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Conformational Changes and Competitive Adsorption between Serum Albumin and Hemoglobin on Bioceramic Substrates

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Traditional methods to analyze interactions and conformational changes of proteins adsorbed onto biomaterials are limited by the protein's associations with the substrate material and the complexity of the surrounding media. We have used EPR spectroscopy in combination with site-directed spin labeling (SDSL) to investigate single protein and competitive adsorption kinetics of horse hemoglobin (Hgb) and bovine serum albumin (BSA) on a silica–calcium–phosphate bioceramic substrate. Combined continuous wave and pulsed (DEER) EPR techniques were employed to monitor local mobility/flexibility changes within the proteins and tertiary structure dynamics upon ad-

sorption. An alternate labeling technique was introduced to allow for specific quantification of each protein adsorbed to the bioceramic surface. We show that at buffer pH 7.4 and 4.7 the amount of adsorbed hemoglobin was increased by a factor of 4–5 compared with BSA. The tertiary structure of hemoglobin was strongly affected upon adsorption, leading to a dissociation of the tetrameric molecule into monomers or $\alpha\beta$ dimers. When the bioceramic substrate was previously functionalized with a layer of BSA, dissociation was reduced by 71% compared with the untreated surface, indicating a “primer” effect of BSA for better adhesion of the globular hemoglobin.

1. Introduction

Biocompatibility of bioengineered materials is determined by the extent of cellular attachment, migration, and proliferation taking place at their surface. The very first phenomenon that occurs at the surface of a biomaterial when it encounters body fluids is protein adsorption.^[1–3] Upon adsorption, both the total amount of adsorbed proteins as well as the protein structure are of great importance for the subsequent process of cell attachment, and thus affect the biocompatibility of the bioengineered material.^[1,4] Glass ceramics are biomaterials with excellent biocompatibility and have been successfully used as bone implants. Particularly, calcium phosphate (Ca–P)-based systems have received considerable attention over the past decades because of their ability to form bone-like apatite layers in vivo and to promote osteointegration.^[5] The phenomenon of protein adsorption on the surface of these so-called osteoinduc-

tive bioceramics represents a major step in promoting bone regeneration.^[6]

Despite the growing body of literature on protein adsorption at liquid–solid surfaces, studies on the detailed analysis of protein dynamics and conformational changes following the adsorption process are still lacking. The major issue in elucidating such details is the fact that adsorbed proteins are always associated with the substrate and traditional biophysical and biochemical analysis techniques have limitations in distinguishing between proteins and the substrate.^[7] The adsorbed proteins can be quantified either by detaching them from the surface or by determining the net amount of the protein in solution before and after adsorption. A major drawback of these methods is that the amount of adsorbed protein is usually below the limit of detection.^[1,7] Furthermore, the phenomenon of competitive adsorption of proteins to substrate surfaces under physiological conditions has been less exploited, as it is extremely difficult to characterize adsorbed proteins in complex biological media compared with single protein solutions. One of the methods used to quantify adsorbed proteins on solid surfaces and to study competitive adsorption from mixtures of proteins is radiolabeling.^[7] Although radiolabeling is a powerful method for detecting and quantifying biomolecules, it has the disadvantage of using radiolabeling solutions, which are expensive, tedious to handle, and require precautions to ensure the safety of personnel. In addition, the main information gained by this technique is strictly limited to quantitative analysis.

Spin labeling (SL) in combination with EPR spectroscopy is a potent alternative that provides not only detailed informa-

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tion on structure and conformational dynamics of biomolecules, but also allows for quantitative analysis of adsorbed labeled proteins.^[8–10] EPR spectroscopy is complementary to X-ray crystallography and NMR spectroscopy but has the advantage of being more flexible and widely applicable to investigations of protein structure and conformational changes.^[11,12] The procedure of spin labeling involves modifying the sulfhydryl group of a cysteine residue in the protein structure by a nitroxide reagent. The resulting EPR spectra of the nitroxide spin labels can provide information about the nitroxide mobility,^[13] solvent accessibility,^[10] the polarity of the local microenvironment,^[14] and the distance between two spin labels.^[12]

In this study, we use EPR spectroscopy in combination with IAA (*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide) spin labeling (Figure 1A) to investigate the adsorption of two model proteins, horse hemoglobin (Hgb) and bovine serum albumin (BSA), on the surface of a silica–calcium–phosphate substrate. BSA adapts a fast monomer–dimer equilibrium at pH 5.8, with the dimer being the predominant form under the conditions used in this study.^[15] The molecular weight of the monomer is 67 kDa. Horse hemoglobin forms a tetramer with

a molecular weight of 64.5 kDa. Serum albumin is the most abundant plasma protein ($\approx 60\%$ of plasma proteins) and one of the most relevant proteins that adsorbs on the surface of blood contact biomaterials, reducing the acute inflammatory response to the biomaterial.^[16,17] The butterfly-shaped structure of the BSA dimer (Figure 1B, pdb 4F5S^[18]) exhibits a remarkable stability as a consequence of the 17 disulfide bridges within the protein core.^[19] Earlier EPR studies on BSA spin-labeled at position Cys34 revealed a two-state model for the spin-labeled side chain, indicating that the nitroxide moiety is located either in the less restricting environment of the solvent or tethered in the polypeptide chain.^[20–22] The strong interactions between the nitroxide side chain and the protein backbone observed in the tethered state were explained either by formation of hydrogen bonds between a fraction of IAA spin labels and amino acid residues near the attachment site, or as result of the Cys34 location in a hydrophobic crevice of 9.5–10 Å depth (Figure 1C).^[21–23] In our experiments, we used the IAA spin label to specifically label the cysteine residues located at position 34 of each monomer, which contains a unique free thiol group not involved in a disulfide bridge.

