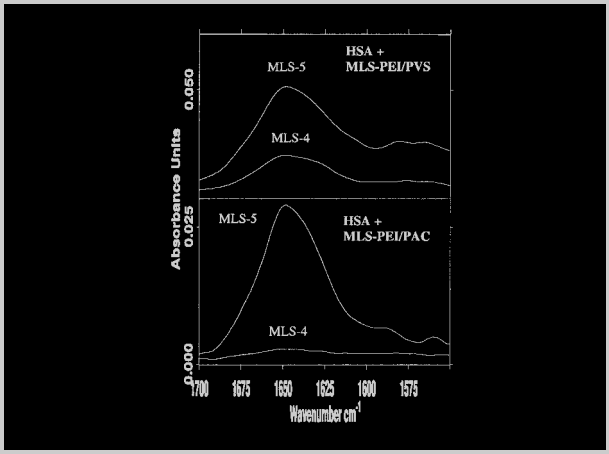
Communication: Protein adsorption was studied by in-situ ATR-FT-IR spectroscopy of consecutively deposited polyelectrolyte multilayer systems terminated either with poly(ethyleneimine) (PEI) or polyanions, such as poly(acrylic acid) (PAC), poly(maleic acid-*co*-propylene) (PMA-P) or poly(vinyl sulfate) (PVS). The influence of the polyanion type, pH and ionic strength was investigated. Negatively charged human serum albumin (HSA) was strongly repelled by multilayers terminated with weak polyanions (PAC, PMA-P), whereas moderate attraction was observed for those terminated with the strong polyanion PVS. Changing the pH from 7.4 to 5 resulted in enhanced HSA adsorption onto PAC-terminated multilayers. An increase in ionic strength diminished the attractive HSA adsorption onto PEI-terminated multilayers. For the PEI/PAC system, the biomedically relevant adsorption of human fibrinogen (FGN) is determined via its isoelectric point in accordance with three other proteins.



ATR-FT-IR spectra of HSA being adsorbed on the two multilayer systems PEI/PAC (bottom) and PEI/PVS (top) for the attractive (MLS-5) and the repulsive case (MLS-4) at pD 7.4 (buffer).

Selective Interaction Between Proteins and the Outermost Surface of Polyelectrolyte Multilayers: Influence of the Polyanion Type, pH and Salt

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Introduction

The surface modification of polymer or inorganic bulk materials that come into contact with biological fluids, is of interest for the food or medical device technology (e.g., membranes, tubing, storage containers), since bioadhesion may alter the surface properties and lower the quality of these materials. One way to generate bioinert surfaces is to block the protein adsorption, since adsorbed protein layers serve as adhesion promotors for the further binding of higher biological functional units, such as cells. Different methods of surface modification have been followed in the past years to obtain protein inert surfaces, among which the creation of hydrophilic surfaces by immobilizing poly(ethylene glycol)[1,2] or the use of self-assembled monolayers consisting of attached oligomeric chains of ethylene glycols (OEG-SAMs)[3] are the most prominent. Other reports include the graft polymerization of ionic and non-ionic monomers onto PET fibers[4] to lower the protein adsorption, and the polymerization of acrylic acid monomers surface-initiated by macromonomers,[5] which also improved the frictional properties of biomaterials (catheters).

We here report on the application of a wet chemical surface modification technology, initiated by Decher,[6] whereby oppositely charged polyelectrolytes (PEL) are alternately adsorbed to form multilayer assemblies, whose surface properties are mainly determined by the topmost layer. There is a growing interest in the application of polyelectrolyte complex (PEC) layers to non-planar geometries, e.g. for microcapsules,[7,8] novel hollow sphere nanoparticles,[9] for the modification of poly(propylene) microfiltration membranes[10] and tissue engineering.[11]

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Since further studies on planar substrates[12–14] have proven that polyelectrolyte multilayer assemblies provide selective charge-driven control of protein interaction according to the choice of either polycation (PC) or polyanion (PA) as the last adsorbed PEL, we were further interested in the influence of parameters which determine the protein adsorption on these charged surfaces. Hence, we varied (i) polyanion type (weak vs strong), (ii) pH value and (iii) ionic strength. We have chosen a 4-layer system (PC/PA/PC/PA) as the repulsive system and a 5-layer system (PC/PA/PC/PA/PC) as the attractive one for interaction with the negatively charged plasma protein human serum albumin (HSA). Previously, we studied the adsorption of three proteins differing in their isoelectric points at repulsive and attractive MLS.[13] Here we also include adsorption data for human fibrinogen (FGN), which might be relevant for studying the thrombogenicity of polyelectrolyte MLS.

