A Polymer Surfactant Corona Dynamically Replaces Water in Solvent-Free Protein Liquids and Ensures Macromolecular Flexibility and Activity

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S *Supporting Information*

ABSTRACT: The observation of biological activity in solvent-free protein−polymer surfactant hybrids challenges the view of aqueous and nonaqueous solvents being unique promoters of protein dynamics linked to function. Here, we combine elastic incoherent neutron scattering and specific deuterium labeling to separately study protein and polymer motions in solvent-free hybrids. Myoglobin motions within the hybrid are found to closely resemble those of a hydrated protein, and motions of the polymer surfactant coating are similar to those of the hydration water, leading to the conclusion that the polymer surfactant coating plasticizes protein structures in a way similar to hydration water.

At the surface of soluble proteins, hydration water forms a mobile hydrogen-bonding network and permits sufficient macromolecular flexibility for proteins to attain full activity.[1] A loss of flexibility, either through reduction in temperature[2−4] or loss of hydration water,[5,6] is known to impair protein function[7,8] because protein motions necessary to access different conformational states[9] are suppressed. The coupling of hydration-water dynamics and protein dynamics is currently the subject of controversial discussions. In particular, the question of whether hydration-water dynamics enslave protein motions in a unilateral way,[10] merely act as a plasticizer that provides conformational freedom for the protein,[11] or couple differently with different classes of biological macromolecules[12,13] is currently a matter of considerable interest. It is known that water can in certain cases be substituted as a solvent by organic compounds[14] and that a large number of proteins function as part of cellular membranes where they are often at least partially surrounded by lipids rather than water. However, all functional proteins have, until recently, been thought to require solvent for function. This view has been challenged by the creation of solvent-free protein−polymer surfactant nano-hybrids (hereafter referred to as "hybrids").[15,16] Specifically, a myoglobin hybrid has been shown to be able to reversibly bind oxygen.[16] However, the physical basis for the activity of the hybrid is unknown; if dynamics underpin protein function and solvent plays an essential role in the dynamical behavior of protein molecules, how can a solvent-free protein be biologically active? In this context, the present study addresses two specific questions: do the dynamics of the protein moiety of the hybrid resemble the dynamics of a hydrated protein, and do the dynamics of polymer surfactant coating resemble hydration-water dynamics? Answering these questions by studying this unique experimental system has the potential to shed light on the interplay between protein solvation, dynamics, and function.

Incoherent neutron scattering (INS) is widely used to study protein dynamics and probes atomic motions on the ns−ps time scales and Å length scales.[17] We applied elastic INS (EINS) to study the dynamic behavior of a solvent-free protein−polymer surfactant nanohybrid for the first time. By deuterating either the protein or the polymer moiety of the hybrid, we were able to separately characterize the temperature-dependent atomic mean square displacements (MSD) of the polymer and protein motions, respectively. Our results indicate that the dynamics of myoglobin within the hybrid closely resemble those of hydrated myoglobin and that the dynamics of the polymer corona are similar to those of typical protein hydration water, leading to the conclusion that the polymer

coating can plasticize protein structures in a similar way to hydration water.

The hybrids used in this work were synthesized by electrostatically grafting anionic poly(ethylene oxide)-based polymer surfactant chains to the surface of myoglobin that has been cationized (C-Mb) with N,N-dimethyl-1,3-propane-diamine (DMPA) (Figure 1a)[18] before extraction of solvent
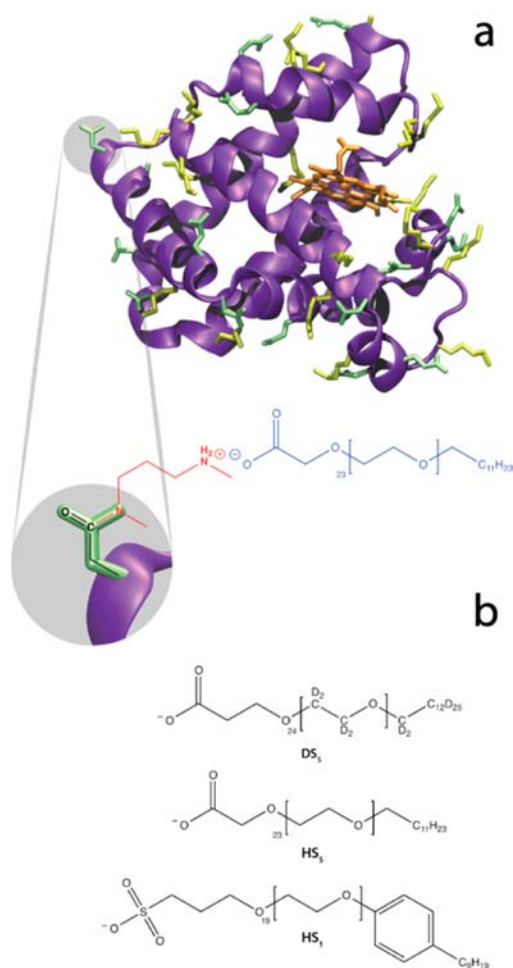

