Both Poly(ethylene glycol) and Poly(methyl ethylene phosphate)
Guide Oriented Adsorption of Specific Proteins

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ABSTRACT: Developing new functional biomaterials requires the ability to simultaneously repel unwanted and guide wanted protein adsorption. Here, we systematically interrogate the factors determining the protein adsorption by comparing the behaviors of different polymeric surfaces, poly(ethylene glycol) and a poly(phosphoester), and five different natural proteins. Interestingly we observe that, at densities comparable to those used in nanocarrier functionalization, the same proteins are either adsorbed (fibrinogen, human serum albumin, and transferrin) or repelled (immunoglobulin G and lysozyme) by both polymers. However, when adsorption takes place, the specific surface dictates the amount and orientation of each protein.

INTRODUCTION

The adsorption of biomolecules to specific surfaces is important for biomedical areas as diverse as replacement surgery, pathogen screening, biomolecular sensing, and drug delivery. The need to control, prevent, and/or sense the adsorption of biomolecules to the surface of materials has fueled synthetic efforts to find better materials capable of doing so.1 Different avenues can be followed to this end through the use of biological molecules (biocides), surface patterning (superhydrophobicity), or polymers characterized by high hydration or high exclusion volumes.2 In the biomedical field, nanoparticles have potential application as both drug nanocarriers and biosensors.3 However, despite their potential, both applications often suffer from non-specific protein adsorption at the surface of the nano-object. These non-specifically bound proteins form the so-called protein corona, which can lead to blockage of the binding sites and, consequently, decrease the efficacy or sensitivity.4,5 Thus, a major challenge is the control of the surface properties. In particular, their optimization relies on two major factors: (1) promoting specific binding of the desired detection entity, with a preferential directionality, while (2) preventing non-specific protein adsorption.

On the one hand, specific binding can be favored by the use of both covalent and non-covalent immobilization of proteins at the surface of the biosensors.6,9,10 In addition to immobilization, the binding sites of the proteins need to be accessible for proper targeting.4,9,10 Thus, the orientation of the immobilized proteins at the surface is of paramount importance11 as recently shown, by comparing nanoparticles covered by “favorably” orientated physisorbed proteins with “unfavorably” chemisorbed proteins.4,11 On the other hand, non-specific protein adsorption is commonly reduced by additional protein-repelling functionalization, for instance, using poly(ethylene glycol) (PEG).5,9,10,12 However, even though unspecific protein adsorption is reduced with PEG, it is known that the protein adsorption is not fully prevented at the surface of PEGylated nanoparticles.13 Furthermore, we have recently shown that the PEG packing density controls not only the quantity of adsorbed fibrinogen (Fbg) but also its orientation at the surface.14

Here, we compare the behavior of chemically different polymeric surfaces, PEG and a member of the poly-(phosphoester) (PPE) family, namely, poly(methyl ethylene phosphate) (PMEP). In fact, PPEs have been established as good alternatives to PEG functionalization for nanoparticles to reduce protein adsorption.13,15 We have found that both PEG and PMEP seem to be unable to fully prevent Fbg and albumin adsorption, when used at surface densities commonly used in nanoparticle coatings, but rather promote the formation of a protein corona.14,16 More specifically, we compare and contrast PEG and PMEP in terms of their interactions with proteins (adsorption versus repulsion) and screen for different proteins to understand whether the protein repelling ability resides in the polymers or proteins.

EXPERIMENTAL SECTION

In our experiments, we use model systems consisting of polymers, modified with a long (C18) alkyl chain to render them surface-active, so that monolayers can be formed at the air/aqueous phosphate-
buffered saline (PBS) subphase. The non-ionic surfactants used in this work are schematically shown in panels a and b of Scheme 1 and are a PEG derivative, Lutensol AT50 (BASF Germany), and a PMEP surfactant, which was synthesized and characterized according to refs 16–18, which will be referred to, in the remainder of this work, as C18-PEG and C18-PMEP, respectively. We use Brewster angle microscopy (BAM) (see section S1A of the Supporting Information) and vibrational sum-frequency generation (SFG) spectroscopy to study the conformation and hydration of the polymeric monolayers, as described in ref 16, respectively. To investigate the surfactant–protein interactions, small volumes of concentrated human fibrinogen (Fbg), human serum albumin (HSA), transferrin (Tf), IgG, and Lys from left to right a,b, c.

