Adsorption of Bovine Hemoglobin onto Spherical Polyelectrolyte Brushes Monitored by Small-Angle X-ray Scattering and Fourier Transform Infrared Spectroscopy

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The adsorption of bovine hemoglobin (BHb) onto colloidal spherical polyelectrolyte brushes (SPBs) is studied by a combination of small-angle X-ray scattering (SAXS) and Fourier transform infrared spectroscopy (FTIR). The SPBs consist of a polystyrene core onto which long chains of poly(styrene sulfonic acid) are grafted. Hemoglobin is a tetrameric protein that disassembles at low pH's and high ionic strengths. The protein is embedded into the brush layer composed of strong polyacids. Thus, the protein is subjected to a pH and ionic strength that largely differs from the bulk solution. At low ionic strengths up to 650 mg of BHb per gram of SPB could be immobilized. The analysis of the particles loaded with protein by SAXS demonstrates that the protein enters deeply into the brush. A large fraction of hemoglobin is bound at the surface of the polystyrene core. We attribute this strong affinity to hydrophobic interactions between the protein and the polystyrene core. The other protein molecules are closely correlated with the polyelectrolyte chains. The secondary structure of the protein within the brush was studied by FTIR spectroscopy. The analysis revealed a significant disturbance of the secondary structure of the tetrameric protein. The content of α-helix is significantly lowered compared to the native conformation. Moreover, there is an increase of β-sheet structure as compared to the native conformation. The partial loss of the structural integrity of the hydrophobic protein is due to hydrophobic interactions with the hydrophobic polystyrene core. Hydrophobic interactions with the phenyl groups of the poly(styrene sulfonate) chains influence the secondary structure as well. These findings indicate that changes of the secondary structure play a role in the uptake of hemoglobin into the poly(styrene sulfonate) brushes.

Introduction

The interaction of dissolved proteins with planar and curved surfaces has been one of the most active fields in biotechnological research over the past few decades.[1−7] While the immobilization of proteins, especially enzymes, is required in biotechnological applications, in many cases the adsorption of proteins must be prevented (biofouling).[7] Hence, numerous efforts were made to realize protein-resistant interfaces.[8] In general, protein adsorption is attributed to electrostatic, van der Waals, and hydrophobic interactions.[9] Hydrophobic interactions are often enhanced by surface-induced conformational changes, which may lead to a loss of the native function of the protein.[5,6] A full understanding of all factors that lead to adsorption of a given protein to a given surface is therefore an important goal.

Grafting long polymer chains at one end to a surface opens new avenues for creating smart surfaces.[11] Recently, we have shown that spherical polyelectrolyte brushes (SPBs, Figure 1) consisting of a solid polystyrene core onto which long polyelectrolyte chains are grafted present a new class of carrier particles for proteins and enzymes.[12−16] Adsorption on these SPBs takes place if the ionic strength is low despite the fact that protein and carrier particles are like-charged. However, little or no protein is bound at high ionic strengths.[12,17] The surprising fact that protein molecules are attracted to the like-charged brush layer is ascribed to the polyelectrolyte-mediated protein adsorption (PMPA). The driving forces of the PMPA are charge reversal of the protein and counterion release: The local pH within the brush may be lower than the isoelectric point (pI) of the protein. Hence, the net charge of the protein might be reversed, and a strong attraction between oppositely charged objects becomes operative.[12,18] Second, positively charged patches on the surface of the protein act as multivalent counterions of the polyelectrolyte chains. Thus, a concomitant

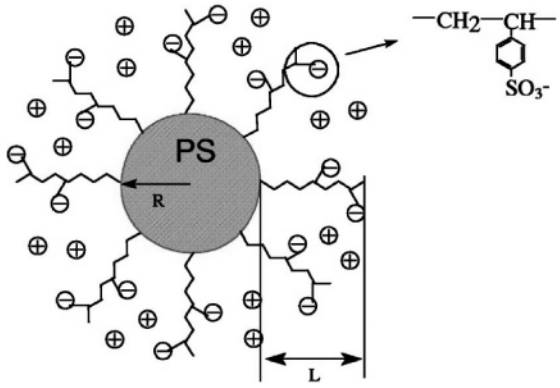

Figure 1. Schematic representation of the spherical polyelectrolyte brushes used in this study. The colloidal particles consist of a polystyrene core of a radius *R* onto which poly(styrene sulfonic acid) chains are densely grafted. The thickness of the brush given by the length *L* depends on the ionic strength in the system.[10]

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number of counterions is thereby released, leading to a gain in entropy of the entire system.[12] A theoretical modeling of the PMPA based on the "counterion release force" is presented in ref 19.