Figure 1. (a) Model illustrating the carbodiimide coupling of N,N'-dimethyl-1,3-propanediamine (DMPA; red) to an aspartic acid side chain (green) of myoglobin to yield cationized myoglobin (C-Mb), followed by electrostatic coupling with ethylene glycol ethoxylate lauryl ether (HS$_5$; blue) to give HMb/HS$_5$. After cationization there were ~41 surfactant binding sites on C-Mb, including modified glutamic (green) and aspartic (green) acid as well as arginine (yellow) and lysine (yellow). (b) Anionic polymer surfactants: α-deuterated tridecanol propionic acid-terminated deuterated poly(ethylene glycol) (DS$_5$), ethylene glycol ethoxylate lauryl ether (HS$_5$), and poly-(ethylene glycol) 4-nonylphenyl 3-sulfopropyl ether (HS$_1$).

to a final hydration level (h) of 0.0035 g of H$_2$O per gram of myoglobin hybrid, which corresponded to ~13 waters per hybrid molecule, or 4 waters per protein molecule by mass. This level of hydration is far below the several hundred water molecules needed to form a full hydration shell around small globular proteins such as myoglobin,[7] and is similar to that of a lyophilized protein powder after drying over P$_2$O$_5$ for several days.[19] As described previously, the protein moiety of these hybrids retains significant secondary structure, heme binding,

and oxygen binding/rebinding capacity[16] and can unfold and refold in response to changes in temperature.[20]

Hybrids of hydrogenated horse myoglobin or deuterated sperm whale myoglobin and the hydrogenated surfactants glycolic acid ethoxylate lauryl ether (HS$_5$) or poly(ethylene glycol) 4-nonylphenyl 3-sulfopropyl ether (HS$_1$) or α-deuterated tridecanol propionic acid-terminated deuterated poly(ethylene glycol) (DS$_5$) were synthesized (Figure 1b; see Materials and Methods in the Supporting Information). Expression and large-scale production of deuterated sperm whale myoglobin were carried out in the ILL-EMBL Deuteration Laboratory (Grenoble, France), and the synthesis of glycolic acid ethoxylate lauryl ether (HS$_5$) was done at the School of Chemistry, University of Bristol. The samples were lyophilized extensively and kept under nitrogen in a desiccator until required. Four different myoglobin/polymer hybrids were synthesized (Figure 1): (i) hydrogenated myoglobin/hydrogenated polymer HS$_5$ (HMb/HS$_5$), (ii) hydrogenated myoglobin/hydrogenated polymer HS$_1$ (HMb/HS$_1$), (iii) hydrogenated myoglobin/deuterated polymer DS$_5$ (HMb/DS$_5$), and (iv) deuterated myoglobin/hydrogenated polymer HS$_5$ (DMb/HS$_5$). The polymer surfactant occupancies of HMb/HS$_5$, HMb/HS$_1$, and DMb/HS$_5$ were approximately 100% (i.e., approximately 41 polymer surfactant chains per C-Mb molecule), while HMb/DS$_5$ had 43% occupancy (18 polymer surfactant chains per C-Mb). Control samples of hydrogenated horse myoglobin powder, hydrated with D$_2$O at 0.43 g D$_2$O/g Mb (HMb/D$_2$O), and hydrogenated horse myoglobin, dried over P$_2$O$_5$ (HMb-dry), were also prepared.

Given that the polymer surfactant used in this study (HS$_5$) differed slightly from those described previously (HS$_1$[16] and HS$_2$[20]), the samples containing HS$_5$ were characterized to ensure they retained the same characteristics as the hybrids previously constructed. Synchrotron radiation circular dichroism (SRCD) experiments performed on HMb/HS$_5$ at 25 °C yielded spectra that are consistent with previous work on HMb/HS$_2$, confirming that, although there is a reduction in the level of α-helical structure (as compared to hydrated myoglobin), the secondary structure of the protein is still intact (Figure S1). Results from thermal denaturation SRCD experiments (Figure S2) performed on HMb/HS$_5$ are also largely identical to those of HMb/HS$_2$; i.e., reversible unfolding of the protein is observed with a half-denaturation temperature ($T_{1/2}$) of 163 °C and a refolding efficiency of 95% from 155 °C (Figure S3). Finally, equilibrium gas binding experiments performed on HMb/HS$_5$ are consistent with previous work on HMb/HS$_1$,[16] showing reversible dioxygen binding with an oxygen affinity ($P_{1/2}$) of 3.4(0.2) torr with no cooperativity (Figure S4).