Hemoglobin is a globular, α -helical protein, composed of four polypeptide chains: two alpha subunits (141 amino acids each) and two beta subunits (146 amino acids each) (Figure 1D, pdb 1NS9^[24]). This protein is crucial for all tetrapods to transport oxygen from the lungs to all other tissues, as each protein subunit contains a heme group the four nitrogen atoms of which chelate an iron ion, Fe^{2+} , that can bind oxygen. Horse hemoglobin possesses four native cysteines. Two of them are in position 93 of each beta subunit (further denoted as position β -93) and the other two in position 104 of each alpha subunit. The crystal structure of horse hemoglobin shows that the cysteines of the alpha chains are buried within the protein domains and therefore not accessible for the spin label.^[25] Thus, in the present approach, the cysteine residues from positions β -93 of horse hemoglobin were labeled with the IAA spin label. It has been shown that IAA spin labels attached to position β -93 in horse hemoglobin display two rotameric states with unique micro-environments for each state. One state corresponds to the spin labels that are oriented towards the molecule surface, allowing relatively free reorientational motion of the nitroxide moiety, whereas the other state denotes strongly immobilized nitroxides occupying the tyrosine pocket Tyr HC2(145) β in the C-terminus of the β -chain (Figure 1E).^[25,26] Previous studies with human hemoglobin by Banham et al.^[27] have shown that spin labels tend to adopt a surface exposed conformation in solution, but are positioned in the tyrosine pocket in the frozen state. The distance between the two β -93 native cysteines in human hemoglobin is 25.3 Å, as determined by methanethiosulphonate (MTS) spin labels.^[27] Previous EPR experiments performed by our group monitored the influence of the ionic strength and substrate surface modification on the adsorption of horse hemoglobin. This study revealed a possible partial unfolding of the helix containing the β -93 position after adsorption.^[26]

The combination of spin labeling (SL) with EPR spectroscopy has been traditionally used to explore the structure and dy-

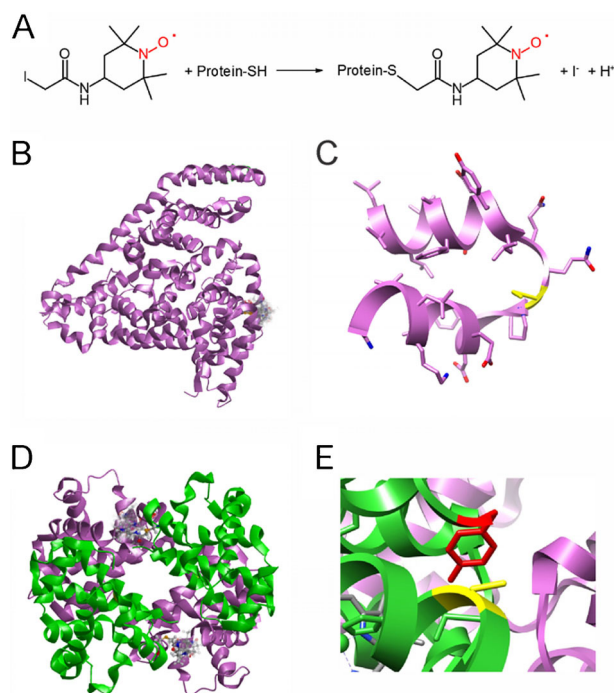


Figure 1. Strategic spin labeling of bovine serum albumin (BSA) and horse hemoglobin. A) Reaction of the IAA spin label with the sulfhydryl group of a cysteine side chain to generate the spin-labeled protein. B) Crystal structure of bovine serum albumin (PDB accession code 4F5S). The 16 possible rotamers of the IAA spin label attached to position 34 are shown as a probability density cloud (populations were calculated for a temperature of 298 K). C) Zoom in to the crevice containing the spin label binding site. The native cysteine at position 34 is colored in yellow. D) Crystal structure of horse hemoglobin (PDB accession code 1NS9). The α chains are colored in purple and the β chains are colored in green. The 16 possible rotamers of the IAA spin label attached to each β -93 position are shown as a probability density cloud (populations were calculated for a temperature of 298 K). E) Close-up view of the spin label binding site. The native cysteine at position β -93 is marked in yellow and the tyrosine β -145 located in the pocket close to the C-terminus of the β -chain is marked in red.

dynamic of proteins in their native or physiological environments. Recently published studies reported that this technique was successfully used for proteins encapsulated or adsorbed onto solid surfaces.^[28–31] In our experiments, continuous-wave (cw) EPR spectroscopy was used to investigate spin label dynamics at room temperature and to identify possible conformational changes induced in the surroundings of the spin label upon adsorption on the Si–Ca–P bioceramic substrate. The amount of adsorbed proteins on the substrate surface was quantified by using the area of the cw-EPR absorption spectrum, which is directly proportional to the spin concentration in the sample. One of the advantages of using this labeling procedure is that it enables the study of a specific protein by following structural and conformational changes within complex biological media or after adsorption. In the present study, the adsorption process was quantitatively monitored as a function of the type of solution used (single protein solution versus mixture of two proteins) and of the pH. Distances between spin labels of different monomers in a dimer (BSA), or between different dimers in a tetramer (hemoglobin) were determined by the double electron–electron resonance (DEER) pulsed EPR method. DEER experiments conducted at temperatures below the glass transition (50 K) allowed quantification of interspin distances in the range 20–80 Å.^[12,32] From the obtained distances, we could extract information about global changes in protein dynamics and tertiary structure. In addition, the relationship between the modulation depth of the DEER data and the average number of interacting spins^[32–34] was used to address the adsorption effect on the proteins' quaternary structure.