The PMPA is not restricted to spherical brushes. A strong adsorption of proteins onto planar brushes was observed as well.[20−22] Moreover, the PMPA is strongly related to the formation of complexes between free polyelectrolytes and proteins.[12,23,24] Both effects can take place even though the protein and the polyelectrolyte are like-charged. Moreover, both effects are traced back to the presence of oppositely charged patches on the surface of the protein[25] and to the release of counterions.[26]

Hemoglobin and myoglobin play a fundamental role in the absorption, transport, and storage of oxygen in vertebrates.[27] The use of tetrameric hemoglobin as a substitute for red blood cells is of great interest because the supply with fresh blood is limited.[28] However, the direct application of hemoglobin can induce adverse effects. Renal toxicity might be elicited because the protein is rapidly filtered and removed by the kidneys.[29] Moreover, the free protein may cause vasoconstriction because it penetrates through capillary walls and accumulates in muscle cells.[29] To overcome these adverse effects associated with the free protein, much work has been devoted to blood substitutes based on immobilized hemoglobin. These substitutes encompass cross-linked or polymerized hemoglobin, polymer-conjugated hemoglobin, hydrogels, and liposomes entrapping hemoglobin.[30−33]

The PMPA presents a novel promising strategy to obtain hemoglobin carriers. Up to now, the embedding of solely monomeric proteins into polyelectrolyte brushes was studied.[13−15,17,34] Here the uptake of the tetrameric bovine hemoglobin (BHb) is presented. This protein is well-known to disintegrate into its subunits by many factors, such as pH and ionic strength.[27] We consider the uptake into a brush composed of the strong polyacid poly(styrene sulfonate). The counterions of the polyelectrolyte chains contribute to the local ionic strength within the brush.[35] Thus, the ionic strength is significantly higher within the brush compared to the bulk value as long as the concentration of added salt is kept low. Moreover, protons as counterions of the polyelectrolytes lead to a lower pH inside the brush. Hence, if we embed hemoglobin into the microenvironment of the brush, then the quaternary structure of the tetrameric protein may be affected.

To analyze the uptake of hemoglobin into a polyelectrolyte brush in detail, we combine for the first time both small-angle X-ray scattering (SAXS)[14] and Fourier transform infrared spectroscopy (FTIR).[13] FTIR spectroscopy reveals changes in the secondary structure of the protein inside SPBs. SAXS allows us to determine the location of the hemoglobin molecules within the brush. Hence, from the combination of both methods, we obtain the full information about the location of the protein within the brush layer as well as the information about its secondary structure. The combination of all the information leads to an advanced understanding of structural changes that occur during embedding into the microenvironment. This knowledge in turn is central when considering possible future applications of these systems.

Materials and Methods

Materials. Bovine hemoglobin (BHb) was purchased from Fluka (blood, 94 wt % protein content, 0.31 wt % Fe; product no. 51290; lot no. S 13114) and used without further purification. The tetramer has a molecular weight of $M_w = 64\,500$ Da and an isoelectric point (pI) in the range of 6.8−7.0.[36] Its adiabatic compressibility is 1.09×10^{13} cm^2 dyn^{-1}.[37] The molecular weight was verified by SAXS. The density of the native protein was measured with a DMA 60/602 densitometer (Paar, Graz, Austria) to be $\rho_T = 1.30 \pm 0.03$ g/cm^3.

The SPBs have been synthesized as described in ref 10. The SPBs consist of a polystyrene (PS) core onto which poly(styrene sulfonic acid) (PSS) chains are densely grafted by photoemulsion polymerization. Figure 1 displays the investigated brush in a schematic fashion. The grafting density of the polyelectrolyte chains on the PS core is on the order of 0.1 nm^{-2}. The system was purified through extensive ultrafiltration against pure water. Afterward the pH was adjusted to 7.2 by ultrafiltration against aqueous 10 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS, Merck, purity 99%) buffer solution containing 2 mM NaN_3 (Merck, p.A.) to prevent microbial growth. The density of the particles was determined to be $\rho_T = 1.18 \pm 0.02$ g/cm^3. By small-angle X-ray measurements, the radius R of the PS core and the thickness L of the PSS shell were determined to $R = 45$ nm and $L = 57$ nm in the buffer medium. Experimental errors in radius and length are 5% and 26%.

Adsorption Experiments. The adsorption of hemoglobin onto the spherical polyelectrolyte brushes was carried out as described in ref 15. Given amounts of hemoglobin were dissolved in 10 mM MOPS buffer. A suspension of SPBs in MOPS buffer of the same ionic strength is added. All experiments were carried out at pH 7.2. The samples containing 1 wt % SPB were equilibrated for 24 h under gentle stirring at 4 °C. The fraction of non-adsorbed protein was removed by ultrafiltration. It needs to be mentioned that the ultrafiltration is done with a large excess of buffer solution. The amount of non-adsorbed hemoglobin was determined by the extinction of the eluate at a wavelength of 278 nm (Lambda 25, Perkin-Elmer, software UV Win). The amount of adsorbed protein is the outcome of the difference between the protein added to the suspension and the non-adsorbed protein.

Small-Angle X-ray Scattering. SAXS measurements were performed using a home-built Kratky compact camera. Details of the measurements and data treatment can be found in ref 38. The scattering intensities of the solvent $I_{LM}(q)$ and the empty capillary $I_{cap}(q)$ were subtracted from the scattering intensity of each sample $I_{sample}(q)$. The scattering intensity of the pure particles $I(q)$ is given by $I(q) = I_{sample}(q) - (1 - \phi)I_{LM}(q) - \phi I_{cap}(q)$ (where ϕ is the volume fraction of the dispersed particles). All data discussed here represent absolute intensities.