Experimental Part

Surface

As model surfaces for the polyelectrolyte multilayer modification, plasma cleaned (plasma chamber PDC-32G, Harrick (distributed by Starna, Pfungstadt), 1 Torr, 2 min, 100 W) internal reflection elements (IRE) of Si ($n_1 = 3.5$) were used.

Polyelectrolyte Multilayers

Branched polycation poly(ethyleneimine) (PEI) (Aldrich, $\overline{M}_w$ = 750000 g/mol); and polyanions poly(maleic acid-*co*-propylene) (PMA-P) (Leuna, $\overline{M}_w$ = 23000 g/mol); poly(acrylic acid) (PAC) (Sigma, $\overline{M}_w$ = 90000 g/mol); and poly(vinylsulfate) (PVS) (Serva, $\overline{M}_w$ = 162000 g/mol) were used without further purification. All the polyelectrolytes were dissolved in deionized water (Millipore, 18.2 MΩ) to a concentration of 10 mmol/l. The multilayers of oppositely charged polyelectrolytes were fabricated by consecutive deposition/rinsing cycles of these unbuffered polyelectrolyte solutions above the Si-IRE in the sample compartment of the ATR-IR sorption cell (IPF Dresden) according to the stream coating procedure described in the literature.[12, 13] After each polyelectrolyte addition, the sorption cell was carefully rinsed with Millipore water.

Protein Adsorption

Human serum albumin (HSA, $\overline{M}_r \approx 66000$) and human fibrinogen (FGN, $\overline{M}_r \approx 340000$) were supplied by Sigma and were dissolved in PBS buffer (Sigma, pH 7.4, 0.001 M phosphate, 0.00027 M KCl, 0.0137 M NaCl). The protein adsorption measurements were performed directly on freshly prepared multilayers in the ATR-IR sorption cell as described elsewhere.[12, 13] Typically, the protein adsorption was followed by IR spectroscopy for 5 h. The sample cell was then rinsed with buffer of given pD values, and additional IR spectra were recorded.

ATR-FT-IR Spectroscopy

The in-situ ATR-FT-IR apparatus for sorption measurements[15] (OPTISPEC, Zürich), consisting of a special mirror setup and the in-situ sorption cell (IPF Dresden) was used on a commercial rapid scan FT-IR spectrometer (IFS 28, BRUKER) equipped with globar® source and MCT detector, as described elsewhere.[13] ATR-FT-IR absorbance spectra were recorded by means of the SBSR (single beam sample reference) method, whereby single channel spectra $I_{S,R}$ were recorded of both the upper (S) and lower (R) half of the Si-IRE ($50 \times 20 \times 2$ mm^3). Above the sample and reference half two liquid chambers are placed (S, R), sealed with O-rings (Viton), which are filled with polyelectrolyte or protein solution (S chamber) and with the solvent (R chamber), respectively. Transforming of the single channel spectra according to $A_{SBSR} = -\log(I_S/I_R)$ resulted in absorbance spectra (A_{SBSR}), which exhibited a proper compensation of the background absorption due to the SiO_x layer, solvent (water, buffer), water vapor (spectrometer) and the ice on the MCT detector window.