2. Results and Discussion

2.1. Spin Labeling of BSA

As position Cys34 is situated in a partially occluded site (i.e. not solvent-accessible), some difficulties were expected concerning the labeling efficiency. Indeed, the spin labels' access to the Cys34 location was found to be limited at 4 °C, and we could achieve a spin labeling efficiency of less than 30% after 24 h incubation. We assume that at this temperature the access to the thiol group inside the crevice is restricted by the more compact local environment. This inconvenience can be overcome by increasing the temperature during the labeling procedure, which could induce transient local unfolding in close vicinity of the Cys34 residue. The crystal structure of BSA revealed that the unique free thiol of Cys34 resides in a partially protected loop situated between two helices (Figure 1C). Previous EPR studies using nitroxide compounds of varying lengths have shown that the hydrophobic crevice surrounding Cys34 is accessible only to compounds with a Stokes radius smaller than approximately 10 Å.^[23,36]

It was previously shown that albumin is very temperature-tolerant and that the effects of heating to 45 °C^[37,38] appear to be fully reversible. However, the influence of a possible change in pH also has to be considered when targeting cysteines for SL, especially because it was shown that up to three additional disulfide bonds are reducible in BSA when changing the buffer

conditions from pH 7 to pH 10.^[19] The Ellman test^[39] performed on BSA kept for up to 96 h at two different temperatures (4 °C and 37 °C) in phosphate buffered saline (PBS) solutions with pH values of 6.8 and 7.4, indicated that the best conditions for labelling only Cys34 were a buffer of pH 6.8 and a temperature of 4 °C (Figure S3 in the Supporting Information), yielding a spin labeling efficiency of 46% after 96 h of incubation.

The cw-EPR spectrum of spin-labeled BSA reveals the coexistence of two spectral components because of the thermodynamic equilibrium between two nitroxide populations with different mobilities. It was previously shown by Anjos et al.^[22] that the strongly immobilized spectral component *I* (Figure 2A) arises from a fraction of the nitroxide population attached to Cys34, which is in contact with the lateral polypeptide chain of the SH group cavity by means of a hydrogen bond formed between the nitroxide oxygen atom and the protein.^[22] The mobile spectral component *M*, characterized by narrow line-widths of the central line, is associated with more flexible nitroxide side chains, which are exposed to the aqueous environment.

Upon increase of the temperature during spin labeling, the equilibrium between the two states is driven towards the

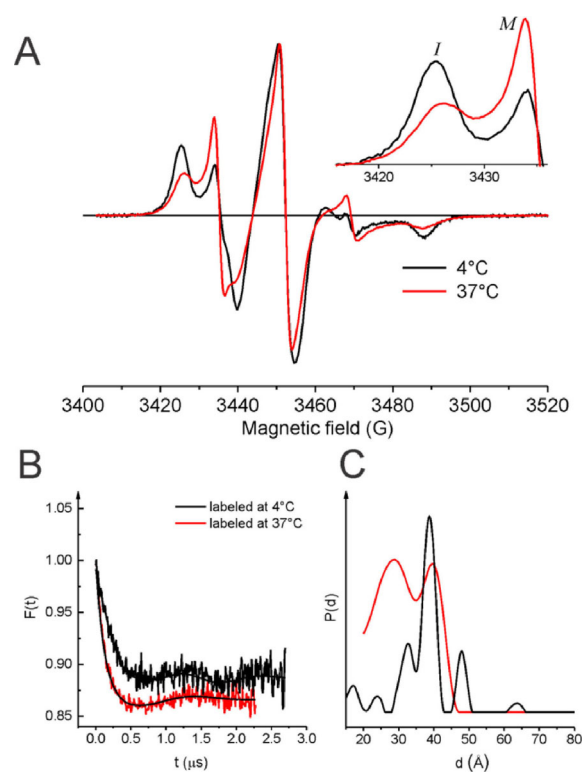


Figure 2. Influence of temperature on cysteine accessibility and spin label mobility in BSA. A) Room-temperature cw-EPR spectra obtained for BSA spin-labeled with IAA at 4 °C (black lines) and 37 °C (red lines). The immobile and mobile components are denoted as *I* and *M*. Inset: the low-field spectral line, showing the shift of the equilibrium in favor of the mobile component upon raising the labeling temperature. B) DEER characterization of BSA spin-labeled with IAA at 4 °C (black lines) and 37 °C (red lines). Data analysis was performed by Tikhonov regularization.^[35] Shown is the background-corrected dipolar evolution data together with the fits of the experimental data. C) Comparison of the distance distributions obtained for BSA spin-labeled at the two temperatures.

mobile *M* component. This observation is consistent with the hypothesis that more than one position is labeled in BSA. Previous work by Hinderberger and co-workers highlighted the similarities between the crystal structure of HSA and the solution structure of BSA.^[40] Their study envisions that the BSA conformational ensemble in solution seems to be less flexible compared with HSA and that the core of both proteins is rather rigid. Considering that the labeling site used in our study (Cys34) is situated in a crevice inside the BSA protein, we expect that this region would be less affected by motional changes of the individual proteins. Thus, we assume that another, more flexible position than Cys34 is labeled as well with the degree of labeling of this position being increased at elevated labeling temperatures. This conclusion is also supported by the distance distributions obtained from the DEER traces (Figure 2B,C), which show two major peaks (at 29 and 40 Å) for the samples labeled at 37 °C and pH 7.4, implying that more than one cysteine is labeled in this case. Additional validation experiments were performed by using the DeerAnalysis2016 software (Figure S4 in the Supporting Information). The resulting data show that the two peaks observed for BSA spin-labeled at 37 °C are not fitting artifacts, that is, variations of the background or the dimensionality do not significantly affect the distance distribution (Figure S4A in the Supporting Information). Only one major peak (at 39 Å) was observed for the sample labeled at pH 6.8 and 4 °C (Figure 2C and Figure S4B in the Supporting Information), which we associate with the distance between the two Cys34 in the BSA dimer. The additional small peaks observed could indicate that even under these labelling conditions additional binding sites per protein monomer may be accessible for spin labeling, however, to lesser extent compared with at 37 °C and pH 7.4.