FTIR Spectroscopy. The analysis of the secondary structure of hemoglobin was performed using a FTIR spectroscopy setup developed for protein analytics (Bruker Optik Confocheck). Four solutions of native BHb with concentrations in the range of 5−10 mg/mL were prepared using 10 mM MOPS buffer. For each sample, at least three measurements of 128 scans at 20 kHz were performed as described in ref 13. Minute amounts of the suspension of SPBs loaded with BHb were diluted to obtain protein concentrations in the same range. The concentrations of SPB in these samples were in the range of 1.5−2.5 wt %. The spectra of pure SPB were recorded at a concentration of 2 wt %. All spectra were collected in transmission mode and analyzed via the software OPUS. The derivation of the α-helix and β-sheet contents from the spectra is based on a protein database (Bruker Optik Bruker Protein Library). Cross-validation of the reference spectra contained in the database was done with data taken from the RCSB Protein Data Bank (http://www.pdb.org/). The absolute experimental errors were 4% for α-helix and 3% for β-sheet structure, respectively.[13] Detailed information about the experimental setup and data treatment is given in ref 13.

Results and Discussion

Adsorption of Hemoglobin onto the Polyelectrolyte Brush. Hemoglobin has an isoelectric point (pI) in the range of 6.8−7.0.[36] At the pI the overall electrostatic repulsion among the

Table 1. Protein Adsorption on SPB[a]

method	adsorbed amount of BHb (mg/g SPB)		
UV−vis (total amount)	416 ± 20	540 ± 30	649 ± 30
SAXS (total amount)	414	538	648
SAXS (amount at the PS core)	112	145	149

[a] The amount of adsorbed protein per unit mass of particles is given. The values determined by fitting the SAXS intensities of the particles loaded with BHb are in accordance with the results obtained by UV−vis spectroscopy. In addition, the amount of adsorbed BHb inside the first 2 nm of the brush layer is obtained from SAXS fits.

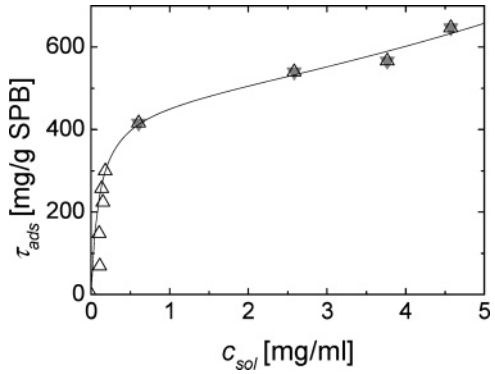

Figure 2. Adsorbed amount of BHb per unit mass of SPB, τ_{ads}, as function of the concentration of protein, c_{sol}, left free in solution. The experimental data (open triangles) is fitted with the expression described in ref 12 (line). The filled symbols represent the samples analyzed by SAXS and FTIR spectroscopy.

protein molecules and the substrate will vanish. Thus, a high amount of bound protein is expected if the adsorption is carried out close to the pI of hemoglobin. Therefore, the adsorption was done in MOPS buffer at pH 7.2. The unbound protein is removed by exhaustive ultrafiltration against the buffer solution. As mentioned above, the amount of bound protein is given as the difference of protein added and the amount of non-adsorbed protein as determined by UV−vis spectroscopy (cf. Table 1). It needs to be noted that no BHb is liberated from the SPB during the ultrafiltration. This points to a nonequilibrium process as discussed in ref 12.

In Figure 2, the amount of absorbed BHb per unit weight of SPB, τ_{ads}, is plotted as function of the concentration of unbound protein in solution, c_{sol}. At low c_{sol} the adsorption is so strong that almost no protein is left in solution. In this case the curve shoots up at the origin and bends over only at high adsorption degrees. Up to 650 mg of BHb per gram of SPB could be immobilized at the given ionic strength of the buffer medium. This large amount of adsorbed protein has to be attributed to several layers of protein within the brush. The increase of the adsorption degree at high c_{sol} values underlines the adsorption into multiple layers inasmuch the large amount of unbound protein promotes further adsorption. In this case proteins may bind to the periphery of the polyelectrolyte layer although the adsorption energy is lower than that of proteins in inner layers. Recently, we could demonstrate that the adsorption can be indeed described using an expression similar to the Brunauer−Emmett−Teller (BET) isotherm derived for the equilibrium adsorption of multilayers.[12,15]

Localization of the Adsorbed Protein. Having discussed the dependence of τ_{ads} as a function of c_{sol}, we now study the location of protein inside the polyelectrolyte brush by SAXS. The scattering intensities are given by $I(q)$. $|q| = (4\pi/\lambda)\sin(\theta/2)$ is the magnitude of the scattering vector, where θ is the scattering angle and λ is the wavelength of radiation. The core−shell morphology of the SPB can be well-analyzed as the contrast of the PS core (7 excess electrons/nm^3) largely differs from the contrast of the PSS brush layer (136 excess electrons/nm^3 for a pure PSS system in water). Moreover, proteins in general exhibit a good contrast in water.[39] For BHb (tetramer in water) the contrast is 85 excess electrons per nm^3. Thus, the distribution of the protein molecules within the brush layer can be derived from the scattering intensities in situ.

The scattering function of the loaded spherical polyelectrolyte brush follows from the excess electron densities of the individual components, SPB and BHb. The experimental intensities were described using the model of a multishell sphere, i.e., a sphere consisting of several subsequent shells.[14] In the following, a SPB particle is modeled as a large sphere with five subsequent shells. The electron density of these shells may be varied to take adsorbed protein into account. In addition, a contribution due to much smaller entities is needed to describe the adsorption of the protein molecules at higher q values. The small entities are homogenously distributed inside the individual shells and modeled as small spheres (cf. Figure 3 right-hand picture). The scattering intensity of these entities corresponds to the scattering of free protein in solution. Further details of the model are discussed in ref 14.