EINS experiments were carried out on the backscattering spectrometers IN16[21] (Institut Laue-Langevin, Grenoble, France) and SPHERES[22] (Jülich Centre for Neutron Science at FRMII, Garching, Germany). Incoherent neutron scattering from biological samples is dominated by the signal from hydrogen nuclei, whose incoherent scattering cross section is ~40 times larger than those of deuterium and other atoms in the sample.[17] Consequently, for the HMb/HS$_1$ sample, 26% of the EINS signal is from the protein and 74% from the polymer surfactant HS$_1$. In the case of the HMb/HS$_5$ sample, 23% of the signal arises from the protein and 77% from the polymer surfactant HS$_5$. For the DMb/HS$_5$ sample, 11% of the signal is from protein and 89% is from the polymer surfactant. Thus, in these three samples, the signal is dominated by the polymer

contribution. In contrast, for HMb/DS$_5$, 91% of the signal is from the protein contribution and 9% from the polymer surfactant HS$_5$. Finally, in HMb/D$_2$O (HMb-dry), 98% (100%) of the signal is from protein and 2% (0%) from the D$_2$O hydration water (see Materials and Methods for detailed contributions to EINS signal).

The temperature dependence of the EINS was measured from 20 to 300 K for all samples, and atomic MSD (Figure 2) were obtained by linear regressions of the logarithm of the elastic intensity as a function of the scattering vector Q^2 (Figure SI Methods 10). Experiments on the fully hydrogenated hybrids HMb/HS$_5$ and HMb/HS$_1$ provide MSD averaged over both the Mb and the polymer moieties (Figure 2a). Up to ∼120 K, MSD increase linearly due to harmonic motions. At ∼120 K, the MSD start to increase at a higher rate, due to methyl rotations entering the experimental window of the neutron spectrometer.[11,24−26] A dynamical transition[3] is observed at temperatures ∼250 K, marking the onset of anharmonic motions. A similar dynamical transition is also apparent in the HMb/D$_2$O sample (Figure 2a), which has been linked to the onset of biological activity in myoglobin.[8] We conclude that the dynamical behavior of the hybrid is similar to that of hydrated myoglobin and is largely independent of the nature of the polymer employed (HS$_1$[16] or HS$_5$).

The polymer surfactant occupancy of the HMb/DS$_5$ sample was reduced due to an unknown reason related to polymer deuteration. Nevertheless, the temperature dependence of its MSD qualitatively and quantitatively resembles the MSD of a hydrated protein, and is significantly different from those of a dry myoglobin powder (Figure 2b). In particular, the MSD go through a dynamical transition at ∼250 K that is absent in the dry protein and are of a similar absolute value at 300 K to those of the HMb/D$_2$O sample (Figure 2b). Flash photolysis experiments have shown that a heme-bound ligand cannot leave the protein after photodissociation if myoglobin is either hydrated below h = 0.15,[6] embedded in a solid,[2] or below 230 K.[2] Thus, the anharmonic equilibrium fluctuations present in the hybrids above 250 K (Figure 2a,b) provide a physical explanation for the observed reversible oxygen binding activity in the hybrids (Figure S4). We suspect that the MSD of the protein moiety of a HMb/DS$_5$ sample with full polymer occupancy would be even closer to those of HMb/D$_2$O. However, we cannot exclude the possibility that the reduction in MSD of HMb/DS$_5$ with respect to HMb/D$_2$O is a result of the polymer surfactant molecules providing a somehow less plasticizing environment compared to aqueous solvent.