2.2. Spin Labeling of Horse Hemoglobin

The spectral components visible in the cw-EPR spectrum of horse hemoglobin (Figure 3) arise from two nitroxide popula-

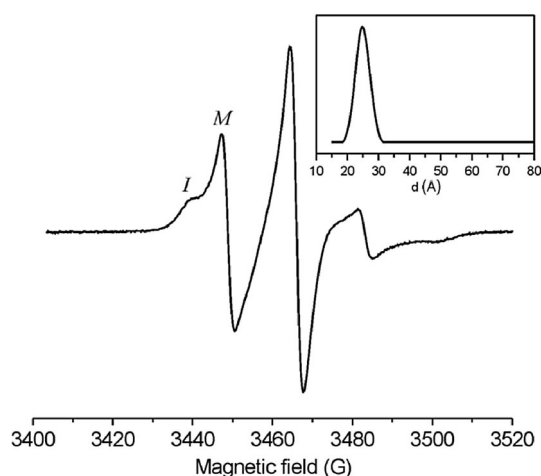


Figure 3. Cw-EPR spectrum and DEER traces of spin-labeled horse hemoglobin. The protein was spin-labeled with IAA at position β -93. Inset: the distance distribution obtained from DEER traces, based on data analysis performed by Tikhonov^[35] regularization.

tions experiencing different environments. Component *I* was found to correspond to a fraction of spin labels trapped in the tyrosine pocket Tyr145 β from the C-terminus of the β -chain (Figure 1E), whereas component *M* is associated with nitroxides oriented towards the surface of the molecule.^[25,26] According to Moffat's interpretation, the spin labels trapped in the protein pocket point towards each other, leading to shorter distances, whereas the surface exposed spin labels point away from each other, leading to longer distances.^[25] Distance measurements performed at 50 K by DEER analysis revealed the presence of a singular major peak at 25 Å, with a standard deviation of 2.3 Å (inset of Figure 3). This value is shorter than the distance calculated by the rotamer library analysis between the two labeled β -93 cysteines in horse hemoglobin, which was found to be 28 Å, but almost equal to the distance between the OH groups of the Tyr145 β residues (24 Å according to the crystal structure^[24]). This suggests that in the frozen state, the spin labels are buried within the protein. This observation is also in good agreement with the results presented by Banham et al.^[27] on human hemoglobin. Its amino acid sequence and structure displays a large homology^[41] to horse hemoglobin, and in the frozen state the spin labels occupy the tyrosine pocket within human hemoglobin, leading to an inter-nitroxide distance of approximately 26 ± 3 Å.

2.3. Protein Adsorption—Spin Label Dynamics

Upon adsorption on the bioceramic (BC) surface, the cw-EPR spectra of both proteins showed shifts of their equilibria towards the immobile component *I*, indicating local conformational changes (Figure 4). The linewidth increase observed in the lower field spectral line for component *I* of both proteins after adsorption suggests that the movement of the entire protein is restricted after interaction with the BC substrate.

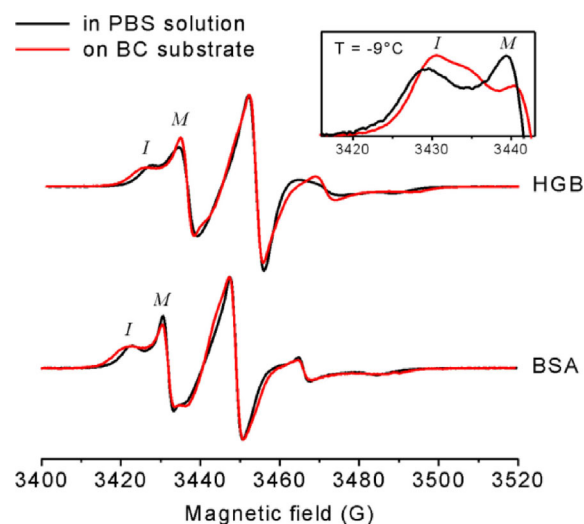


Figure 4. Changes in spin label mobility in HGB and BSA upon adsorption to BC substrate. Proteins were spin-labeled in position β -93* and 34* and recorded in solution (black) and in the adsorbed state (red). Inset: the mobile (*M*) and immobile (*I*) components visible in the lower field spectral lines of the EPR spectrum recorded for horse hemoglobin at -9 °C.

However, the nitroxide spin labels exposed to the aqueous environment exhibit a significantly altered mobility in the two proteins after adsorption.