Figure 3 displays the scattering intensities of a SPB with and without adsorbed protein. The calculation is done for the investigated brush loaded with 648 mg of BHb per gram of SPB. In addition, the corresponding intensity of a solution of free protein (same concentration as the adsorbed amount) is given. The intensities of the free protein dominate the scattering of the loaded brush at high q values. Due to the good contrast, the proteins contribute also to the scattering intensity of the loaded SPB at small q values. At low q vectors, there is no resolution with regard to the size of the protein. Thus, the bound BHb increases the electron density of the shells but is not visible as individual molecules (cf. black dotted line in Figure 3). Further details concerning the model are discussed in ref 14.

The scattering intensity of the loaded particles can be described by the following expression[14]

$$I(q) = B_{\text{large}}{}^2(q) + \sum_i B_{\text{small}}{}^2(q) + \sum_i B_{\text{small}}(q)B_{\text{large}}(q)\frac{\sin qd}{qd} + 2\sum_{i\neq j} B_{\text{small}}(q)B_{\text{small}}(q)\frac{\sin qd_{ij}}{qd_{ij}} \approx B_{\text{large}}{}^2(q) + \sum_i B_{\text{small}}{}^2(q) \quad (1)$$

where $B(q)$ denotes the scattering amplitude of the respective small or large spheres. The distance of the protein molecules to the center of PS core of the SPB is given by d. The mutual distances of the adsorbed proteins are denoted as d_{ij}. The first two terms of eq 1 refer to the scattering functions of isolated large (SPB) and small (BHb) spheres, respectively. The third term is the cross-term describing the interference of the BHb molecules with the SPB particles, whereas the fourth term is related to the mutual interference of the protein alone. Only the first and the second terms contribute noticeably to the measured scattering intensity due to the polydispersity of the system and due to the random distribution of the BHb molecules (cf. discussion in ref 14 and Figure 3).

Figure 4 demonstrates the change in the scattering intensity upon adsorption of 648 mg of BHb per gram of SPB. All intensities have been normalized to the weight fraction of the carrier particles. The fits are based on the radial distribution of the excess electron densities shown on the right-hand side of

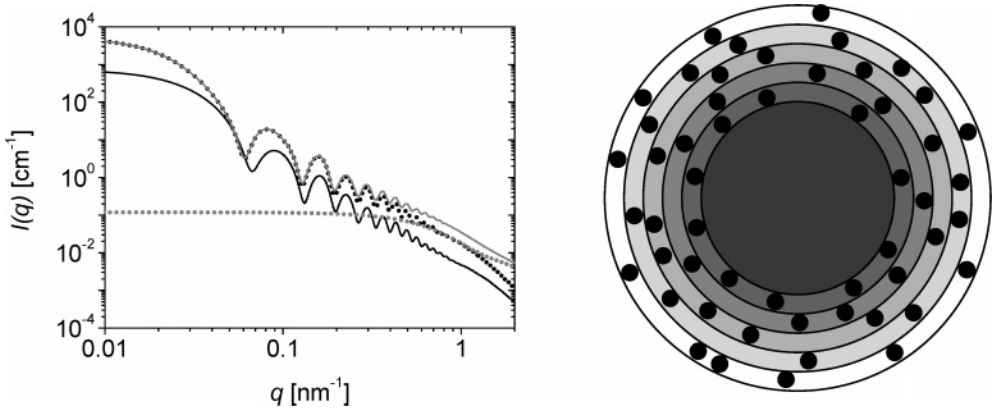

Figure 3. Left-hand panel: SAXS intensities of unloaded SPB (black line) and loaded SPB (gray line) with individual components (dotted lines). The black dotted line refers to the contribution of the brush taking higher electron densities of the individual shells due to the adsorbed protein into account (term 1 of eq 1). The additional contribution due to the smaller entities is given as the gray dotted line (term 2 of eq 1). The geometric parameters of the brush were considered, and the amount of adsorbed BHb was set to the experimental value of 648 mg/g SPB. Right-hand panel: Schematic representation of the multishell sphere model (not to scale) that was used for the calculation of the scattering intensities. Further information is given in the text and in ref 14.

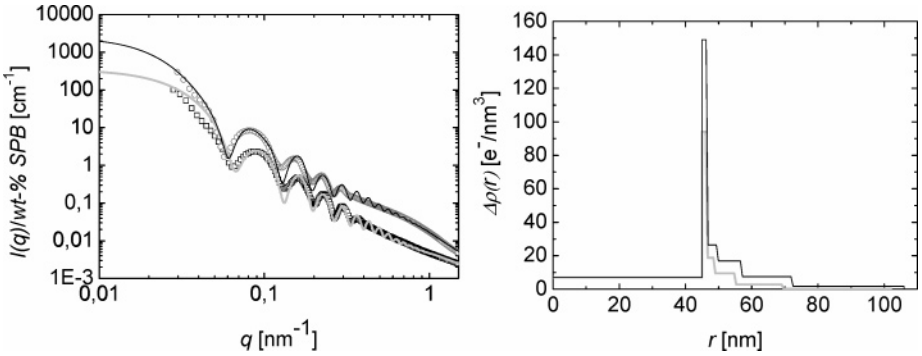

Figure 4. Adsorption of BHb into the SPB. Left-hand panel: Measured scattering intensities (symbols) at different amounts of adsorbed BHb (square, 0 mg BHb/g SPB; circle, 648 mg BHb/g SPB). All intensities are normalized to the weight fraction of the SPB. The respective fits (lines) using the model described recently in ref 14 are based on the radial distribution of the excess electron density of the brush shown on the right (gray line, 0 mg BHb/g SPB; black line, 648 mg BHb/g SPB).

