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Protein adsorption to oligo(ethylene glycol) self-assembled monolayers: Experiments with fibrinogen, heparinized plasma, and serum

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Abstract—Low protein adsorption is believed advantageous for blood-contacting materials and ethylene glycols (EG)-based polymeric compounds are often attached to surfaces for this purpose. In the present study, the adsorption of fibrinogen, serum, and plasma were studied by ellipsometry on a series of well-defined oligo(EG) terminated alkane-thiols self-assembled on gold. The layers were prepared with compounds of the general structure HS-$(CH_2)_{15}$-CONH-EG_n, where $n = 2$, 4, and 6. Methoxy-terminated tri(EG) undecanethiol and hydroxyl-terminated hexadecanethiol self-assembled monolayers (SAMs) were used as references.

The results clearly demonstrate that the adsorption depends on the experimental conditions with small amounts of fibrinogen adsorbing from a single protein solution, but larger amounts of proteins from serum and plasma. The adsorption of fibrinogen and blood plasma decreased with an increasing number of EG repeats and was temperature-dependent. Significantly less serum adsorbed to methoxy tri(EG) than to hexa(EG) and more proteins remained on the latter surface after incubation in a sodium dodecyl sulfate (SDS) solution, indicating a looser protein binding to the methoxy-terminated surface. All surfaces adsorbed complement factor 3 (C3) from serum and plasma, although no surface-mediated complement activation was observed. The present study points to the importance of a careful choice of the protein model system before general statements regarding the protein repellant properties of potential surfaces can be made.

Key words: Self-assembled monolayers; oligo(ethylene glycol); protein adsorption; fibrinogen; heparinized plasma; serum; complement.

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1. INTRODUCTION

It is frequently observed that humoral and cellular systems become activated when blood contacts large surface areas in, for example, oxygenators and dialysis apparatuses [1]. In these applications a low non-specific or specific adsorption of proteins is desirable. Other biomaterials applications with similar requirements are tissue- or blood-contacting biosensors, blood collection devices using narrow syringes, arterial stents, and artificial heart valves. Also, a non-specific protein adsorption may obscure results during *in vitro* analysis of serum and plasma [2]. For example, fibrinogen and complement factor 3 (C3) are often observed at surfaces and both are considered important because platelets and neutrophils carry receptors that may participate in the activation of the cells through complex protein–surface interactions. Thus, there seems to exist a general consensus within the biomaterials community saying that non-specific adsorption of proteins is one of the critical issues that must be carefully addressed during the design and development of advanced devices for blood-contacting applications.

The aim of the present work was to study adsorption from single and complex protein solutions to oligo(ethylene glycol) (OEG) self-assembled monolayers (SAMs) on gold. The ethylene glycol termination of surface-bound molecules is of great interest because it possesses properties such as charge neutrality, high water retention capacity, and/or conformational flexibility [3]. Considerable efforts have been paid by others to prepare EG-containing interfaces and a number of studies have demonstrated their excellent protein resistance in various single protein systems [4–11]. However, very little is known about the resistance in complex media, such as blood serum or plasma, or at room temperature (22°C) vs physiological temperature (37°C). It is, for example, not well understood if the nonspecific adsorption of fibrinogen to EGs varies with temperature. The molecular flux towards surfaces is certainly higher at 37°C but simultaneously the Brownian motion of proteins and surfaces increases and may counteract adsorption. Also, if and when activated, the humoral cascade systems such as the complement are believed to proceed quicker at the higher temperature [12], which in turn may facilitate the deposition of proteins to surfaces. With the above in mind, the adsorption of fibrinogen and plasma to a number of different functionalized OEG-SAMs were tested at 22 and 37°C, although the serum experiments were only undertaken at the higher temperature.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were used as-received and purchased from Merck, Germany, if not otherwise stated. The solvents used for contact angle measurements were from Sigma, Sweden and the polyclonal antibodies from Dako, Denmark, except anti-fibrinogen that was from Binding Site, UK. Fibrinogen was purchased from

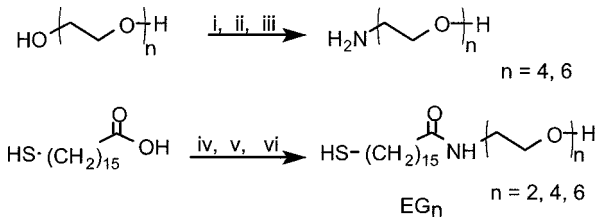

Figure 1. The synthesis scheme for the present OEG alkane thiols with amide linkage: (i) MsCl, TEA, CH_2Cl_2; (ii) NaN_3, EtOH; (iii) Ph_3P/H_2O, THF; (iv) Ac_2O, pyridine; (v) H_2N-$(CH_2$-CH_2-$O)_n$-H (n = 2, 4, 6), EDC/HOBt, DMF; and (vi) NaOMe, MeOH, Dowex H^+.