Results and Discussion

Reproducibility of Multilayer Deposition

A prerequisite for comparing the adsorption phenomena of the same multilayer system (MLS) is the reproducibility of the deposition procedure, since different samples of the same MLS type have to be used. We checked this by investigating the multilayer growth of, e.g. PEI/PAC, by ATR-FT-IR spectroscopy, using the diagnostic $\nu_{as}(COO^-)$ and ν(C═O) bands of poly(acrylic acid) (PAC) and the ν(OH) band due to desorbed water, as we published previously.[12] In Figure 1A, a typical series of ATR-FT-IR spectra on the stepwise multilayer deposition of the 6-layered PEI/PAC-system (MLS-PEI/PAC-6) is shown. The integrated areas of $\nu(COO^-)$ and ν(OH) absorptions for the consecutive preparation of MLS-4, MLS-5 and MLS-6 of PEI/PAC are plotted against the adsorption step in Figure 1B (for each MLS, two deposition series are shown). Evidently, a rather good reproducibility of all the multilayer preparations was obtained, with a deviation of <5% with respect to the integrated areas, which is sufficient for the direct comparison of protein adsorption data measured for different sample preparations (e.g., sample of MLS-5 compared to MLS-4, or comparison of two samples of the same MLS-X), as referred to in the following sections. Furthermore, it became evident that in the odd numbered multilayer spectra (MLS-3, MLS-5), the ν(C═O) band due to the COOH groups disappeared, suggesting predominant salt bridge formation in the bulk phase of the multilayer.

The reproducibility of preparing both MLS-PEI/PVS (ν_{as}(O═S═O) at about 1250 cm^{-1} was used as the diagnostic band) and MLS-PEI/PMA-P systems ($\nu(COO^-)$ was used as the probe) were similarly satisfactory. These deposition series are not shown here.

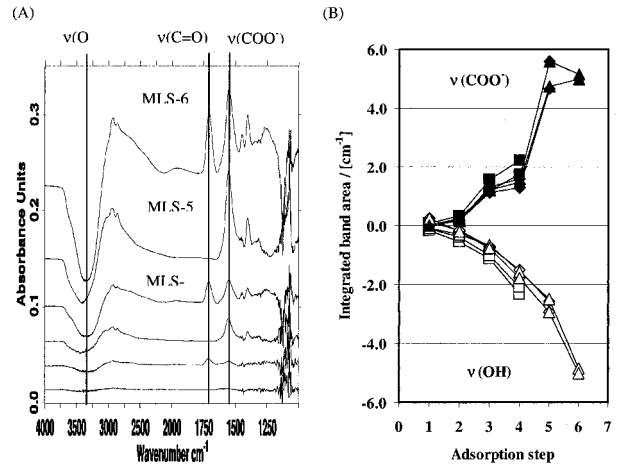

Figure 1. (A) ATR-FT-IR spectra on the consecutive built-up of PEI/PAC multilayers (MLS-1 to MLS-6), which are used as surfaces for protein adsorption experiments. (B) Adsorption step versus integrated areas of the $\nu(COO^-)$ and the $\nu(OH)$ (scaled with a factor of 0.1) band monitoring the multilayer deposition for two samples each of MLS-4, MLS-5 and MLS-6, respectively.

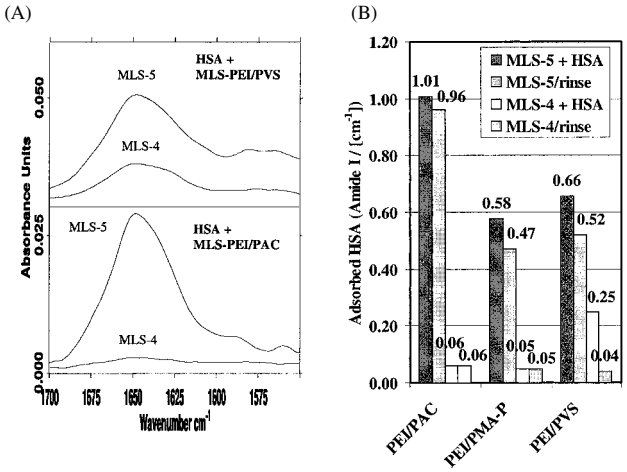

Figure 2. (A) ATR-FT-IR spectra of HSA being adsorbed on the two multilayer systems PEI/PAC (bottom) and PEI/PVS (top) for the attractive (MLS-5) and the repulsive case (MLS-4) at pD 7.4 (buffer). (B) Bar graph of the amide I band integrals; scaling with the adsorbed amounts of HSA ($\int A_{\text{amide I}}$), onto three MLS, containing PEI as the fixed polycation component and PAC, PMA-P, PVS as the variable polyanion components. The given amide I band integrals were measured after an adsorption time of 5 h before (first and third column) and after rinsing with buffer (second and fourth column).