Figure 4. An increase in the overall size of the loaded SPB is monitored due to the uptake of the protein molecules. Upon adsorption of BHb, the maxima of $I(q)$ are increased and shifted to smaller q values due to the increase in electron density in the brush layer. The scattering intensity of a brush loaded with protein is dominated by the scattering contributions of the adsorbed protein molecules at higher q values. To calculate the scattering contribution of the adsorbed BHb at high q values, the proteins are rendered as homogeneous spheres with a radius of 3.6 nm. The model has a restricted resolution at high q values. Hence, small changes of the average radius due to partial denaturation of protein molecules can be neglected.

Figure 5 gives a schematic rendition of the final result derived from the SAXS fits. First of all, the protein BHb enters deeply into the brush. Thus, there is no steric penalty for BHb despite the densely packed polyelectrolyte layer. The amount of adsorbed protein that has been derived from the fit of the SAXS data is in excellent agreement with the amount determined by UV−vis spectroscopy (Table 1). About 30% of the adsorbed amount of hemoglobin is located directly at the surface of the polystyrene core, i.e., within the first 2 nm of the 61-nm-thick brush layer. The radius of gyration of the native BHb (tetramer in solution) is 2.8 nm. For spherical particles, this value corresponds to a radius of 3.6 nm.[40] It should be noted that the thickness of the first protein layer at the surface of the PS core is only 2 nm. Model calculations concerning the accuracy of the determination of the first brush layer are given in the Appendix. It is also shown in the Appendix that an accumulation of protein in outer layers of the brush can be ruled out. The accumulation of protein at the core surface indicates either decomposition of the tetramer into subunits or unfolding of the protein induced by adsorption onto the hydrophobic PS core. This is discussed in the following.

The counterions of the polyelectrolyte chains are strongly confined within the brush.[41] As a consequence, the ionic strength within the polyelectrolyte brush is significantly higher than that in the bulk solution.[10,41] The ionic strength increases with decreasing distance to the polystyrene core due to the spherical geometry of the brushes. Assuming totally stretched chains of the strong polyelectrolyte PSS, the ionic strength inside the first 2 nm of the brush is on the order of 0.4 M. This value is significantly higher than the ionic strength at the periphery of the brush, which is given by the ionic strength of the buffer solution (0.007 M). For the same reason, protons as counterions decrease the pH within the brush compared to the pH of the buffer medium. Native hemoglobin is known to dissociate into dimers or even monomers at low pH's or high ionic strengths.[27,42] No tetramers are found below pH 5[42] and above an ionic strength of 0.5 M.[27] Thus, hemoglobin molecules may indeed dissociate inside the brush close to the core surface.

An additional explanation for the accumulation of BHb at the solid polystyrene core is located in hydrophobic interactions of protein molecules with the core involving surface-induced conformational changes. This is corroborated by the fact that the amount of protein located within a 2 nm distance from the core is very high (cf. Table 1). Calculations based on the radius

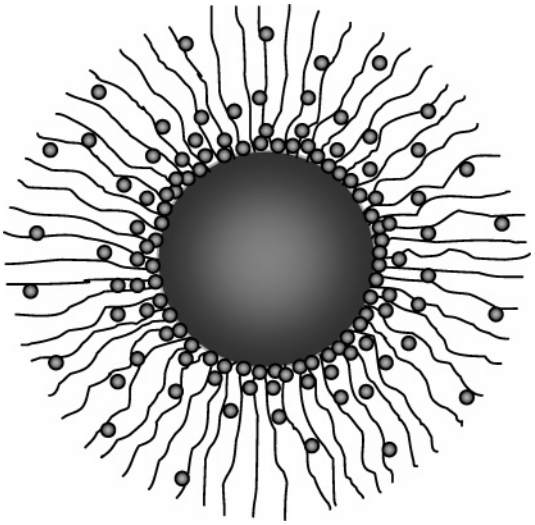

Figure 5. Schematic representation of hemoglobin molecules embedded into the spherical polyelectrolyte brush (PS/PSS) as derived from SAXS measurements (648 mg of BHb per gram of SPB). The size of the SPB is to scale. The illustration of the proteins is based on the radius of gyration of the free protein, which corresponds to a radius of 3.6 nm for tetrameric BHb. However, hemoglobin molecules bound in close proximity to the PS core are disintegrated into subunits because of high ionic strengths and low pH's in inner layers of the brush. See text for further explanation.

of the PS core of 45 nm and spherical protein molecules of radius 3.6 nm predict that 170 mg of protein per gram of PS core are necessary to completely cover the hydrophobic core with hemoglobin. Thus, the amount of BHb in close proximity to the core might refer to protein that is adsorbed onto the PS core due to hydrophobic interactions.