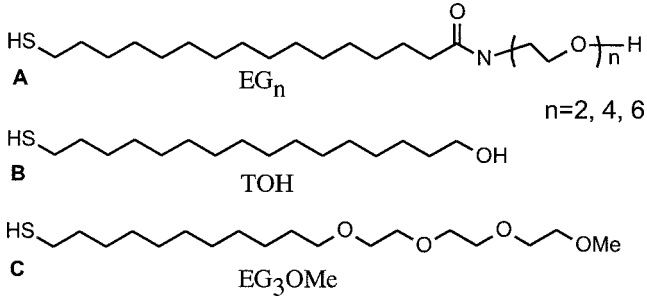

Figure 2. General structure of the alkanethiols from which the SAMs on gold were prepared: (A) OEG amido alkanethiols, n = 2, 4, and 6, (EG_n); (B) mercaptohexadecanol (TOH); and (C) methoxy tri(ethyleneglycol)ether undecanethiol (EG_3OMe).

Chromogenix AB, Sweden (38 wt% human fibrinogen, 16 wt% sodium chloride, 38 wt% sodium citrate, and 8 wt% citric acid).

Di-, tetra- and hexameric OEG-terminated alkanethiols (EG_2, EG_4, and EG_6) with the general structure HS-$(CH_2)_{15}$-CONH-$(CH_2$-CH_2-$O)_n$-H were synthesized according to the route in Fig. 1. The synthesis details are described elsewhere [13]. The molecules were purified by reverse-phase HPLC ($MeOH/H_2O$) and elemental analysis and HPLC (at 230 nm) proved a high purity (>99.5%). The methoxy-terminated tri(ethylene glycol) derivative with the structure HS-$(CH_2)_{11}$-O-$(CH_2$-CH_2-$O)_3$-CH_3 (EG_3OMe) was prepared according to a previously published procedure [14]. The 16-mercaptohexadecanol with the structure HS-$(CH_2)_{16}$-OH (TOH) was a kind gift from Biacore AB, Sweden. The chemical structures of the present alkanethiols are shown in Fig. 2.

2.2. Surface preparations

Glass substrates coated with 2000 Å gold were obtained as a kind gift from Biacore AB, Sweden. The gold surfaces were washed twice in five parts high purity water (Milli-Q, 18 MΩ) with one part 30 wt% hydrogen peroxide and one part 25 wt% ammonia at 80°C for 5 min. The substrate refractive index was determined by null ellipsometry immediately after cleaning. The surfaces were subsequently incubated in 20 μM thiol solutions in 99.5% ethanol for at least 48 h at room temperature and

stored in the solution until use. The samples were then rinsed in ethanol (99%), sonicated for 3 min in ethanol, rinsed in ethanol, blown dry with nitrogen, and put in a cuvette for the subsequent protein adsorption studies.

2.3. Buffers

Two different buffers were used in this study. Veronal buffer (VB) was prepared by addition to distilled water (Milli-Q, 18 MΩ) of 5 mM 5,5′-dietylbarbituric acid, its corresponding sodium salt and 145 mM sodium chloride. VB^{2+} buffer was prepared by the addition of 0.5 mM magnesium dichloride and 0.15 mM calcium dichloride to VB. Phosphate buffered saline (PBS) was prepared by the addition to distilled water of 10 mM sodium hydrogen phosphate, 10 mM potassium dihydrogen phosphate, and 150 mM sodium chloride. All buffers were adjusted to pH 7.4 by addition of a few drops of 1 M sodium hydroxide in distilled water.

2.4. Surface characterization

The self assembled surfaces were rinsed, sonicated for 3 min, and rinsed again in 99% ethanol, and the quality of the SAMs were verified with infrared reflection adsorption-spectroscopy (IRAS). A Bruker 66 aligned at 85 deg was used [14]. Null ellipsometry was used to determine the thickness of the thiol layers with an assumed film refractive index of $n_f = 1.5$ [15] (see below for experimental details).

Surface/water contact angles were determined with the sessile drop technique at room conditions (Ramé–Hart goniometer). Contact angles were also measured for a series of high purity solvents. These solvents were chosen to cover a wide range of surface tensions: glycerol (64 mN m^{-1}), ethyleneglycol (48 mN m^{-1}), bicyclohexyl (33 mN m^{-1}), and nonane (23 mN m^{-1}). The solvents were applied as 2 μl droplets with a pipette. Before use, alumina adsorption chromatography was used to remove unsaturated hydrocarbons from bicyclohexyl and nonane.

2.5. In situ *ellipsometry*

The ellipsometric measurements were performed with a Rudolph Research AutoEL III null ellipsometer at 70 deg. All protein adsorption measurements were made *in situ* [16]. The assumed refractive indices at 632.8 nm were $n_{protein} = 1.465$ [17, 18] and $n_{buffer} = 1.335$, respectively. Changes in the refractive indices due to change in the temperature from 22°C to 37°C were not accounted for but were estimated not to qualitatively change the results.