Scheme 1. Chemical Structures of (a) C18-PEG and (b) C18-PMEP Surfactant and (c) Spacefill Representation of Fbg, HSA, Tf, IgG, and Lys from Left to Right a,b, c.

Table 1. Protein Characteristics

| protein | MW (kDa) | isoelectric point (IEP) | estimated charge at pH 7.4 | PDB code |
|---------|----------|-------------------------|-----------------------------|----------|
| Fbg     | 325      | 5.5                     | −15.2                       | 3GHG     |
| HSA     | 65       | 4.8                     | −16.1                       | 1AO6     |
| Tf      | 75       | 6                       | −3.0                        | 2HAV     |
| IgG     | 150      | 7.5                     | +0.2                        | 1HTG     |
| Lys     | 15       | 11                      | +7.3                        | 1DPX     |

“Approximated. a Calculated with the Protein Calculator, version 3.4 (http://protcalc.sourceforge.net/).

we use a chemically modified cationic human serum albumin (cHSA), obtained as described in ref 19 (see also section S1B of the Supporting Information). To investigate the protein adsorption behavior onto the two different surfactant monolayers C18-PEG and C18-PMEP, we combine surface pressure measurements and SFG. The surface pressure measurements are sensitive to the amount of adsorbed protein at the interface, while SFG spectroscopy is a second-order nonlinear optical spectroscopy, which is surface-selective as a result of symmetry selection rules and has been frequently used to obtain information on the conformation and orientation of proteins at interfaces.20–28 More details about the sample preparation and the experiments can be found below and in section S1 of the Supporting Information.

Monolayer Preparation and Surface Pressure Measurements. The C18-PMEP surfactant and C18-PEG are dissolved in a 9:1 (v/v) mixture of high-purity chloroform (VWR Chemicals) and methanol (VWR Chemicals) to obtain solutions with 50 μM concentration. Subsequently, the surfactants are spread on a PBS (pH 7.4, Sigma-Aldrich) subphase. The mean molecular area per surfactant at the surface for all measurements is ~5 nm² and was calculated by tracking the number of molecules present at the surface and by knowing the total area available to them. For the protein adsorption studies, ~100 μL of highly concentrated solutions of either Fbg (Sigma-Aldrich), HSA (Sigma-Aldrich), Tf (Sigma-Aldrich), IgG (antibodies-online GmbH) or Lys (Roche) dissolved in PBS buffer solution is injected into the subphase to reach a final protein concentration of ~0.1 mg/mL. The proteins and PBS tablets are used as received. During the SFG experiments, the surface pressure is simultaneously monitored during all measurements using a DeltaPi tensiometer (Kibron, Finland).

