were incubated with the protein solutions for 5 min and carefully rinsed with unfluorescent buffer solution before imaging. Intensity profiles along the dashed lines in the fluorescence micrographs (scale bar 50 μm) are given below the images. The bright spots in the images a, b, and c are microtubules floating freely in solution.
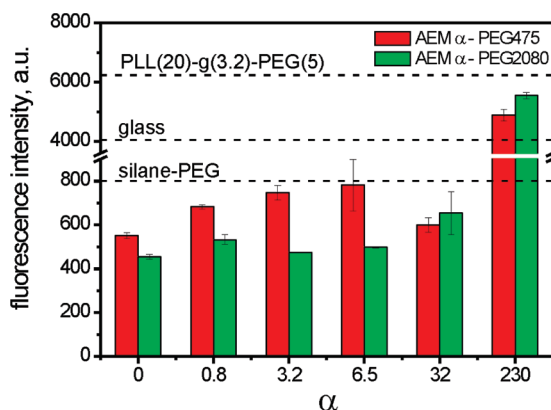

Figure 5. Background fluorescence intensities of rhodamine-labeled tubulin adsorbed on glass substrates treated with different AEM-PEG copolymers (evaluated from images as exemplarily shown in Figure 4). Intensity values on piranha-cleaned as well as silane-PEG and PLL(20)-g(3.2)-PEG(5)-coated glass surfaces are given for reference.

Figure 6. Surface patterning of kinesin-1 using AEM-PEG. Before the whole flow cell was incubated with kinesin-1 motors, half of the glass surfaces were treated with AEM6.5-PEG2080. Rhodamine-labeled microtubules were then gliding on the areas formerly not-covered by AEM-PEG, while no motility was observed on the areas with adsorbed polymer.

protein. In microtubule motility assays we then observed a well-defined border between the untreated and the formerly polymer-covered areas.

In conclusion, we designed novel polymers, which are highly suitable to prevent the adsorption of proteins on glass surfaces. The proposed polymers show unique combination of advantages (such as low price, simplicity of use, applicability in water solutions) over widely used PLL-PEG, silane-PEG, and PEG brushes prepared by surface-initiated polymerization. We believe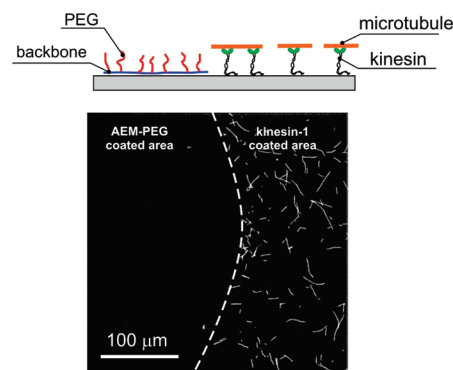 that these advantages will make the developed polymers attractive for many future applications including the chemical structuring of surfaces in microfluidic devices and the design of bioanalitical chips in general.

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