First, the buffer was added into the cuvette and the system allowed to equilibrate for at least 10 min at the desired temperature (22 or 37°C). The effective substrate (gold plus SAM) refractive index was then determined. The buffer was replaced with respective protein solution, and the thickness of the adsorbed layer determined 5 min after the triplicate buffer rinsing. After the plasma or serum incubations, specific antibodies at 1 : 50 dilutions in buffer were introduced into the cuvette and

incubated for 15 min. The surfaces were then rinsed three times and the total layer thickness was finally measured. The average thickness of the adsorbed protein layers were calculated according to the McCrackin algorithm [19]. From this value the average adsorbed mass density was calculated according to de Feijter *et al.* [20] assuming $dn/dc = 0.18\ cm^3\ g^{-1}$.

2.6. Protein experiments

Fibrinogen was dissolved in VB^{2+} buffer with or without added 1.7 mM $CaCl_2$, or in PBS buffer, at least 30 min prior to the surface incubations for 15 min. The final fibrinogen concentration was 0.4 mg ml^{-1}. The experiments were performed at 22 and 37°C.

Heparinized plasma was pooled from two apparently healthy donors, aliquoted in 2 ml vials and stored at −80°C until use. Upon use, the plasma was thawed for 3 min in a water bath at 37°C to avoid precipitations (of fibrinogen) and then transferred into a water bath at the desired temperature, 22 or 37°C, and temperature-equilibrated for 2 min. The plasma was then transferred to the cuvette with the surface to be studied and incubated at a final concentration of 67% in VB^{2+} for 10 min. A similar procedure was performed after 2.5 mM $MgCl_2$ and 10 mM ethylene glycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA) were added to the plasma 2 min prior to the incubations, except that the plasma was diluted in VB buffer (EGTA plasma). After incubation in plasma at 37°C, the surfaces were rinsed and then incubated in polyclonal anti-C3c or anti-fibrinogen solutions (see above).

Human serum was pooled from two apparently healthy donors, and stored and handled similarly to the heparinized plasma. The incubations and ellipsometric measurements were performed as above except that the serum incubation time was 30 min. The subsequent antibody incubations were performed with polyclonal anti-C3c, a marker of surface bound C3, anti-C3d that indicates proteolytic degradation of C3 and anti-properdin (anti-P) that suggests the presence of surface-bound functional C3 convertases (C3bBbP).

The adsorption or binding strength of the deposited serum layers on the different surfaces was tested using the anionic detergent sodium dodecyl sulfate (SDS) (cmc = 8.3 mM) that removes proteins from both hydrophilic and hydrophobic surfaces [21]. The SDS incubation time was 30 min in 10.4 mM concentration in PBS, pH 7.4, at 37 °C.

2.7. Statistical methods

The results were analyzed using the two-tailed Student's *t*-test with the null hypothesis that the differences were equal to zero. When the probability that the null hypothesis was false was 95% or more the probability of the error in stating that a difference existed was indicated (p). Each measurement was repeated at least nine times ($m = 9$) with surfaces from at least three different thiol solutions, if not

otherwise stated. The error bars in the figures indicate one standard error of the mean (SEM).

3. RESULTS

3.1. Surface characterization

The ellipsometric thicknesses and water-contact angles of the investigated SAMs were similar to those reported in recent publications (Table 1) [4, 11, 14]. Unfortunately, the contact angles with different solvents (Fig. 3) did not allow discrimination between the different OH-terminated OEG-SAMs. This is probably due to similarities in polarity, mobility, and exposure of the terminal portions of the OEG tails. Observation of complete wetting of curfaces coated with OH-terminated EG derivatives indicated that the OEG really faced outward from the surface and strongly interacted with this solvent possessing a similar chemistry. The more hydrophobic EG_3OMe SAM displayed larger contact angles for all solvents, except nonane, (Fig. 3) and could easily be discriminated from the others.

All SAMs showed CH_2 asymmetric and symmetric stretching peaks at 2918–2920 and 2850 cm^{-1}, respectively, suggesting a densely packed alkyl part of the assembly [4, 14]. The EG_6 spectrum also display a number of sharp features in the fingerprint region that are assigned to CH_2 wagging at 1349 cm^{-1}, twisting at 1244 cm^{-1}, rocking at 964 cm^{-1}, and COC stretching at 1114 cm^{-1}, respectively. All these peaks are characteristic for a 7/2 helical crystalline phase (Table 2) [14]. In contrast, the shorter EG_2 and EG_4 tails assemble in a planar all-*trans* conformation. The different conformations, helical and all-*trans*, coexist with the amorphous phase in dry SAMs at room temperature. Recent studies, however, have shown that the helical conformation is affected upon exposure to liquid water. Water penetrating into the OEG-SAMs seems to induce a disordering of the OEG tails [22]. Thus, an OEG-SAM model assuming structurally intact (crystalline) OEG tails in water (or a biological fluid) is an oversimplification.