In the case of BSA, the spectral component M associated with this nitroxide population decreases after adsorption, whereas for hemoglobin it presents smaller linewidths and higher amplitude, reflecting a higher degree of mobility. For human hemoglobin, it has previously been shown by Banham et al.^[27] that, in contrast to the solution-phase EPR where both positions are occupied, upon freezing the preferred spin label conformation is the buried one (into the Tyr145 β tyrosine pocket), corresponding to strong immobilization of the spin label. Therefore, additional cw-EPR experiments were conducted also in the frozen state (-9°C) both in solution and after adsorption. The room-temperature results revealed a higher amplitude of the component M after adsorption on the BC substrate than in solution. This can be a result of the smaller linewidth, resulting from a higher mobility of the M state, but it can also suggest that the fraction of mobile spin labels is higher than in solution, on the account of the restricted ones. A shoulder between the I and M peaks of the spectrum recorded for the BC substrate at -9°C also indicates the presence of less restricted spin-labeled side chains (see inset of Figure 4). This behavior suggests that the structure in the vicinity of position β -93 in hemoglobin changes upon adsorption in a way that would allow for a fraction of spin labels to avoid the tyrosine pocket. As the equilibrium between the two spin label mobility states in horse hemoglobin is strongly affected by the α - β subunit interactions, we assume that these changes are either associated with an opening of the protein domains, or even dissociation of the tetramers into constitutive dimers or monomers, as a consequence of protein-surface interactions. To verify this assumption, possible changes induced at the level of the tertiary structure upon adsorption were investigated by DEER experiments.

2.4. Protein Adsorption—Distances from DEER

For the BSA dimer, the modulation depth undergoes only minor changes upon adsorption on the BC substrate (Figure 5A). As previously discussed, the possibility of two accessible binding sites per protein cannot be excluded. However, the major maximum visible in the distance distribution curve for BSA in PBS solution, which we attributed to the interspin distance in the BSA dimer, appears also after adsorption, but it is shifted by approximately 10 Å towards higher values (Figure 5B). The DEER data validation experiments show that the large distance peak at about 4.7 nm of the sample containing BSA attached on the BC substrate is consistent, even if we change the background and the dimensionality (Figure S4C in the Supporting Information). The local environment of the spin label binding site remains largely unchanged upon adsorption as shown by the cw-EPR spectra (Figure 4), whereas the arrangement of the dimer seems to be altered as evident from the increased interspin distance.

An evaluation of the DEER traces of horse hemoglobin reveals a significant decrease of the modulation depth upon ad-

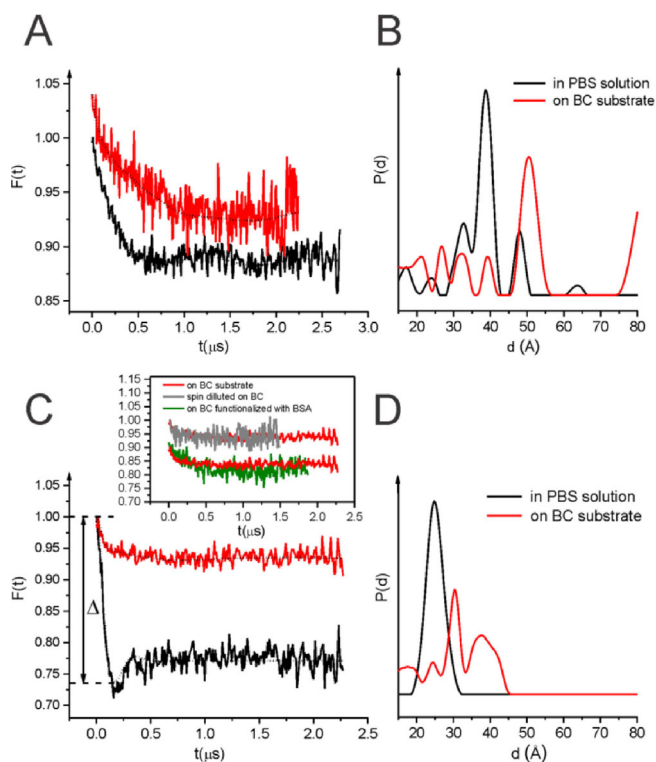


Figure 5. DEER measurements of spin-labeled BSA and horse hemoglobin before (black) and after adsorption (red) on the BC substrate. Data analysis was performed by Tikhonov regularization.^[35] A) Background-corrected dipolar evolution data together with the fits of the experimental data for BSA. B) Comparison of the derived distance distributions for BSA. C) Background-corrected dipolar evolution data together with the fits of the experimental data for hemoglobin. Inset: DEER dipolar evolution time traces recorded for hemoglobin (red) and spin diluted hemoglobin (grey) attached on BC. The green line displays the DEER dipolar evolution time traces recorded for hemoglobin attached on the BC substrate previously functionalized with unlabeled BSA. D) Comparison of the derived distance distributions. All distance distributions were area normalized.

sorption on the BC (Figure 5C,D). This behavior is associated with a decreased number of dipolar coupled spin pairs and can be explained either by dissociation occurring for most of the hemoglobin molecules involved in the adsorption process, or by an extremely crowded protein layer on the BC surface, leading to homogenous intermolecular spin-spin interactions between the spin labels. Control DEER experiments conducted with spin-diluted hemoglobin (inset of Figure 5C) exhibited the same behavior, suggesting that the agglomeration of protein molecules on the BC surface cannot account for the low intramolecular spin-spin interaction observed in our experiments. Consequently, we have to interpret the low number of interacting spin pairs to be a consequence of protein dissociation on the BC surface. A similar dissociation has been observed at extreme pH values (below 6.5 and above 8.0), or for low sample concentrations ($< 100 \mu\text{M}$).^[42]

Pulse EPR distance measurements also provide information about the number of interacting spins, which can further be used for structure determination. The modulation depth of the background-corrected DEER time-domain data is directly related to the number of interacting spins per protein molecule,^[33]

providing thus valuable information on the proteins' quaternary structure. Other parameters, such as spin labeling efficiency or experimental conditions, can also influence the modulation depth of the DEER signal. This inconvenience was overcome in our study by keeping constant all the experimental conditions from our DEER experiments and by preparing for each protein only one sample of spin-labeled solution that was further used both in solution and adsorption studies. The modulation depth values obtained from the DEER experiments of our two proteins in different media are presented in Table 1.

