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Authors
Gruian, Cristina Mihaela
Rickert, Christian
Nicklisch, Sascha CT
et al.

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

Cristina Mihaela Gruian,[a, b] Christian Rickert,[b] Sascha C. T. Nicklisch,[c] Emilia Vanea,[a, b] Heinz-Jürgen Steinhoff,[b] and Simion Simon*[a]

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 adsorption. 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 αβ 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.[1–6] 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.[10] The phenomenon of protein adsorption on the surface of these so-called osteoinductive bioceramics represents a major step in promoting bone regeneration.[11]

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.[12] 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-
tion on structure and conformational dynamics of biomolecules, but also allows for quantitative analysis of adsorbed labeled proteins. 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. 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, solvent accessibility, the polarity of the local microenvironment, and the distance between two spin labels.

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 1 A) 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. 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 (≈ 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. The butterfly-shaped structure of the BSA dimer (Figure 1B, pdb 4F5S) exhibits a remarkable stability as a consequence of the 17 disulfide bridges within the protein core. Earlier EPR studies on BSA spin-labeled at position Cys 34 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. 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 Cys 34 location in a hydrophobic crevice of 9.5–10 Å depth (Figure 1 C). 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 1 D, pdb 1NS9). 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, Fe²⁺, 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. 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 1 E). Previous studies with human hemoglobin by Banham et al. 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. 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. The combination of spin labeling (SL) with EPR spectroscopy has been traditionally used to explore the structure and dy-

![Figure 1](image-url)
namic 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. 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 absorption. 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. 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 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. 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 strongly immobilized spectral component \( I \) (Figure 2A) arising 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.

Upon increase of the temperature during spin labeling, the equilibrium between the two states is driven towards the conditions from pH 7 to pH 10. The Ellman test 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 labeling 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. 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.

The mobile spectral component \( M \), characterized by narrow linewidths 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. 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.](image-url)
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.\(^{[25]}\) 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 (Cys 34) 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 Cys 34 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 Cys 34 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 populations experiencing different environments. Component $I$ was found to correspond to a fraction of spin labels trapped in the tyrosine pocket Tyr145β 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 Tyr145β residues (24 Å according to the crystal structure).\(^{[26]}\) 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\(^{[28]}\) to horse hemoglobin, and in the frozen state the spin labels occupy the tyrosine pocket within human hemoglobin, leading to an internitroxide 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 3](image3.png)

**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\(^{[20]}\) regularization.

![Figure 4](image4.png)

**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.\textsuperscript{[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 Tyr 145β 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 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 μM).\textsuperscript{[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.\textsuperscript{[31]}

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 Tickhonov regularization.\textsuperscript{[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.
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.

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 αβ dimers but not of α1α1 or β1β1 dimers. This means that dissociation happens either at the αβ2/αβ1 or at the α1β1/α1β2 sliding interfaces. The α and β subunits interact along the αβ1 (αβ1) packing interface by forming tight contacts involving approximately 34 residues, whereas the αβ2 (αβ1) sliding interface involves only about 19 residues.[32] The formation of αβ1 and αβ2 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
changes induced in the protein. The Mer prevented a clear conclusion regarding the structural data obtained at 550 °C for several hours and further heat treatment 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, Carringtwonhill, 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, Carrington, W. 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 mgmL-1 for single spin-labeled proteins (further denoted as Hgb* and BSA*);
2) 2 mgmL-1 of spin-labeled BSA mixed with 2 mgmL-1 of non-labeled hemoglobin (further denoted as BSA*-Hgb);
3) 2 mgmL-1 of non-labeled BSA mixed with 2 mgmL-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 x 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 Elexys 580 spectrometer equipped with a Bruker Flexible split-ring resonator ER 4118XMS3 and a continuous-flow helium cryostat (ESR900, Oxford Instruments). Prior to freezing the protein solution (500 μL) to each sample as a cryoprotectant. All data were collected at 50 K with observer pulse lengths of 16 ns for π/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 values, starting at t= 400 ns and incrementing by Δτ = 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. Parameter validations for the Tikhonov regularizations of the DEER experiments with BSA were performed as implemented in DeerAnalysis2016 (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.

Keywords: bioceramics · bovine serum albumin · EPR spectroscopy · horse hemoglobin · spin labeling

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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.