Table 1.
Ellipsometric thickness (d) and advancing (θ_a) and receding (θ_r) water contact angles of the studied SAMs, standard deviations within parentheses ($n \geqslant 6$)

	d (Å)	θ_a (deg)	θ_r (deg)
TOH	23 (3)	18 (4)	12 (2)
EG_2	26 (1)	27 (1)	23 (1)
EG_4	33 (3)	29 (1)	27 (1)
EG_6	38 (2)	28 (2)	25 (2)
EG_3OMe	25 (1)	63 (2)	61 (2)

3.2. Fibrinogen

Fibrinogen adsorbed to all EGs at 22°C. The lowest amounts were observed at the EG_4 and EG_6 surfaces, whereas, intermediate amounts adsorbed onto EG_3OMe compared to the others (Fig. 4). The hydroxyl-terminated hexadecanethiol (TOH) was the most adsorbing at approximately 75 ng cm^{-2}. The film thicknesses on EG_4 and EG_6 were close to the detection limit of the present ellipsometric set-up, 5 ng cm^{-2}, and it was therefore not possible to ascertain differences between them.

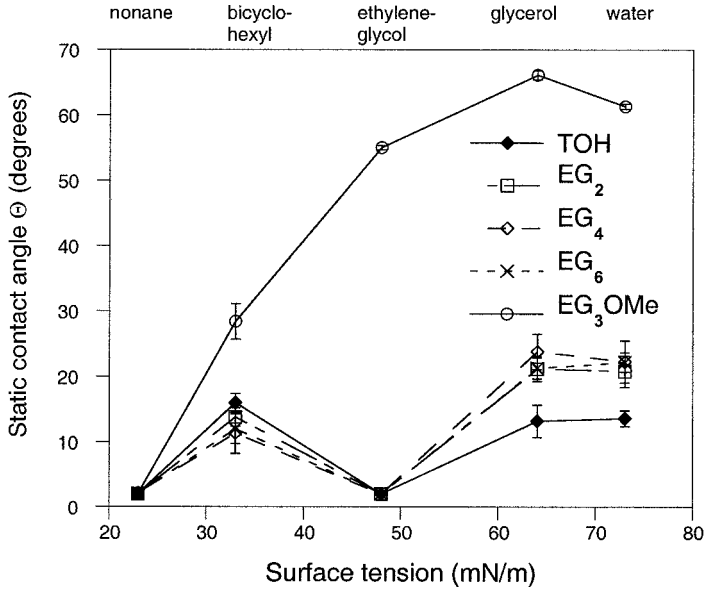

Figure 3. Static contact angles for the different SAMs and solvents with different surface tensions: water (73 mN m^{-1}), glycerol (64 mN m^{-1}), ethyleneglycol (48 mN m^{-1}), bicyclohexyl (33 mN m^{-1}), and nonane (23 mN m^{-1}) ($m \geqslant 3$). The curves were drawn as guides for the eye.

Table 2.
Mode and phase assignments for the OEG specific IR peaks for the present OEG-SAMs in vacuum (cm^{-1}) (in part modified from Valiokas *et al.* [14, 25] and Harder *et al.* [4])

Mode assignments	IR peak positions (cm^{-1})				Phase
	EG_2	EG_4	EG_6	EG_3OMe	
CH_2 symm. stretch			2893		α-helix
CH_2 wag			1349	1350	α-helix
CH_2 twist			1244		α-helix
C-O, C-C stretch	1143	1146	1146^{sh}	1146^{sh}	all-*trans*
			1114	1116^{sh}	α-helix
CH_2 rock, twist			964		α-helix

Notation: sh — shoulder.

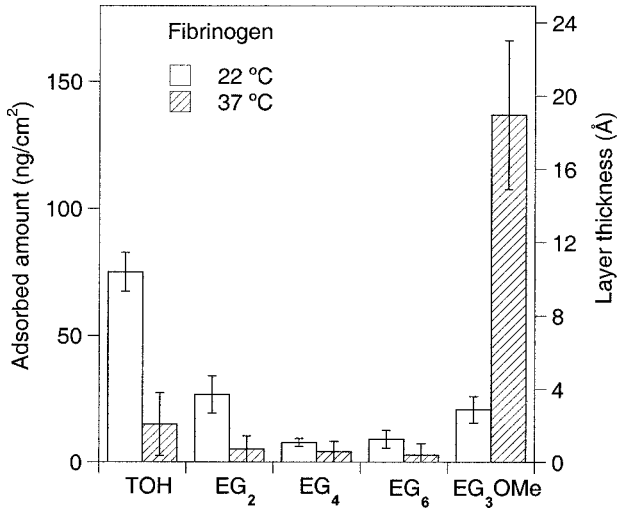

Figure 4. Fibrinogen adsorption onto TOH, EG_3OMe and the OH-terminated OEG-SAMs at 22 and 37°C after 15 min of incubation in 0.4 mg ml^{-1} fibrinogen in VB^{2+} buffer ($m \geqslant 9$).

Surprisingly, when the experiments were undertaken at 37°C *less* fibrinogen remained on the EG and TOH surfaces after rinsing in pure PBS buffer ($p < 0.0005$), whereas *more* fibrinogen remained on the EG_3OMe surface.

Since the EG_n-SAMs showed comparable fibrinogen adsorption at 37°C, the Ca^{2+} dependence of fibrinogen adsorption was studied for the EG_6, TOH, and EG_3OMe surfaces. Diverging adsorption effects were observed for the three buffer systems used, namely PBS buffer (without calcium) for VB^{2+} buffer (with calcium) with or without an extra 1.7 mM $CaCl_2$ added. A significant increase, ~15% ($p < 0.05$), was found for EG_3OMe and TOH surfaces, and a corresponding decrease on EG_6 (not shown). Thus unexpectedly, the presence of Ca^{2+} did not result in a clear cut increase in the adsorption.