Influence of the Polyanion Type

We studied the three multilayer systems poly(ethyleneimine)/poly(acrylate) (PEI/PAC), PEI/poly(maleic acid-*co*-propylene) (PEI/PMA-P) and PEI/poly(vinylsulfate) (PEI/PVS), all having branched PEI as the polycation component. MLS-PEI/PAC was used as the standard system in previous publications.[12, 13] In Figure 2A, in-situ ATR-FT-IR spectra displaying the adsorption of human serum albumin (HSA) onto the MLS-4 and MLS-5 of PEI/PAC and PEI/PVS are shown in the spectral range of the amide I band. The integrated amide I band area ($\int A_{\text{amide I}}$) is a linear measure for the amount of protein adsorbed.[16] The bar plot of Figure 2B gives the amide I areas, which were measured after an adsorption time of 5 h before and after rinsing with D_2O for the three different odd and even numbered multilayer systems PEI/PAC, PEI/PMA-P and PEI/PVS, respectively. Generally, MLS-PEI/PMA-P showed the same trend as MLS-PEI/PAC: polycation-terminated MLS-5 revealed a very high amount of adsorbed protein compared to the very low protein binding to polyanion terminated MLS-4.[12] This was attributed to either charge attraction or repulsion between the negatively charged HSA (isoelectric point (IEP) of 4.7) and the outermost polycation or polyanion layer, respectively. However, 'repulsive' MLS-4 of PEI/PVS terminated with PVS revealed a significantly higher amount of adsorbed protein ($\int A_{\text{amide I}} = 0.25\ \text{cm}^{-1}$) compared to the MLS-4 of PEI/PAC and PEI/PMA-P terminated either with PAC ($\int A_{\text{amide I}} = 0.06\ \text{cm}^{-1}$) or PMA-P ($\int A_{\text{amide I}} = 0.05\ \text{cm}^{-1}$). This was found reproducibly for all four measured series, in case the protein covered surface was not rinsed with buffer. After rinsing with buffer, we reproducibly observed the same low adsorption levels ($A_{\text{amide I}} = 0.04\ \text{cm}^{-1}$) as for the MLS-4 of PEI/PAC and PEI/PVS, within the error of the experiment. Obviously, an unspecifically and loosely bound adsorption layer was deposited at the outermost PVS layer, which could be removed by rinsing with buffer and could not be observed when PAC or PMA-P were the outermost polyanion layers. If we consider electrostatic repulsion as the main contribution to the interaction between negatively charged HSA and the outermost polyanion layer, this unspecific protein binding was very surprising, since PVS represents a 'strong', fully dissociated polyanion with a higher linear (negative) charge density compared to the 'weak' and incompletely dissociated polyanions PAC and PMA-P. The pK_s values of the amino acid polyanions are listed in Table 1.[17, 18] Hence, there must be some additional contribution to the interaction between HSA and the outermost polyelectrolyte layer. HSA is known to have more than 200 charged amino acids distributed over the whole protein, giving rise to both positively and negatively charged domains[19] made up by basic ($^+NR_4$) and acidic (COO^-) amino acid side chains, respectively, with the acidic ones being in the majority. One may speculate that a strong polyanion, such as PVS, in the outermost layer might orient and bind HSA through patches, which are positively charged, although the net charge of HSA is negative. On the other hand, the topmost PAC (PMA-P) layer in MLS-PEI/PAC (PEI/PMA-P) is charged too weakly to arrange HSA in a similar way, thus 'repelling' the HSA via its negatively charged patches. Certain evi-