Vibrational SFG Spectroscopy. For our experiments, we use a SFG setup in reflection geometry to measure the amide I and OH stretch signals at the monolayer/PBS interface in the absence and presence of proteins in the subphase. For the SFG process, we overlap a spectrally tunable broadband femtosecond infrared (IR) pulse in space and time with a spectrally narrow visible (VIS) pulse. The visible pulses at 800 nm wavelength (~40 fs pulse duration) are generated by a regenerative Ti:sapphire amplifier (Spitfire Ace, Spectra Physics, Santa Clara, CA, U.S.A.) with a repetition rate of 1 kHz. A beam splitter divides the VIS beam into two parts. One of them is spectrally narrowed by an etalon (SLS Optics, Ltd.) to a full width at half maximum (fwhm) of ~15 cm⁻¹. The pulse energy of the VIS after the etalon is ~20 μJ. The other part of the VIS beam is used to pump an optical parametric amplifier (TOPAS-C, Spectra Physics, Santa Clara, CA, U.S.A.), which employs an additional difference frequency generation scheme to generate the broadband tunable IR pulses. The IR pulse energies within this work are in the range of ~2.5–5 μJ. Then, both beams are focused onto the monolayer/PBS interface, and the generated SFG signal is collimated with a lens, dispersed with a spectograph (Acton SpectraPro 300i, Princeton Instruments, Trenton, NJ, U.S.A.), and finally collected with an electron-multiplied charge-coupled device camera (Newton EMCCD 971P-BV, Andor Technology, Ltd., U.K.). The polarization state of the IR, VIS, and SFG beam is controlled by polarizers and half-wave plates in the setup. The experimental stage is flushed with nitrogen during all measurements in the amide I and free OH region, to suppress IR absorption from water in the atmosphere. The monolayers are spread on the PBS subphase in a homemade circular Teflon-coated trough (diameter, ~8 cm; volume, ~20 mL). The trough is rotated during the measurements to prevent laser-heating-induced displacement of the surfactants.29 Unless otherwise stated, all spectra are collected in the ssp (s-polarized SFG signal, s-polarized VIS beam, and p-polarized IR beam) polarization combination. The average accumulation time for one SFG spectra is 10 min. Afterward, the spectra are background-subtracted and normalized to account for the spectral shape of the IR beam with a non-resonant reference signal from z-cut quartz. The room temperature for all measurements is controlled to be (22 ± 1) °C.

RESULTS AND DISCUSSION

We prepare monolayers of C18-PEG and C18-PMEP on a PBS subphase using a mean molecular area of 5 nm², comparable to the area per polymeric chain used at the surface of nanoparticles.5,15 Notwithstanding the differences in their chemical structures, in terms of their conformation and hydration, the two guiding parameters determining the protein-repelling ability of the polymers,10–34 PEG and PMEP, seem to be quite similar. In fact, characterization of the monolayers via BAM (ref 16 and sections S1A and S2A of...
the Supporting Information) suggests that, while the C$_{18}$-PEG monolayer appears thicker than the monolayer formed by C$_{18}$-PMEP and even though the packing density for both polymers is expected to be beyond the mushroom-to-brush conformational transition, they are unlikely in a fully extended brush conformation. In fact, in a previous study comparing different PPEs at the same surface density used in this work, PMEP seemed to adopt a more mushroom-like conformation compared to other PPEs.$^{16}$ Also in terms of hydration, the two monolayers show similar characteristics (see section S2B of the Supporting Information for more details). If the polymer parameters would be the only factor determining the adsorption/repulsion of the proteins then, on the basis of the above considerations, one would expect that the two polymers would similarly adsorb or repel indiscriminately all proteins. To verify such a hypothesis, we inject small volumes of different protein solutions into the subphase.

**Adsorption of Specific Proteins.** Figure 1 shows the time evolution of the surface pressure at the C$_{18}$-PMEP and C$_{18}$-PEG monolayers upon protein injection. Fbg, HSA, or Tf induce an increase in the surface pressure over time, suggesting adsorption of these proteins onto both C$_{18}$-PMEP and C$_{18}$-PEG monolayers. Furthermore, IgG and Lys seem to be repelled by both PEG and PMEP because no increase in the surface pressure is observed in the 45 min following the injection. Thus, the ability to adsorb or repel the protein, in this case, does not seem polymer-specific but rather protein-specific. The results obtained on C$_{18}$-PEG monolayers are in line with those observed for nanoparticles, where enrichment in the protein corona of some plasma proteins was observed.$^{13}$ This agreement validates our strategy to model the nanoparticle surface with a planar polymer—aqueous solution interface.