3.3. Heparinized plasma

All surfaces adsorbed more plasma at 37 than 22°C ($p \ll 0.0005$ for all SAMs). The amounts varied between 300 and 600 ng cm^{-2} at 37°C, and 40 and 380 ng cm^{-2} at 22°C (Fig. 5). Again, the adsorbed amounts decreased with increasing number of EG units at both temperatures. Note that the effect of temperature was the reverse to that observed for the adsorption of fibrinogen (Fig. 4). All surfaces bound anti-C3c into the plasma layer although the deposition was significantly lower to EG_4 and EG_6 than to the others ($p < 0.0005$, Fig. 6). Upon addition of 10 mM EGTA and 2.5 mM $MgCl_2$ to the plasma, the adsorbed amounts decreased by 50, 85, and 60% on the TOH, EG_6, and EG_3OMe SAMs, respectively (Fig. 7), i.e. a significant calcium dependency for the adsorption of plasma was observed. Only small amounts of anti-C3c bound into these protein layers.

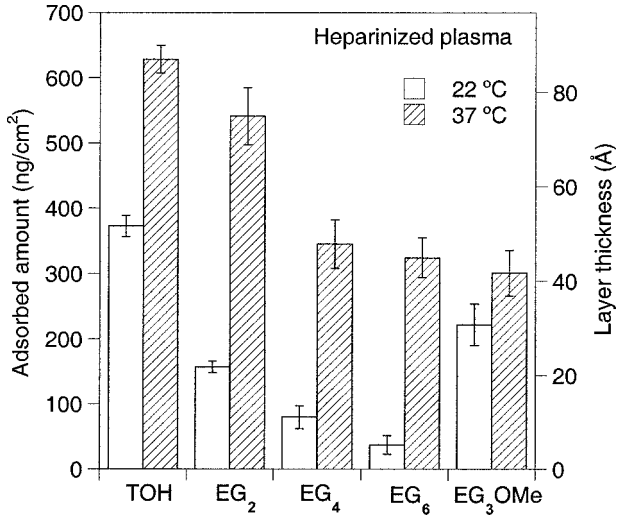

Figure 5. Adsorption of 67% heparin plasma in VB^{2+} buffer to TOH, EG_3OMe and OEG-SAMs at 22 and 37°C after 10 min of incubation ($m \geqslant 9$).

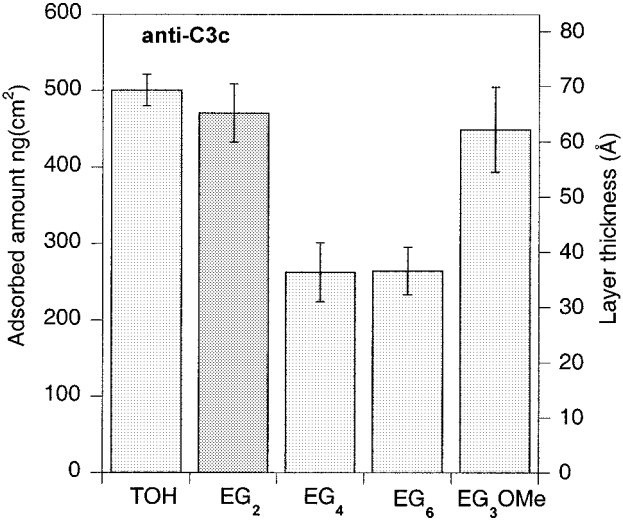

Figure 6. Net binding of polyclonal anti-C3c to the surfaces after 10 min of incubation in heparin plasma at 37°C in VB^{2+} buffer followed by 30 min incubation in antibodies at 1 : 50 dilution in the same buffer ($m \geqslant 9$).

3.4. Serum

Approximately three times more serum adsorbed to hydroxyl terminated OEG-SAMs than to the methoxy-terminated at 37°C. Apparently, the serum deposition was independent of the number of EG units (Fig. 8). The large serum deposition ($\sim$800 ng cm^{-2}) indicates that a humoral system was activated by the surfaces. The comparatively low serum adsorption ($\sim$300 ng cm^{-2}) to EG_3OMe is therefore

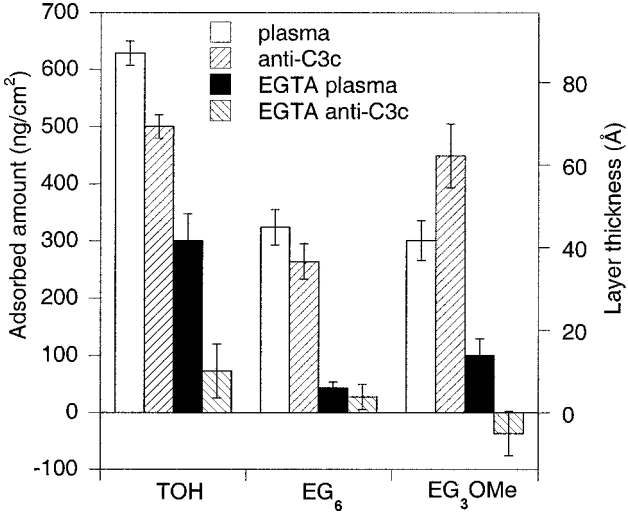

Figure 7. Adsorption of heparin plasma and subsequent binding of anti-C3c onto TOH, EG_6, and EG_3OMe at 37°C after 10 min of incubation in 67% plasma in VB^{2+} or VB buffer with 10 mM EGTA and 2.5 mM $CaCl_2$ added and followed by 30 min incubation in anti-C3c at 1 : 50 dilution in respective buffer ($m \geqslant 9$).