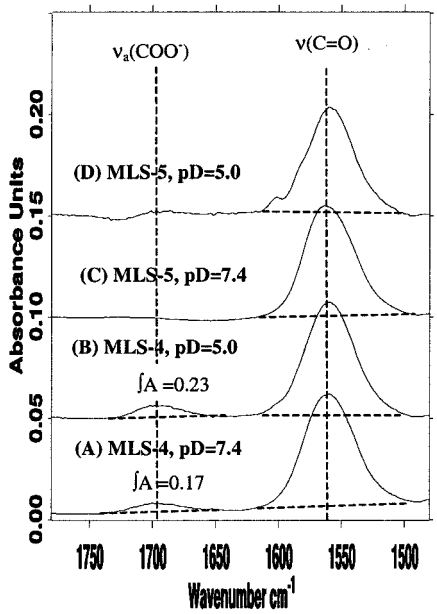

Figure 3. ATR-FT-IR spectra of the PEI/PAC multilayer system MLS-4 in the presence of D_2O at pD 7.4 (A) and pD 5.0 (B) and of the MLS-5 at pD 7.4 and pD 5.0. The spectra (B, C, D) were normalized in that way, that the integral $\int A$ (1610–1490 cm^{-1}) of the $\nu_a(COO^-)$ bands approximately equal ($\Delta\int A = \pm 0.05$ cm^{-1}) the integral of spectrum A, which was $\int A = 2.65$ cm^{-1}.

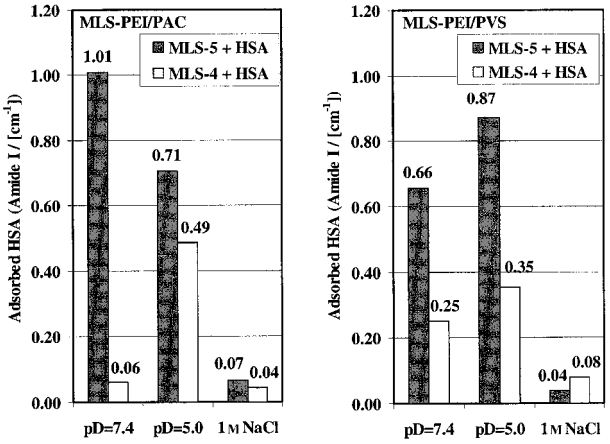

Figure 4. (A) Bar graph of the amounts of HSA adsorbed onto MLS-PEI/PAC-4 and MLS-PEI/PAC-5 at pH 7.4 (left column), at pH 5 (middle column) and in the presence of 1 M NaCl. (B) Bar graph of the amounts of HSA adsorbed onto MLS-PEI/PVS-4 and MLS-PEI/PVS-5 at pH 7.4 (left column), at pH 5 (middle column) and in the presence of 1 M NaCl.

dence for the incomplete dissociation of the carboxylic groups (i.e. minor charge density) of PAC is given by the ATR-FT-IR spectrum of MLS-PEI/PAC-4 at pD 7.4 in Figure 3A, indicating that, obviously, the topmost PAC layer is not completely dissociated. The proof that this spectrum is due to the outermost layer is given by the ATR-FT-IR spectrum of MLS-5 at pD 7.4 (Figure 3C), in which nearly no COOH groups were detected either on the surface or in the bulk zone of MLS.

On the other hand, different induced secondary structures of adsorbed HSA onto the MLS-4 of either PEI/PAC or PEI/PVS could play a role, which might be detectable by means of ATR-FT-IR spectroscopy.[16, 20] We checked this, but the comparison to the HSA conformation adopted at the MLS-4 of PEI/PVS to that at the MLS-4 of PEI/PAC was not possible, since the amide I band was very weak in the latter case (see Figure 2, bottom).

Influence of pH

The dependence of protein adsorption onto multilayer systems on the parameters pH and ionic strength was studied. The first and second column of Figure 4A show data on HSA adsorption onto the PEI/PAC system under neutral (pD 7.4) and acidic (pD 5.0) conditions without rinsing (no significant difference was observed before and after rinsing). Interestingly, the adsorption level for repulsive MLS-PEI/PAC-4 at pD 5.0 ($\int A_{\text{amide I}} = 0.49$ cm^{-1}) was significantly higher than in the neutral case ($\int A_{\text{amide I}} = 0.06$ cm^{-1}). Both HSA and the outermost PAC layer lost negative charge due to protonation under these acidic conditions, causing decreased repulsion. Here adsorption might be governed by hydrophobic interaction.