Protein	State	Δ
BSA	in PBS solution	0.12
	adsorbed on BC	0.11
Horse hemoglobin	in PBS solution	0.26
	adsorbed on BC	0.05
	adsorbed on BC coated with BSA	0.09

The modulation depth of the DEER signal is significantly lowered in horse hemoglobin upon adsorption on the bare BC substrate, indicating that the quaternary structure of hemoglobin is strongly affected by the protein–substrate interaction: most of the hemoglobin tetramers dissociate as a consequence of the adsorption process. This effect could also be observed by cw-EPR analysis in the frozen state, indicating a conformational change of this protein upon adsorption on the BC surface. A partial unfolding of the hemoglobin molecule upon adsorption is reflected in the respective distance distribution curve showing distance peaks centered at 30 and 43 Å for the molecules that did not seem to have dissociated. This indicates that after adsorption the tetrameric structure adopts a conformation in which the two β chains are more separated as a consequence of the interactions between protein and BC surface.

The four subunits in hemoglobin are held together in the globular tetramer configuration by non-covalent bonds. The small modulation depth observed for the spin labels bound to position β -93 suggests that hemoglobin dissociation from tetramer to dimer can only result in the formation of $\alpha\beta$ dimers but not of $\alpha_1\alpha_2$ or $\beta_1\beta_2$ dimers. This means that dissociation happens either at the $\alpha_1\beta_2/\alpha_2\beta_1$ or at the $\alpha_1\beta_1/\alpha_2\beta_2$ sliding interfaces. The α and β subunits interact along the $\alpha_1\beta_1$ ($\alpha_2\beta_2$) packing interface by forming tight contacts involving approximately 34 residues, whereas the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) sliding interface involves only about 19 residues.^[42] The formation of $\alpha_1\beta_1$ and $\alpha_2\beta_2$ is therefore more likely.^[42,43] We tried to reduce the number of dissociated hemoglobin dimers by using an organic layer as a buffer between the substrate and the proteins. For this, the BC substrate was first coated with unlabeled BSA molecules and then dried at room temperature for 24 h prior to hemoglobin adsorption. The DEER experiments conducted on this sample revealed a slight increase in the modulation depth (see inset of Figure 5C and Table 1) and, implicitly, less dissociated dimers compared with hemoglobin on the BC alone. It

seems that the BSA layer coated onto the BC substrate directly facilitates the binding of hemoglobin to BSA and thus indirectly to the BC substrate. Thereby, the intermediate BSA layer reduces the impact of the adsorption process on the hemoglobin's tertiary and quaternary structure.

2.5. Competitive Adsorption—Quantitative EPR Spectroscopy

An interesting aspect of protein adsorption onto bioceramics is the "Vroman effect", which consists of simultaneous and competitive adsorption between two or more distinct protein molecules on the substrate surface. Protein concentration, size, shape, and biochemistry are the most important factors influencing the competitive adsorption process.^[44,45] However, in the case of protein–bioceramic interactions, their mutual affinity is driven by electrostatic interactions,^[1] with the net charge of the protein and bioceramics surface having a major impact on the amount of adsorbed protein.^[46,47] In the present study, a competitive adsorption study was carried out at two different pH values: 7.4 (physiological pH) and 4.7 (corresponding to the isoelectric point of BSA). Both proteins had equal concentrations in the multicomponent protein solutions used for this purpose (see Experimental Section). The results show that from both single and double component solutions the amount of irreversibly attached hemoglobin is higher than that of BSA (Figure 6). This suggests that hemoglobin has a higher affinity towards the substrate surface than BSA. Dissociation of hemoglobin oligomers upon adsorption could also contribute to this observation, as the smaller sized protein monomers can easily adapt to surface irregularities.

Concerning the influence of pH, Figure 6 shows that both proteins attach to the BC substrate preferably at lower pH. At pH 4.7 the single protein adsorption increases by a factor of 3

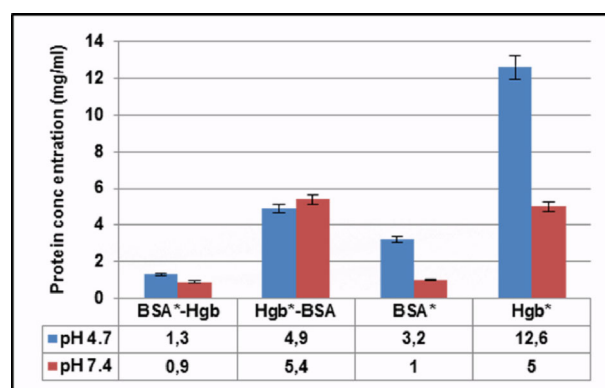


Figure 6. Concentrations of spin-labeled horse hemoglobin and BSA adsorbed onto the BC substrate from one- and two-component solutions at pH 4.7 and 7.4. The symbol * denotes the labeled protein in the mixture (see Experimental Section). Each protein concentration value obtained within the competitive adsorption study represents the mean of three independent measurements. Each protein concentration obtained within the adsorption study is the mean value of three independently prepared and measured experiments. Errors are estimated to be $\pm 5\%$ owing to experimental settings and uncertainties in baseline subtraction.