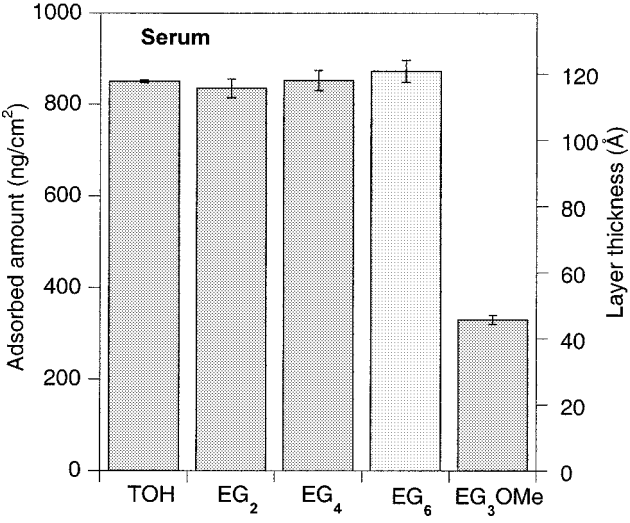

Figure 8. Protein deposition to the surfaces at 37°C after 30 min incubation in 67% human serum in VB^{2+} buffer ($m \geqslant 27$).

interesting and suggests a lower activation of the humoral system by this particular surface.

Similar amounts of anti-C3c bound to all the serum-coated EG_n-terminated surfaces, but significantly more to the EG_3OMe surface ($p < 0.005$, Fig. 9). Also, comparable amounts of anti-C3d bound to all serum-coated surfaces, indicating that

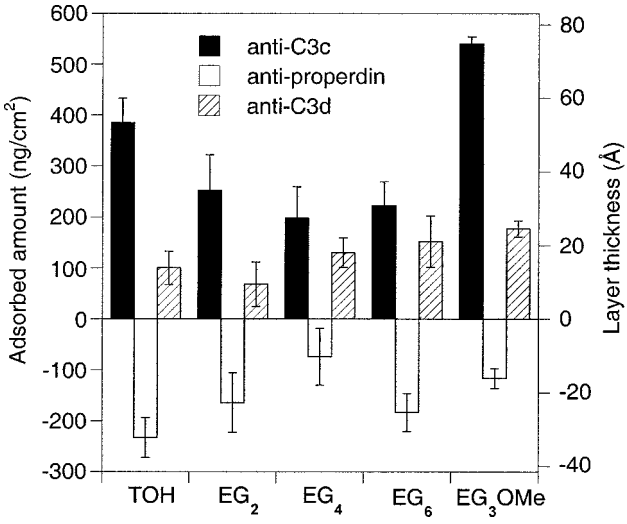

Figure 9. Net anti-C3c, anti-C3d and anti-properdin binding after 30 min of incubation of the surfaces in 67% human serum at 37°C in VB^{2+} buffer ($m \geqslant 9$).

a large fraction of the surface-bound C3b molecules were proteolytically degraded to form C3d during the 30 min incubation in serum.

Large adsorbed protein removal effects were observed on all serum-coated surfaces after incubations in anti-properdin (Fig. 9). Notably, approximately one third of the rinsing effect occurred during the antibody incubation itself and two thirds during the final rinse in buffer (not shown). Properdin was probed for because surface-bound C3 convertases become stabilized by properdin. The active convertases with attached properdin are firmly attached to such surfaces via thioester bindings between C3b and surface nucleophilic groups. In our experiments, as anti-properdin did not bind to the surfaces, the indication is that no functional C3 convertases, and hence no complement activation occurred during the serum incubations.