ATR-FT-IR spectra of MLS-4 consisting of PEI/PAC exposed to pD 7.4 (a) and pD 5.0 (b) are shown in Figure 3. If we define the ratio $Q = \int A_{\nu(C=O)}/\int A_{\nu(COO-)}$ as a measure for the reciprocal dissociation degree, we observe $Q = 0.06$ for pD 7.4 and $Q = 0.09$ for pD 5.0, which means that at pD 5.0, 50% more COOH groups are obtained, resulting in the mentioned loss of surface charge. Furthermore, ATR-FT-IR spectra of MLS-5 of PEI/PAC at pD 7.4 (C) and pD 5.0 (D) are given, respectively. Evidently, at pD 7.4, no ν(C═O) peak was obtained showing that most of the COO^- groups of PAC (surface and bulk) were salt-bridged with the ammonium groups of PEI and could not be protonated. Changing the pD to 5.0 led, therefore, only to a marginal increase of $\int A_{\nu(C=O)}$ within the error.

For the attractive PEI/PAC-MLS-5 at pD 5.0, we observed an adsorption level ($\int A_{\text{amide I}} = 0.71$ cm^{-1}), which is 30% of that under neutral conditions ($\int A_{\text{amide I}} = 1.01$ cm^{-1}). We assume that at pD 5, the net charge of HSA was diminished by protonation of its dissociable amino acid side chains (Asp, Glu), resulting in slightly lower attractive protein/surface interaction, as compared to the case at pD 7.4.

pH-induced adsorption of HSA onto repulsive MLS-PEI/PVS-4, which exposes a strong polyanion, is shown in Figure 4B. We obtained a moderate adsorption level of $\int A_{\text{amide I}} = 0.35$ cm^{-1}, which was slightly higher than that in the neutral case ($\int A_{\text{amide I}} = 0.25$ cm^{-1}). The loss of

Table 1. pK_s values of the polyanions in comparison to those of carboxyl group-containing amino acids of proteins.

Component	pK_s
PAC[17]	≈ 4.5
PMA-P	≈ 4
PVS	≈ 1
Asp, Glu[18]	≈ 4

negative charge caused by HSA led to moderate adsorption as noted above. However, at pD 5.0, HSA adsorption on MLS-PEI/PVS-4 ($\int A_{\text{amide I}} = 0.35\ \text{cm}^{-1}$) was lower than on MLS-PEI/PAC-4 ($\int A_{\text{amide I}} = 0.49\ \text{cm}^{-1}$), which might be due to the fact that, in contrast to the PAC layer, the outermost PVS layer did not loose negative charge due to protonation according to its smaller pK_s value (Table 1).

Influence of Ionic Strength

Generally, increasing ionic strength caused a diminution in charge driven effects with respect to the adsorption level of HSA onto PEI/PAC-MLS and PEI/PVS-MLS, as shown in the third column of Figure 4A and 4B, respectively. The addition of 1 M NaCl resulted in a decrease of HSA adsorbed in the attractive case (outermost PEI layer) by a factor of more than 10 for both MLS. This is due to the screening effect of low-molecular salts (decrease of the Debye length) and further suggests that protein adsorption on these multilayer surfaces is mainly governed by electrostatic interaction.

In the repulsive case (outermost polyanion layer), no difference in HSA adsorption could be observed in the absence versus the presence of salt for both MLS (PEI/PAC and PEI/PVS) within the error of the experiment ($\Delta\int A_{\text{amide I}} = 0.05\ \text{cm}^{-1}$).