Polymer surfactant motions in the hybrid were probed with the DMb/HS$_5$ sample. The MSD are almost identical to those of the fully hydrogenated hybrids and are qualitatively similar to the MSD of hydration water on the surface of deuterated maltose binding protein[23] (Figure 2c). Dynamics on the ns−ps time scale of the polymer surfactant coating and of hydration water are thus very similar, suggesting that the corona dynamically replaces water in these hybrids and creates a local environment in which proteins can be biologically active. On a microscopic scale, it remains unclear how the partially hydrophobic polymer surfactant interacts with the protein surface. In contrast to water molecules that hydrogen-bond with polar groups on the protein surface or cluster around apolar groups, the polymer surfactant can also engage in hydrophobic and electrostatic interactions. Electrostatic interactions via the DMPA linker might contribute to the dynamical coupling between the polymer surfactant and protein moieties
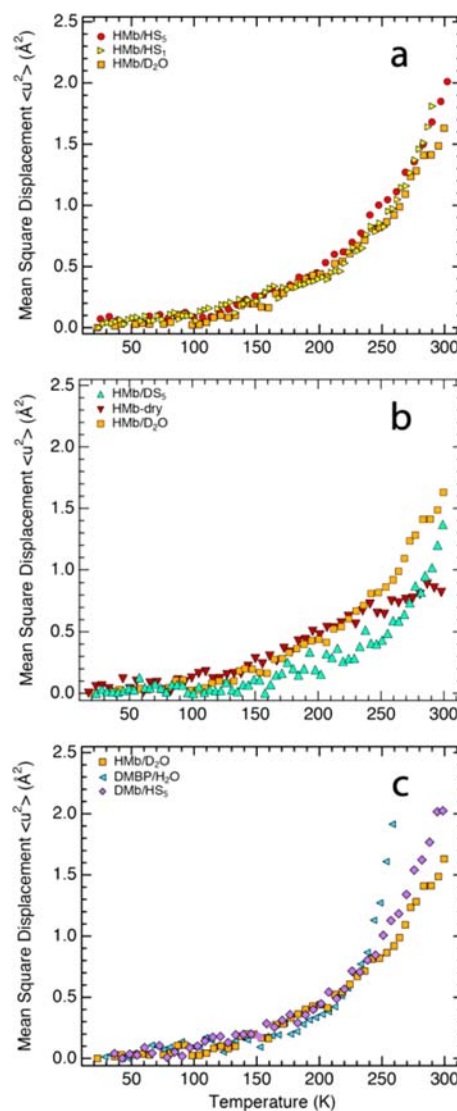

Figure 2. Atomic mean square displacements (MSD) as a function of temperature of solvent-free myoglobin−polymer surfactant hybrids and of their components. (a) Cationized, hydrogenated horse myoglobin complexed with the hydrogenated polymer surfactant HS$_1$ (HMb/HS$_1$; yellow triangles) or with the hydrogenated polymer surfactant HS$_5$ (HMb/HS$_5$; red circles), hydrogenated horse myoglobin in a powder hydrated at 0.43 g D$_2$O/g myoglobin[13] (HMb/D$_2$O; orange squares). (b) Cationized, hydrogenated horse myoglobin complexed with the deuterated polymer surfactant DS$_5$ (HMb/DS$_5$; green triangles), hydrogenated dry Mb powder (HMb-dry; red triangles), and HMb/D$_2$O[13] (orange squares). (c) Cationized, deuterated sperm whale myoglobin complexed with hydrogenated polymer surfactant HS$_5$ (DMb/HS$_5$; violet diamonds), hydration water in a powder of deuterated maltose binding protein (MBP) hydrated at 0.44 g H$_2$O/g MBP (blue triangles),[23] and HMb/D$_2$O[13] (orange squares). MSD were extracted in the range 0.20 < Q^2 < 1.40 Å^{-2} for the HMb/HS$_5$, HMb/DS$_5$, and HMb/D$_2$O samples, in the range 0.18 < Q^2 < 1.33 Å^{-2} for the HMb-dry sample, and in the range 0.20 < Q^2 < 1.13 Å^{-2} for the DMb/HS$_5$ sample. Published neutron data of DMBP/H$_2$O[23] were re-examined for MSD extraction in the Q^2-range 0.19 < Q^2 < 1.13 Å^{-2}. All data were collected on IN16[21] and SPHERES[22] and were processed in exactly the same way.

and might themselves modify the dynamics of the protein. The decreased number of hydrogen-bonding donors and acceptors in the polymer surfactant corona within a hybrid as compared

to those in the first water layer in fully hydrated proteins might lead to more electrostatic and polar interactions among surface side chains. As a result, the number of hydrogen-bonding partners per amino acid side chain might be similar in polymer surfactant-coated and hydration-water-covered proteins. Molecular dynamics simulations of a solvent-free protein–polymer surfactant hybrid might shed some light on microscopic details of polymer surfactant–protein interactions.

The observed similarities between the MSD of the polymer surfactant coating in these hybrids and protein hydration water highlight the importance of flexibility in the molecular matrix encapsulating biologically active proteins. We propose that this similarity is the basis of ligand-binding activity in solvent-free liquid myoglobins. Our results indicate that electrostatically linking polymer surfactant molecules to the surface of myoglobin produces a novel class of solvent-free biomolecular liquids in which the attached coronal layer functionally and dynamically substitutes for a hydration shell of water molecules and ensures macromolecular flexibility and conformational dynamics. The ability to replace the solvation shell of proteins without impairment of molecular dynamics could have considerable potential for the design and development of new functional biohybrid nanomaterials for use in anhydrous environments and other technologically challenging milieux.

ASSOCIATED CONTENT

S **Supporting Information**

Mb deuteration, purification, and characterization; synthesis and characterization of anionic polymer surfactants; Mb functionalization and polymer grafting; neutron scattering experiments; SRCD spectroscopy and thermal denaturation thermodynamics; oxygen binding assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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