(BSA) or 2.5 (hemoglobin) relative to the adsorption at physiological pH (7.4). This was expected as silicate glass surfaces in water have been shown to gain a negative surface charge density, primarily through the dissociation of terminal silanol groups.^[48] As the pH decreases, the positively charged surface of both proteins increases, leading to increased electrostatic interaction with the negatively charged surface. BSA has a pI of 4.7 and horse hemoglobin pI = 6.84, therefore both are expected to be positively charged at pH 4.7, but BSA to a lesser extent. However, in the two-component solutions, BSA shows strong adsorption at pH 4.7 (corresponding to its isoelectric point) competing thus with the high adsorption rate of hemoglobin at this pH. This can explain the decreased amount of adsorbed hemoglobin at pH 4.7 compared with the amount attached at pH 7.4, in the double component solutions. At pH 7.4, however, both proteins are expected to be negatively charged with hemoglobin charged to a lesser extent, leading to decreased interaction with the negatively charged substrate surface. By following the same reasoning, in the two-component solutions at physiological pH (7.4) the amount of hemoglobin adsorbed to the BC substrate is enhanced by a factor of 5 relative to the amount of BSA. Interestingly, at this pH the extent of adsorption of protein from the single component solutions relative to the two-component protein mixture is identical (0.9 mg mL⁻¹ versus 1.0 mg mL⁻¹ for BSA and 5 mg mL⁻¹ versus 5.4 mg mL⁻¹ for hemoglobin).

3. Conclusions

EPR spectroscopy and site-directed spin labeling were employed to explore the interaction between two plasma proteins (horse hemoglobin and bovine serum albumin) with the surface of a bioceramic substrate in terms of quantitative analysis and conformational changes.

The quantitative analysis performed to evaluate the competitive adsorption between the two proteins on the bioceramic substrate showed that the amount of adsorbed hemoglobin is considerably higher than that of BSA independent of the pH of the buffer solution. The results suggest that hemoglobin has more anchoring points towards the BC substrate surface than BSA, and dissociation of hemoglobin in smaller subunits may facilitate the adsorption onto the porous bioceramic surface, for example, by increasing the surface of interacting residues. The solution pH and overall surface charge of protein and bioceramic substrate are important determinants in the adsorption process as they influence both the electrostatic interactions and hydrogen bonds formed between the proteins and the surface. At pH 4.7, both proteins showed the highest adsorption to the bioceramic substrate surface owing to the increase in positive protein surface charge, resulting in stronger electrostatic interactions with the negatively charged surface of the substrate.

The conformations of the two proteins are differently affected by the adsorption process. In the case of BSA, the possibility of having two binding sites for spin labels within the monomer prevented a clear conclusion regarding the structural changes induced in the protein. The ≈ 11 Å shift of the dis-

tribution towards higher values suggests that the BSA dimer adopts a more extended conformation upon adsorption. For horse hemoglobin, the results indicate that most of the globular molecules dissociate into monomers or $\alpha\beta$ dimers upon adsorption. This dissociation can be overcome to some extent if the substrate has been previously coated with an intermediate protein layer (here: BSA) as coating for the substrate surface. Covering the BC surface with a BSA layer seems to act as a molecular "primer" that helps preserve the native oligomeric structure of horse hemoglobin while mediating strong adsorption to the bioceramic surface. By using this priming technique, we observed a 71% decrease in dissociated hemoglobin molecules compared with the untreated surface. More detailed investigations of these aspects need to be performed in future studies to clarify the influence of such protein buffer layers on the interaction between proteins and solid substrates.

Experimental Section

If not noted otherwise, chemicals were obtained from Sigma-Aldrich (Munich, Germany). All pH values were determined at 20 °C.

Bioceramic Substrate Preparation

The 60SiO₂:30CaO×10P₂O₅ (mol%) bioactive glass used as substrate was prepared by using a sol-gel method. As starting reagents, tetraethylorthosilicate (TEOS), calcium nitrate Ca(NO₃)₂·4H₂O, and triethyl phosphate (TEP) were used. The sol was prepared by stirring TEOS in water and alcohol in a 1:2:1 weight ratio catalyzed by HNO₃ and heated at 80 °C for about 30 min in a closed receptacle to achieve complete hydrolysis without lowering the pH to below 3.5. Ca(NO₃)₂·4H₂O and TEP previously dissolved in water were then added to and stirred together for another 30 min and then left to gel at room temperature. The sample was dried at 110 °C for several hours and further heat treated at 550 °C for 2 h. The heat treatment was performed according to thermogravimetric/differential thermal analysis (TG/DTA) analysis results (Figure S1 in the Supporting Information) to remove any unreacted organic components and to stabilize the glass network. The resulting sample was milled to a powder and then sieved to obtain a maximum grain size of 40 μm. The specific textural and morphological characteristics of the bioceramic (BC) substrate are presented in Figure S2 (in the Supporting Information).

Spin Labeling

BSA: Bovine serum albumin solution (100 μM) was prepared from lyophilized protein in phosphate buffer (0.01 M, pH 6.8). For spin labeling, the protein solution was incubated for 48 to 96 h at 37 °C or 4 °C with a ten-fold molar excess of IAA spin label. Unbound IAA was removed by consecutive washing steps with sodium phosphate buffer, pH 7.4, by using centrifugal filter units with 30 kDa molecular weight cut-off (Amicon/Millipore, Carringtonwohill, Co. Cork, Ireland). Labeling efficiency (IAA spin label per Cys 34 residue) for each sample was determined by comparison with a reference spin probe (TEMPO) of 100 μM concentration.