The adsorbed BHb molecules not located at the surface of the core (ca. 70%) are bound in close correlation to the polyelectrolyte chains (cf. Figures 4 and 5). Details concerning the three-dimensional structure of the adsorbed protein molecules cannot be obtained due to the limited resolution. Similar results were obtained for all other samples (cf. Table 1). Thus, the adsorption of BHb onto the SPB can be understood as follows: A fraction of the protein is accumulated onto the hydrophobic PS core, while a second fraction is bound along the polyelectrolyte chains.

These observations for the tetrameric BHb differ from earlier studies for monomeric proteins such as bovine pancreatic ribonuclease A (RNase A) or bovine serum albumin (BSA).[14] Both proteins may enter deeply into a PS/PSS brush as well. However, they are homogeneously distributed along the polyelectrolyte chains.[14] In contrast to BHb, both proteins avoid a direct contact with the hydrophobic PS surface except for high adsorption degrees. For instance, for small amounts of adsorbed RNase A no protein was found within the first 5 nm of the brush layer.[14] Even after adsorption of 500 mg RNase A per gram of SPB, only a few molecules of RNase A are found close to the PS core. However, in the case of the tetramer BHb, which might disintegrate into subunits inside the brush, about 30% of the adsorbed amount is located at the polystyrene core. Thus, in the following the conformation of hemoglobin embedded into the polyelectrolyte brush is studied. This will provide a better understanding of the uptake of the oligomeric protein that is sensitive to decomposition into monomers induced by external stimuli such as ionic strength and pH.

Analysis of the Secondary Structure. Circular dichroism (CD) spectroscopy is a widely used method to detect the secondary structure of proteins. However, this method cannot

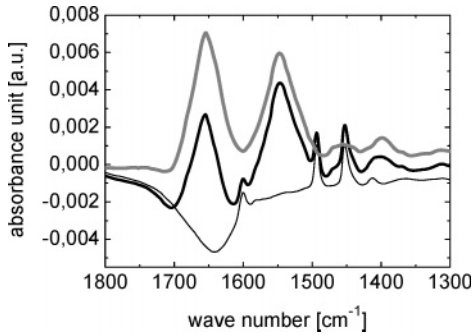

Figure 6. FTIR spectra of SPB before (thin line) and after loading with protein: 538 mg of BHb per gram of SPB (black thick line). The spectrum of adsorbed hemoglobin (gray thick line) is obtained as the difference of the former two. All spectra refer to 1 wt % SPB.

be applied due to the strong absorption and scattering by the SPB.[43] FTIR spectroscopy is also well-suited to analyze the secondary structure of free proteins in solution.[44,45] Moreover, attenuated total reflectance FTIR spectroscopy (ATR-FTIR) can be used to study the secondary structures of proteins bound to planar surfaces. This technique was applied to monitor changes in the secondary structures of proteins embedded into polyelectrolyte multilayers.[46,47] Recently, we showed that in a turbid medium such as the suspension of the SPBs the content of β-sheet and α-helices can be determined by FTIR spectroscopy in transmission mode if high amounts of protein (>400 mg/g SPB) are bound to the carrier particles.[13] A short path length of only 6 μm is used to prevent total absorption by water molecules during the FTIR experiments. As a consequence, quite high concentrations of protein are required to perform the secondary structure analysis.[13] This does not present any limitation in the present case because of the high amounts of hemoglobin bound to the SPB (Figure 2).

Figure 6 shows the FTIR spectra of the SPB before and after loading with hemoglobin. The negative band in the spectra of the bare SPB results from the absence of water at the location of the SPB particles. The signals at 1452 and 1493 cm^{-1} can be traced back to the spherical polyelectrolyte brushes.[13] Subtraction of the two spectra leads to the spectrum of the adsorbed protein, which is dominated by the amide I (1600−1700 cm^{-1}) and amide II (1480−1575 cm^{-1}) bands of the protein. The shape of the amide I band is typical for a helix-rich protein having a maximum at 1656 cm^{-1}. However, the shoulder at 1682 cm^{-1} is a clear indication for the presence of β-sheet structure, which is absent in native hemoglobin.

A comparison of the spectra of the adsorbed hemoglobin with the native protein provides further insight to the conformational changes caused by the interaction with the SPB (Figure 7). The difference in the two protein spectra reveals indeed a significant change in the secondary structure induced by the adsorption. The maxima of the amide I bands are located at 1656 cm^{-1} in both cases. However, the intensity is lower for adsorbed BHb due to a significant loss in α-helix structure during adsorption. This comes along with the formation of β-sheet structure (cf. signals at 1682 and 1636 cm^{-1} of the difference spectra), which is absent in native hemoglobin. In total numbers the helical content decreases from 71% to 34−42% depending on the amount of adsorbed protein. In addition, 10−13% of β-sheet structure is formed upon adsorption. Table 2 summarizes these values and gives further information about parameters influencing the structure of the protein.