Indeed, various properties of both surface and protein are responsible for their interaction, and they can be divided into geometrical, chemical, and electrical interactions. From the protein side, the protein size can be an important factor, because bigger proteins have, in principle, the ability to form more contacts with the surface and small proteins have the possibility to interdigitate within the polymeric coating; another factor is the sequence of amino acids, because charged amino acids are more amphiphilic and, therefore, usually located on the surface of the protein and, thus, more readily available to interact with the surface. Of course, also, the amino acid sequence, directly affecting the distribution of charges, in the protein has an impact, and proteins close to their isoelectric point might be able to adsorb in higher quantities because of the reduction of the electrostatic interprotein repulsion. On the surface side, relevant properties for the interaction with the protein include the roughness, because it can favor interactions with the proteins by increasing the effective surface area, the chemical composition that determines which groups are available for interaction, and finally, the presence of charges or dipoles at the surface and ions in the aqueous solution that modify the liquid phase near the surface and, in turn, affect the interaction between the protein and the surface.$^{35}$ We attempted to rationalize the commonalities between adsorbed and repelled proteins and set out to check whether size and/or charge could be the determining factors. However, in our experiments, we observe that the smallest protein (Lys) is repelled, whereas the largest protein (Fbg) adsorbs, and no pattern based on size can be highlighted because the second biggest (IgG) and second smallest (HSA) protein invert such a trend. A parameter that better correlates with protein adsorption seems to be the isoelectric point (IEP); negatively charged proteins (IEP < 7.4: Fbg, HSA, and Tf) adsorb, while positively charged proteins (IEP > 7.4: IgG and Lys) are repelled. To further test this hypothesis while keeping other parameters constant, we use a chemically modified chSA.$^{19}$ With this modification, the overall changes to the system are minimal, because only the protein surface is changed. Figure 2 shows the surface pressure measurements after injection of chSA into the subphase of the two different monolayers. As seen, the positively charged chSA adsorbs onto both monolayers. Thus, also, the protein charge does not seem to be the driving factor in protein adsorption onto PEG and PMEP.

The fact that protein adsorption is very complex does not allow us to rule out other contributing factors, such as the structural rigidity of the proteins or finer properties, such as the charge and/or the hydrophobicity/hydrophilicity distribution of specific protein domains, and further investigation on such parameters is needed. What we can conclude at this stage is that, independent of the underlying reason, both surfaces present adsorption of specific proteins, which is the sought after property in the development of biomaterials. It is worth noting that the change in pressure induced by chSA is bigger than that for HSA: ∼9-fold on C$_{18}$-PEG and ∼6-fold for C$_{18}$-PMEP. This might suggest a higher affinity of chSA for the...
two polymeric surfaces. However, because the surface and the molecular weight of the two proteins are different, the adsorbed amounts cannot be directly obtained from the surface pressure measurement.

Amount and Orientation of the Adsorbed Protein.

From Figure 1, it is clear that there is a difference in the affinity of HSA and Tf toward PEG and PMEP and that such a difference is not observed for Fbg. Now, we will investigate whether there is also a difference in the orientation induced by these two surfaces on the adsorbed proteins. We do so by collecting the SFG spectra in the amide I region after injection of the proteins in the subphase of the two monolayers.

Figure 3 shows the SFG spectra before (black) and after (colored) injection of either Fbg, HSA, or Tf into the subphase of the C18-PEG and C18-PMEP monolayers, respectively. Here, the general behavior of the proteins at the two different surfaces is very similar. Without proteins present in the subphase, no distinct peak is present in the amide I region between 1600 and 1700 cm\(^{-1}\) for either monolayer. In the presence of Fbg, HSA, and Tf (see Figure S5 of the Supporting Information for a zoom-in of the spectra), a broad peak around 1660 cm\(^{-1}\) appears, which is assigned to \(\alpha\)-helices in the secondary structure of the proteins. Additionally, in the case of Fbg, a shoulder at around 1690 cm\(^{-1}\) is present, which is assigned to \(\beta\)-sheet structures.\(^{16}\) The presence of the amide I peak in the SFG spectra indicates that Fbg, HSA, and Tf not only adsorb onto the polymers but do so in an ordered manner.