Horse hemoglobin: Lyophilized horse hemoglobin was purchased from Sigma-Aldrich, and a protein solution was prepared in phosphate buffer (0.01 M, pH 7.4). Afterwards, the protein was incubat-

ed with 10 mM dithiothreitol (DTT) at 4 °C for 2 h to reduce the cysteine thiol groups. DTT was removed by repeated dilution steps with sodium phosphate buffer, pH 7.4, by using centrifugal filter units with 30 kDa molecular weight cut-off (Amicon/Millipore, Carlingtwhill, Co. Cork, Ireland). For spin labeling, the protein solutions were incubated for 16 h at 4 °C with a ten-fold molar excess of IAA spin label. Unbound spin labels were removed by repeated ultrafiltration steps as described above. Labeling efficiency (IAA spin label per β -93 cysteine) was determined by the comparison with a reference spin sample (TEMPO) of 100 μM concentration and estimated to be 65% for horse hemoglobin.

Protein Adsorption

For cw and DEER experiments, substrate powder samples (75 mg) were incubated for 4 h at room temperature in phosphate buffer solution (500 μL , 0.01 M, pH 7.4) enriched with hemoglobin or BSA (6 mg mL^{-1}). For the competitive adsorption study, the BC powder sample (50 mg) was incubated with 1 mL solution of single or double spin-labeled proteins at two pH values (7.4 and 4.7) with the following concentrations:

- 1) 2 mg mL^{-1} for single spin-labeled proteins (further denoted as Hgb* and BSA*);
- 2) 2 mg mL^{-1} of spin-labeled BSA mixed with 2 mg mL^{-1} of non-labeled hemoglobin (further denoted as BSA*-Hgb);
- 3) 2 mg mL^{-1} of non-labeled BSA mixed with 2 mg mL^{-1} of spin-labeled hemoglobin (further denoted as BSA-Hgb*).

To attach reproducible amounts of proteins on the substrate, each loaded capillary was centrifuged for 10 min at 3725 $\times g$ to ensure equal sample density in each tube. Each protein concentration value obtained within the adsorption study is the mean value of three independently prepared experiments.

Cw-EPR Measurements

X-band cw-EPR experiments were performed by using a custom-built EPR spectrometer equipped with a Bruker dielectric resonator. The microwave power was set to 1.0 mW, the B-field modulation amplitude was 0.15 mT. Glass capillaries of 0.9 mm inner diameter were filled with a sample volume of 15 μL , sufficient for a total active resonator volume of 10 μL .

DEER Measurements

The four-pulse DEER experiments were conducted with an X-band Bruker Elexsys 580 spectrometer equipped with a Bruker Flexline split-ring resonator ER 4118XMS3 and a continuous-flow helium cryostat (ESR900, Oxford Instruments). Prior to freezing the protein in 3 mm inner diameter EPR tubes, 20% (v/v) glycerol was added to each sample as a cryoprotectant. All data were collected at 50 K by following the four-pulse DEER sequence [Eq. (1)].^[32,33]

$$\pi/2(\nu_{\text{obs}}) - \tau_1 - \pi(\nu_{\text{obs}}) - t' - \pi(\nu_{\text{pump}}) - (t_1 + t_2 - t') - \pi(\nu_{\text{obs}}) - \tau_2 - \text{echo} \quad (1)$$

A two-step phase cycling (+ $\langle x \rangle$, - $\langle x \rangle$) was performed on $\pi/2(\nu_{\text{obs}})$. Time t' is varied, whereas τ_1 and τ_2 are kept constant, and the dipolar evolution time is given by $t = t' - t_1$. Data were analyzed only for $t > 0$. The pump frequency ν_{pump} was set to the center of the reso-

nator dip and coincided with the maximum of the nitroxide EPR spectrum, whereas the observer frequency ν_{obs} was ≈ 65 MHz higher, coinciding with the low-field local maximum of the spectrum. All measurements were performed at a temperature of 50 K with observer pulse lengths of 16 ns for $\pi/2$ and 32 ns for π pulses and a pump pulse length of 12 ns. Proton modulation was averaged by adding traces at eight different t_1 values, starting at $\tau_{1,0} = 400$ ns and incrementing by $\Delta\tau = 56$ ns. Data points were collected in 8 ns time steps. The total measurement time for each sample was 8–24 h. Data analysis was performed with DeerAnalysis2011/2013.^[35] Parameter validations for the Tikhonov regularizations of the DEER experiments with BSA were performed as implemented in DeerAnalysis2016^[35] (see Supporting Information). The samples prepared for DEER measurements had the following spin label concentrations: ≈ 170 μM for samples containing horse hemoglobin and ≈ 150 μM for samples containing BSA.

To allow the comparability of the data recorded before and after adsorption, for each protein the experimental conditions were kept constant for all samples.

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Conflict of interest

The authors declare no conflict of interest.

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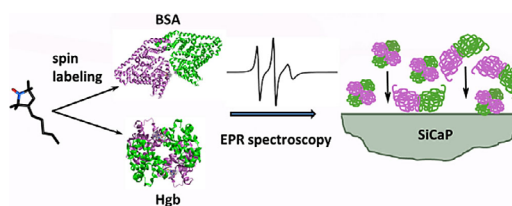
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ARTICLES

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Conformational Changes and Competitive Adsorption between Serum Albumin and Hemoglobin on Bioceramic Substrates



Protein adsorption onto bioceramics:
EPR spectroscopy has been used in combination with site-directed spin labeling (SDSL) to investigate single protein and competitive adsorption kinetics of horse hemoglobin (Hgb) and bovine

serum albumin (BSA) on a silica–calcium–phosphate bioceramic substrate. The dissociation of hemoglobin upon adsorption could be significantly reduced when the substrate was previously “primed” with a layer of BSA.