3.5. Serum after SDS elution

The EG_6 and EG_3OMe serum layers were compared with respect to their SDS serum protein elutability, followed by incubations in anti-C3c (Fig. 10) (since the serum levels on TOH, EG_2, and EG_4 surfaces were similar to that on EG_6 they were excluded). Surfaces that bind proteins strongly should display small protein removal effects in this SDS test. The washing indicated approximately a 15% decrease of the serum layer thickness on EG_6 ($p < 0.01$) and surprisingly a 70% *increase* in the subsequent binding of anti-C3c ($p < 0.01$). Much less serum remained on the EG_3OMe surface (20%, $p \ll 0.0005$) and no anti-C3c bound to this surface after the SDS washing ($p \ll 0.0005$) (Fig. 10). The results indicate a stronger binding of serum proteins to OH terminated EG_6 than to EG_3OMe SAMs, although the mechanisms for this are not understood. The observed increase in anti-C3c binding

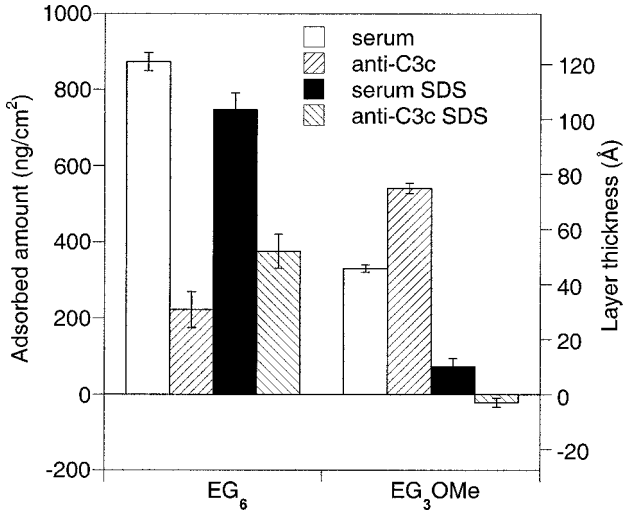

Figure 10. Retention of adsorbed serum proteins and subsequent binding of anti-C3c to EG_6 and EG_3OMe SAMs after incubation (washing) in 10.4 mM SDS in PBS buffer ($m = 9$). The serum and antibody incubations were performed in VB^{2+} buffer.

after the washing of EG_6 was most likely due to removal of loosely associated proteins that otherwise could restrict the access of anti-C3c to adsorbed or bound C3 or its fragments.

4. DISCUSSION

4.1. OEG surfaces

In a recent theoretical work it was suggested that OEG tails in SAMs on gold similar to the ones used here organize themselves to an α-helical conformation thereby possessing energetically-favorable sites that may retain water more strongly than the other conformers [23]. The water-binding capability was introduced as one key interface property for explaining the relatively low protein adsorption from single protein solutions to OEG-containing SAMs [4, 24]. In the present work all the OH-terminated OEG-SAMs displayed similar water contact angles (Fig. 3 and Table 1) suggesting that the OEG tail conformations were very similar in the presence of water. Moreover, it was observed in a recent study that the α-helix conformation of EG_3OMe SAMs disappeared when water was introduced [22]. We lack, however, data that explicitly confirm that the OEG tail conformation for the other OEG-SAMs changes in the presence of water. In yet another study, it was shown that the α-helix conformation shifted towards a more disordered phase when the temperature was increased from 22 to 37°C [25], suggesting that the OEG tail may lose a large portion of its helical content at physiological temperatures and thereby the protein repellent characteristics. Possibly the combination of water

penetration and increased temperature to 37°C efficiently counteract the α-helix conformation of such interfaces. The low contact angles for EG are probably due to structural and chemical similarities between the solvent and EG_n, and this probably also explains the high contact angle for the EG_3OMe SAMs that have a lower H-bonding character.

4.2. Fibrinogen

The present low adsorption of fibrinogen at 22°C is in excellent agreement with other studies although minor differences were observed concerning the adsorption to the EG_3OMe surfaces [4–11] (Fig. 4). This was most likely due to differences in surface preparations, protein incubation protocols, and quantification methods.

The result observed for the EGs and TOH-SAMs can be partly explained by an observed lower rinsing-caused desorption at 22 than 37°C (not shown). The low deposition to the present EGs is in agreement with previous results on similar SAMs [9], but to the best of our knowledge, the high deposition to EG_3OMe at 37°C has not previously been observed. Since EG_6 but not EG_4 exhibited the α-helix conformation in a nitrogen atmosphere at 22°C [14], a lower adsorption onto the former was expected but, surprisingly, similar amounts adsorbed to both. Furthermore this suggests that the α-helix structure of the EG_6 tail was not preserved during the adsorption process or that other mechanisms than molecular helicity determines the adsorption characteristics.

It was recently shown that OEG alkanethiols, similar to the present, and mixed with hydrophobic undecanethiol in SAMs remained protein-resistant in a single protein system [9]. The mixing limit for the preserved protein resistance coincided with the limit for the increase of the water contact angle (approximately 30 deg) [9]. This limit was extended to larger dilutions as the number of EG units increased. It was concluded that the effectiveness by which the OEG tail covered the surface largely determined its repellent properties [9]. It is also known that the OEG tail α-helix conformation may be lost under dry conditions even at moderate dilutions with other thiolated molecules [26]. Thus, these findings suggest that an elevated surface entropy is important for the protein repellence of OEG-SAMs.