Moreover, despite the thus far observed similarities in their behavior toward protein adsorption, PMEP and PEG present a distinct difference in the overall SFG signal intensity in the amide I region generated by HSA and Fbg. This can have several origins, namely, differences in the (1) amount of adsorbed protein, (2) protein orientation, and (3) protein conformation at the two surfaces or any combination of the factors mentioned above. In the case of HSA and Tf, it seems likely that differences in the amount of adsorbed protein are responsible for the differences in the SFG intensities, as also observed in the surface pressure measurements. For Fbg, on the other hand, the initial surface pressure is comparable for C18-PEG and C18-PMEP (∼10.5 mN/m), as is the relative change (∼2.5 mN/m). This suggests that similar amounts of Fbg adsorb onto both surfaces. Consequently, we conclude that the differences in the SFG signal intensities can only be explained by different orientation and/or conformation of Fbg on the two surfaces. Previous studies on the Fbg adsorption on other hydrophilic surfaces showed that Fbg can adopt various conformations upon adsorption.\(^{36−39}\) However, the most common conformations in all of these studies show a structure very close to the crystalline structure. Moreover, changes in the secondary structure of Fbg upon adsorption onto the polymer surfaces should be reflected in changes of the spectral shape of the amide I SFG signal. This is not observed in our study, where the normalized SFG spectra of Fbg adsorbed onto C18-PEG and C18-PMEP have the same shape (see Figure S6 of the Supporting Information). We thus assume in the following that the Fbg conformation is mainly preserved upon adsorption on the two surfaces.

Insights into the protein orientation at the surface can be obtained through the collection of SFG spectra in different polarization combinations,\(^{40,41}\) even more so when this is combined with calculated spectra.\(^{42,43}\) Details on the calculation of the SFG spectra can be found in section S3 of the Supporting Information. Figure 4 shows the experimental and calculated SFG spectra in the amide I region for Fbg adsorbed on C18-PEG and C18-PMEP in ssp, ppp, and sps polarization combinations, respectively. The calculated SFG spectra (on the basis of the PDB structure) reproduce the spectral shape and center position of the experimental peaks very well, consistently with a preserved protein structure upon adsorption. Moreover, the fitted tilt angles of Fbg with respect to the surface plane indicate that the orientation of Fbg is different for the two surfaces: ∼33 and 0 degrees for the C18-PEG and C18-PMEP monolayers, respectively (see section S3 of the Supporting Information). The change in surface pressure and the comparison of the SFG data and calculations indicate that, in the case of C18-PMEP, Fbg adsorbs parallel to the surface and affects the surface pressure because it “pushes” against the monolayer, while in the case of C18-PEG, Fbg is more likely to intercalate with the polymer. Therefore, we conclude that PEG and PMEP do adsorb Fbg in similar amounts but induce different orientations of this protein at the...
Figure 4. SFG spectra in the amide I region of 0.1 mg/mL Fbg at the (a) C16-PEG and (b) C16-PMEP monolayers in ssp (black diamonds), ppp (red circles), and sps (green squares) polarization combinations. The full lines represent the calculated SFG spectra for Fbg at the C16-PEG and C16-PMEP monolayers, respectively. The mean molecular area for both monolayers is 5 nm².

In conclusion, our results suggest that, for the proteins tested in this work, at surface densities relevant in nanoparticle functionalization, the two polymers of interest, PEG and PMEP, are able to adsorb specific proteins, meaning that proteins are not adsorbed or repelled indiscriminately, but also the two polymers adsorb or repel the same proteins. Furthermore, the specific polymeric surface affects the amount of adsorbed protein, such as in the case of HSA and Tf, or the protein orientation, as observed for Fbg. This finding is important for the development of biomaterial surfaces, because it shows that a single surface functionalization can be sufficient to control the quantity and orientation of specific proteins upon adsorption, while at the same time, it can suppress unspecific adsorption of others.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.9b02275.

Materials and methods, supporting measurements, and calculation of vibrational SFG spectra (PDF)

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Notes

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