Variation of Protein

In addition to the data published in a recent paper,[13] we studied the adsorption of human fibrinogen (FGN) as a relevant protein in the framework of creating non-thrombogenic surfaces. The results obtained for FGN fitted well into a plot,[13] presenting attractive and repulsive protein/surface interaction for the proteins lysozyme, human serum albumin and γ-immunoglobuline (Figure 5). Protein adsorption driven by attractive charge interaction towards the surface leads to high adsorbed amounts and depends on the magnitude of |pH-IEP|. However, in the case of repulsive interaction, the adsorption level was low and no significant correlation between |pH-IEP| and the adsorbed amount could be observed. Hence, it appears that when protein and surface charge are alike, there is no adsorption regardless of the IEP of the protein. Contrarily, in the case of unlike charges between protein and surface, attraction strongly depends on the IEP of the proteins,

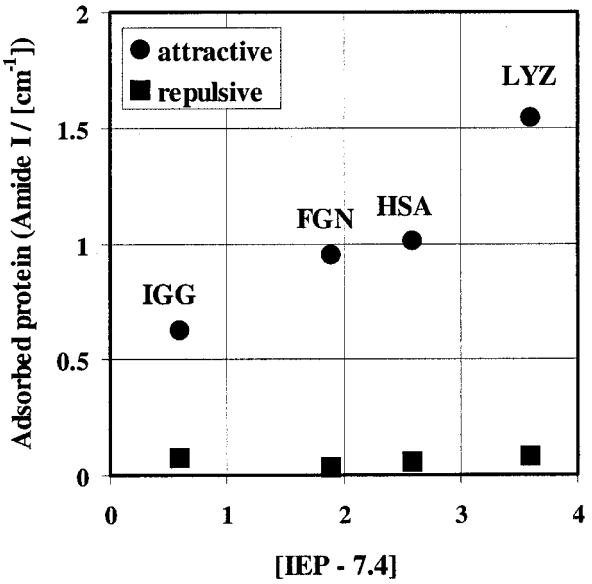

Figure 5. Amounts of FGN, HSA, γ-immunoglobuline (IGG) and lysozyme (LYZ) adsorbed onto MLS-PEI/PAC-4 and MLS-PEI/PAC-5 at pH 7.4 in dependence of the magnitude |pH-IEP|. For additional data, cf. to the literature.[13]

although they are very different in size, shape and amino acid composition.

Furthermore, no secondary structural change of FGN, which could be evidenced from position and shape of the amide I band,[16] could be observed.

Conclusion

In-situ ATR-FT-IR spectroscopy was used to study protein adsorption on four- (MLS-4) and five-layered systems (MLS-5) of branched poly(ethyleneimine) (PEI) alternating with three different polyanions (PAC, PMA-P and PVS). These MLS could be deposited reproducibly, providing well-defined model systems for studying charge-driven protein adsorption, while also showing possibilities of engineering protein resistant surfaces for various biomedical applications. The influence of polyanion structure, pH of the medium and ionic strength on protein adsorption was studied.

There was a strong charge-driven repulsion between negatively charged HSA (IEP = 4.7) with the outermost layer being poly(acrylic acid) (PAC). However, less repulsion was obtained in the case of MLS terminated with poly(vinylsulfate) (PVS). This might be explained in terms of different binding arrangements between HSA and less charged PAC, as compared to the more highly charged PVS in the outermost layer. Changing the pD from 7.4 to 5.0 strongly enhanced HSA adsorption on MLS-PEI/PAC-4 and slightly increased adsorption onto MLS-PEI/PVS-4. This can be explained by the diminished repulsive protein/surface interaction caused by the

loss of negative net charge of both HSA and the repelling outermost polyanion layer as well.

Increasing the ionic strength caused a diminution in HSA adsorption on both MLS-PEI/PAC and MLS-PEI/PVS due to electrostatic screening. This observation, as well as the correlation between the amount of protein adsorbed and the protein's IEP points to a major contribution of electrostatics.

Finally, in comparison to other proteins investigated, FGN was found to adsorb onto the standard MLS-PEI/PAC at the expected level based on its IEP for both the attractive (MLS-5) and repulsive (MLS-4) case. The latter case might be relevant for anti-thrombogenic surface design.

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