4.3. Heparinized plasma

Significantly larger amounts of plasma than fibrinogen adsorbed to all the studied surfaces. Interestingly, when the number of EG units increased from 2 to 6, lower amounts of plasma adsorbed at 22 than 37°C (Fig. 5). The reasons for this are not well understood but may hypothetically be due to increased α-helicity of the EG_6 surface with an increase of the number of tightly bound water molecules in the backbone of the ethylene glycol chain, decrease of Brownian motion of the system, decrease of the surface volume exclusion effect, or combinations thereof. No anti-fibrinogen bound to any of the tested SAMs after plasma incubations at 37°C (data not shown). This is an interesting finding from

a biomaterials point of view since preadsorbed fibrinogen is suggested to amplify the inflammatory response at biomaterials interfaces *in vivo* [27, 28]. Fibrinogen is often deposited from plasma to negatively-charged surfaces but as the present surfaces were charge neutral, neither the adsorption of fibrinogen nor contact activation of coagulation were expected [29]. Also, the accumulation of fibrinogen due to hydrophobic interactions was less likely [30]. The depletion of Ca^{2+} from plasma by EGTA resulted in a large decrease in the total adsorbed amount and the subsequent binding of anti-C3c (Fig. 7). This effect was more prominent on EG_6 and EG_3OMe compared to the TOH surface, suggesting different interactions with the humoral systems [31] and/or Ca^{2+}-chelation properties [32]. In short, it is concluded that many more proteins adsorb from plasma than from a single protein solution of fibrinogen to EG_n surfaces and an increase in temperature and free Ca^{2+} promotes the adsorption.

4.4. Serum

The serum layer on the EG_n surfaces was sixty times thicker than the fibrinogen layer (compare 600 ng cm^{-2} in Fig. 8 with 10 ng cm^{-2} in Fig. 4). The result suggests that different protein–surface interactions take place in single and complex protein solutions. The opsonization of surfaces by complement may compromise surfaces in blood-contacting applications [33] or simply lead to increased protein deposition with poorly understood consequences. Since the complement system is suggested to become activated by OH or NH_2 surface groups (nucleophiles) via the reactive C3b* [34–36], the complement binding to the present surfaces was paid special interest.

Anti-C3c but not anti-properdin deposited onto the serum layers of the present work (Fig. 9). It is, therefore, likely that C3b was bound to the surfaces. C3b then likely formed alternative pathway C3 convertases but these were rapidly down-regulated as properdin, a part of a stabilized native C3 convertase on activator surfaces, was not antibody-detectable [31]. The decrease in the adsorbed amount of protein after anti-properdin incubation was likely due to the removal of antigen–antibody complexes from the protein film or a general detergency effect by the antibodies. Thus is not clear if properdin was present on the surfaces after the serum incubation, although they were definitely not as firmly bound as has been observed at classical complement activator surfaces [31]. The observation that C3d, the degradation product of C3 was formed on the surfaces is a strong indicator that the convertases were downregulated. Also, in a recent study, low C3a plasma levels were observed when serum contacted poly(ethylene oxide) surfaces, indicating a low complement activation by such interfaces [37]. The results are consistent with a low or non-existent binding of anti-properdin to the present plasma or serum-coated OEG-SAMs. Our results also demonstrate the high reactivity followed by a rapid down-regulation of the complement system on most artificial surfaces including EGs. The phenomenon is general and underestimated and originates from the surveillance-like function of the complement system.

4.5. Surface energy and protein adsorption

A previous study with heparinized plasma on methyl/silica gradients suggested a low protein binding strength and contact activation of coagulation at intermediate static water contact angles, between 50 and 70 deg [38]. The present EG_3OMe with a water contact angle close to 60 deg adsorbed significantly less serum than the OH-OEGs and the proteins were easily removed by SDS (Fig. 10). This suggests a low protein binding strength or association to this particular SAM. Thus, the by now classical concept of mixed surface hydrophilicity/hydrophobicity with intermediate surface energy for a minimized biological activity, as described by Baier, still seems also to hold here [39, 40]. The results suggest that surfaces such as the present OEG-SAMs which bind water strongly may not be automatically protein-resistant. It may well be that the least protein-retaining surface is the electrically neutral one that allows some optimal, not maximal, water residence time at physiological temperatures. Finally, it was recently observed that proteins and serum did not bind to rigid brushite crystals which possess a tightly-bound crystal water layer [41]. In this case the water layer was extremely immobile, although the behavior of the surrounding water was not analyzed. The hypothesis of a strong surface water retention for low protein adsorption is still not completely understood.

5. CONCLUSIONS

This study shows that self assembled monolayers with short terminal OEG units bound small amounts of fibrinogen from a single protein solution but large amounts of proteins, among them C3, from serum and plasma. The adsorption of fibrinogen and plasma were temperature-dependent. No correlation was observed between the suggested presence of an α-helix structure in the OEG terminal of the EG-SAMs in the dry state and protein adsorption. We are going to address the influence of water penetrating into helical OEG-SAMs on their protein repellent properties in a subsequent paper.

Washings of the protein-coated surfaces in a SDS solution showed that the proteins were more firmly bound to hexa(EG)-amido-alkanethiol surfaces than to methoxy tri(EG)-ether undecanethiol SAMs, indicating that hydroxyl-terminated SAMs are biologically more active than the methoxy terminated ditto.

The study suggests that it is essential to choose appropriate protein adsorption conditions before conclusions about the protein adsorption properties of materials and surfaces are made.

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