These pronounced conformational changes might be ascribed to the low pH or the high ionic strength within the brush composed of a strong polyacid. Control experiments with

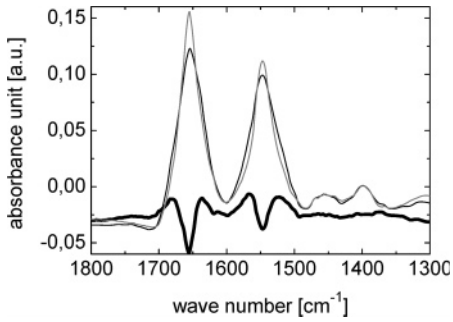

Figure 7. FTIR spectra of native hemoglobin (gray thin line, measured before adsorption) and of adsorbed BHb (black thin line). The IR absorption is plotted in the amide I (1600−1700 cm^{-1}) and amide II region (1480−1575 cm^{-1}). The spectra are scaled to the same concentration of BHb. The difference spectrum of adsorbed and native protein (black thick line) reveals adsorption-induced structural changes.

Table 2. Secondary Structure of Free Hemoglobin and of Hemoglobin Adsorbed onto SPB Consisting of a Polystyrene Core onto Which Linear Chains of Poly(styrene sulfonic acid) Are Grafted[a]

protein	α-helix (%)	β-sheet (%)
native BHb (PDB)	75	0
native BHb (FTIR)	71	0
416 mg BHb/g SPB	34	13
540 mg BHb/g SPB	39	13
566 mg BHb/g SPB	38	11
649 mg BHb/g SPB	42	10

[a] The reference value for native BHb was taken from the RCSB Protein Data Bank (http://www.pdb.org/; PDB code: 1G09). The amounts of adsorbed BHb were determined by UV−vis spectroscopy.

hemoglobin dissolved in MOPS buffer indicate that both the helical and the β-sheet structure remain unaltered as long as the ionic strength does not exceed 0.5 M. Additional control experiments were performed with BHb dissolved in glycin buffer at pH 2.5 to study the influence of pH. These experiments revealed an α-helix content of 60% and 0% β-sheet structure. Thus, there is a slight but significant decrease in α-helix structure at low pH's whereas β-sheet structure is also absent at low pH's. However, it has to be kept in mind that a secondary analysis is not sensitive to the decomposition of the tetramer into monomers. Nonetheless, these experiments demonstrate that the structural changes appearing during adsorption cannot be simply ascribed to the pH and ionic strength within the new microenvironment of the protein.

Hence, the marked structural changes have to be ascribed to the interaction of the BHb with the carrier particles. Hydrophobic interactions with either the PS or the phenyl groups of the PSS chains core might induce such conformational changes. PSS is known to interact with hydrophobic patches of proteins through its hydrophobic backbone.[46,48,49] The careful analysis of the SAXS data clearly demonstrates that there is a large amount of protein bound in very close proximity to the hydrophobic core. Thus, the interaction of BHb with the core surface causes structural changes. Moreover, the protein is enriched at the core surface and thus densely packed. The fraction of β-sheets often increases upon protein association.[50] Thus, "β-aggregation"[51] among the hemoglobin molecules may occur that might explain the formation of β-sheet structure besides the interaction with the hydrophobic surface.

Conformational changes in the secondary structure of hemoglobin take place in a cooperative fashion starting from one subunit to others to optimize the orientations of the binding sites. Such conformational changes can be induced by the contact to the hydrophobic core and interactions with the hydrophobic backbone of PSS as shown by SAXS experiments. In this way the binding energy will be enhanced upon partial unfolding of the protein. The increase in adsorption energy comes along with a loss of the structural integrity of the protein, which should render the adsorption irreversible. Indeed, less than 3% of the adsorbed BHb could be released by an increase of the ionic strength from 7 to 507 mM. This finding has to be related to the fact that 30% of the hemoglobin is bound close to the hydrophobic core (Figure 4) but also to hydrophobic interactions of the other 70% with the hydrophobic backbone of the PSS chains.[48] Otherwise at least the latter fraction should be released at high ionic strengths. Other proteins such as β-lactoglobulin and mEosFP can be almost quantitatively reliberated from SPBs of the same morphology.[13,34] Moreover, previous SAXS experiments could demonstrate that RNase A has no preferential tendency to adsorb in the innermost layer of the PSS brush.[14] Thus, the adsorption of BHb onto the PS/PSS particles is not only related to the counterion release force and charge reversal. Bovine hemoglobin is more hydrophobic than the proteins studied so far.[12] The secondary structure of the adsorbed BHb changes significantly upon adsorption due to hydrophobic interactions. This could be even promoted by the low pH's within the innermost layers of the brush that might decompose the quartary structure into monomers or dimers of hemoglobin so that steric hindrance by the polyelectrolyte layer is reduced. Indeed, SAXS studies showed a preferential binding of hemoglobin to the innermost layer of the brush at the PS surface. The conformational changes induced by hydrophobic interactions present an additional entropic driving force for the adsorption of proteins, which cannot be neglected in the case of hemoglobin.

Conclusion

The adsorption of bovine hemoglobin onto colloidal spherical polyelectrolyte brushes was studied by a combination of SAXS and FTIR spectroscopy. Applying both techniques provided insight on the location of the embedded proteins as well as on their secondary structure within the microenvironment. A preferential accumulation of the protein near the core of the SPB could be demonstrated. Moreover, it was shown that the uptake of hemoglobin into the brush leads to a significant loss in helical structure due to hydrophobic interactions. In addition, a formation of β-sheet has been observed that is ascribed to "β-aggregation". This has to be ascribed to the dense packing of protein molecules near the hydrophobic core revealed by SAXS. Thus, combining both techniques is well-suited to study the embedding of proteins into colloidal polymer nanostructures.

Appendix

In the following, model calculations based on the multishell sphere model described in ref 14 are discussed to testify the accumulation of the protein BHb in the first 2 nm of the brush layer (cf. Figure 4). The amount of adsorbed hemoglobin was set to the experimental value of 648 mg/g SPB (Table 1). The model calculations are accomplished as follows: The scattering intensities are obtained through the contrast of the native tetrameric protein in solution, which is assumed to be equal to the contrast of subunits. The radius of the protein was set to 3.6 nm, and the first model layer was assumed as 2, 3, and 6

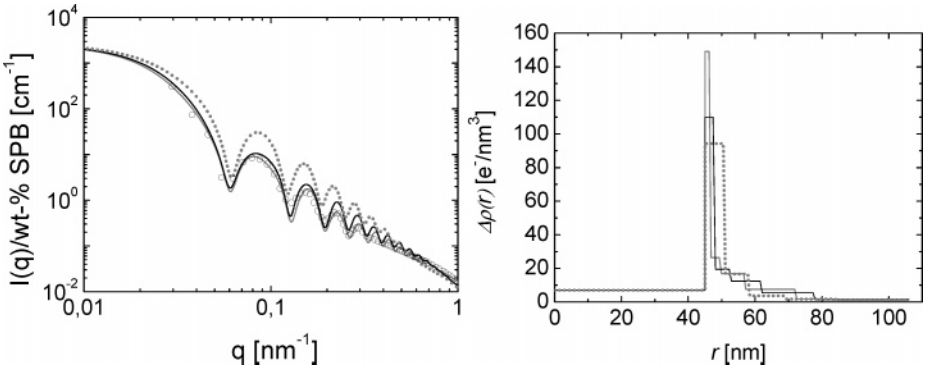

Figure 8. Model calculation. The scattering intensities (left) are based on the distribution of the electron density shown on the right. Only every third experimental data point (circles) is shown for the sake of clarity. In the model calculations different thicknesses of the first model layer were assumed (gray line, 2 nm; black line, 3 nm; gray dots, 6 nm). The amount of adsorbed protein was set to 648 mg of BHb per gram of SPB. Best agreement between experiment and fit of the scattering intensity is obtained assuming a thickness of the first layer of 2 nm. See text for further details.

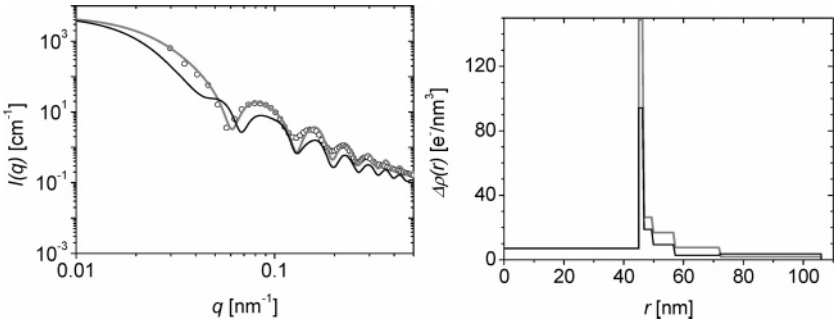

Figure 9. Localization of BHb within the SPB. Circles give the experimental scattering intensity of the SPB loaded with 648 mg of BHb per gram of SPB. The optimal fit of the experimental values (gray line) is taken from Figure 4. The fit obtained for a preferential adsorption of BHb in the outermost parts of the brush shows poor agreement to the experimental data (black line). Hence, this case can be ruled out. See text for further details.

nm (Figure 8). These values are in the range of the size of a BHb tetramer or subunits. The electron density profile was varied until the amount of adsorbed hemoglobin reached the experimental value of 648 ± 25 mg/g SPB.

Figure 8 demonstrates changes in the scattering intensity through variation of the density profile. Evidently, increasing the thickness of the first model layer leads to a significant shift of the maxima of the scattering intensity for $q < 0.4$ nm^{-1}. The small discrepancies in the decay of the intensities at a higher q-range are difficult to detect due to the limited resolution of SAXS. Moreover, additional model calculations where the thickness of the first model layer was varied at constant electron density revealed an overestimation of the amount of bound protein. Thus, this model could be ruled out as well. All data demonstrate that partial disintegration of the native conformation of hemoglobin takes place at the core of the SPB.

Additional model calculations were performed to rule out an accumulation of protein at the outermost layer of the SPB. The calculations were based on the geometric parameters of the unloaded brush. The radius of the protein was set to 3.6 nm, and the amount of adsorbed BHb was fixed to the experimental value of 648 mg per gram of SPB. As a limiting case, all of the protein was assumed to be located in the outermost layer; i.e., the electron densities of the first four layers were kept the same as those for the unloaded SPB. Figure 9 shows the result for this limiting case together with the optimal fit and the experimental data. The fits are based on the radial distribution of the excess electron densities shown on the right-hand side of Figure 9. The increase in intensity and the shift of the maxima is underestimated if preferential adsorption in the outermost part of the brush is assumed. Hence, one can clearly rule out an accumulation of BHb in the outer parts of the brush layer.

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