Liquid Proteins—A New Frontier for Biomolecule-Based Nanoscience

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ABSTRACT Solid, liquid, or gas? The physical states adopted by nano-objects such as proteins are critically dependent on the size and range of the intermolecular attractive forces, and as a consequence, pure liquids comprising solventless melts of structurally intact, discrete functional nanoconstructs are conspicuously absent. Here we describe how globular proteins can be surface-engineered such that anhydrous powders of these nanoscale objects melt at close to room temperature to produce solvent-free liquids comprising exceedingly high concentrations of structurally and functionally intact biomolecules. These liquids offer unprecedented opportunities in bionanomaterials research, represent a new phase for protein chemistry, and challenge the existing paradigm on the role of water molecules in protein folding and function.

It is now generally accepted that materials nanochemistry and bionanotechnology are important engines of discovery in diverse sectors of the global economy.[1−8] Many of these innovations will involve nanoscale objects with discrete and persistent architectures comprising multiple functionalities that can be systematically tuned for enhanced performance, often under adverse conditions. In this regard, it is self-evident that key archetypes of nanoscale miniaturization and functionality are to be found in the recognition, processing, and transmission mechanisms of living organisms—indeed, the emergence and evolution of life may have been contingent on the systems integration of primitive nanostructured objects.[9] As a consequence, the synthetic construction of functional hybrid nano-objects with integrated biological components such as proteins and enzymes is considered to be a key aspiration in many emerging areas of nanoscience research. Proteins are characterized by persistent globular nanoarchitectures that are encoded within linear amino acid sequences and constructed from the three-dimensional folding of secondary structural conformations such as α-helices and β-strands. More complex globular superstructures, such as the hollow spherical architecture of the 24-mer subunit iron storage protein ferritin, are produced from the concerted assembly of folded polypeptide subunits and stabilized by noncovalent interactions. The high informational content of these nanoscale objects and their corresponding uniformity in size, structural topography, chemical site-specificity, ensemble of conformational states, and dynamical responses to energization and small molecule binding far exceeds what is currently achievable in synthetically derived nanoscale objects.

While much attention has been focused on the self-assembly and transformation of hybrid nano-objects prepared under equilibrium and non-equilibrium conditions,[10] the study of the *phase behavior* of nanoscale objects remains virtually unexplored. In general, nanoscale objects as pure materials are limited to the solid state and, therefore, are utilized in the form of dry powders or, more commonly, as solutes dispersed in aqueous or organic solvents. From a practical perspective, these limitations could restrict the use of nanoscale constructs in future markets with regard to handling, storage, transport, safety, and product formulation. For example, the manipulation of low-density freeze-dried proteins is problematic, and obtaining high concentrations of proteins in solution for effective functionality can be difficult due to solubility and aggregation constraints. Significantly, many of these problems could be circumvented if nanoscale objects could be rendered accessible to the *pure liquid phase*, that is, as solventless melts of structurally intact, discrete functional nanoconstructs. As the concept of the solventless liquid phase remains missing from the lexicon of current nanoscience, such a proposal necessitates a fundamental advance in current methodologies of nanoscale construction and design.

Given this context, this Perspective describes recent work that demonstrates the preparation and properties of solventless

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liquids comprising structurally and functionally intact proteins.[11,12] This work represents a new frontier in protein-based nanomaterials chemistry and challenges current paradigms concerning the role of water in protein structure and function. We begin with a short discussion concerning the states of nanomatter: why do proteins, like many other nanoscale objects, have no pure liquid phase? To address this, we discuss underlying concepts in relation to the nature of the forces between nanoscale objects and how they depend on the separation distance. In so doing, we are able to consider why if enough thermal energy is supplied to a dry protein powder, the protein solid phase will decompose rather than melt, but if the surface of the biomolecule is modified with an appropriate, soft coronal layer, then it should be possible to prepare freeze-dried proteins that transform into a solventless liquid phase at ambient temperatures. We then describe how these concepts have been translated into experimental methodologies that have successfully produced solventless liquids of ferritin and the dioxygen binding protein myoglobin. Finally, we review the associated structure and properties of these novel nanomaterials, emphasizing the importance of their stoichiometric composition and high thermal stability, retention of near-native globular nanoarchitectures, and surprising capacity of the liquid myoglobin construct to bind dioxygen reversibly and to undergo thermally induced unfolding and refolding in the absence of water.

States of Nanomatter. In general, the realization of matter in different physical states such as solid, liquid, or gas is a direct consequence of the presence or absence of intermolecular forces that sustain strong, medium, or weak attractive correlations, respectively, between the thermally induced motions in an ensemble of molecular-scale components. The strength of these interactions is based on the nature of the forces and their dependence on intermolecular distance and is usually represented by Lennard-Jones or Morse potential energy curves. Importantly, the depth and width of the energy well associated with these force–distance profiles are influenced by the size of the interacting components, and as a consequence, profound differences in phase behavior are predicted when comparing interactions between molecules with those between nano-objects (Figure 1a,b). Doye *et al.* used cluster simulations to examine the effect of varying the range of the attractive force field on the shape of Morse potential energy curves[13] and showed that as the range of the intermolecular forces was reduced for a given molecule/particle size, the attractive potential energy well deepened and narrowed. This in turn implies that a large energetic penalty exists for small deviations from the equilibrium particle–particle separation, and as a consequence, the transition to the less-ordered liquid phase becomes progressively inhibited. In terms of the corresponding phase diagram, reducing the range of the interparticle forces predicts an increase in the critical temperature (T_C) and a decrease in the solid–fluid coexistence temperature (T_{SC}) such that the solid–liquid transition disappears under most pressure regimes (Figure 1c). This is consistent with the experimentally derived phase diagram for C_{60}: heating a dry powder of the pure material results in sublimation rather than melting, and subsequent cooling of the gaseous C_{60} nanoparticles reinstates the solid phase.[14]

The absence of the liquid phase for nanoscale objects is predicted to be a general and widespread phenomenon because the size of these constructs is typically larger than the range of the attractive interactions. Moreover, in many cases, sintering or degradation of the nano-objects is expected when the thermal energy required to melt the solid phase exceeds the free energy of formation of the construct. This is particularly relevant to globular proteins in the freeze-dried state. In the absence of water, the nanoscale objects are held together by strongly attractive intermolecular forces generated from an ensemble of hydrogen bonding, electrostatic ion pairing, and van der Waals interactions that operate over distances significantly shorter than the size of the globular architecture. As a consequence, increasing the temperature at ambient pressure denatures the protein, causing unfolding of the globular structure followed by decomposition by bond breaking in the polypeptide chain. Only when the freeze-dried solid is heated at low pressures will the protein molecules separate without degradation. However, because the interparticle forces are so weak at any separation distance greater than the size of the protein molecules, sublimation rather than melting occurs.

As a corollary to the above discussion, it seems conceptually straightforward that successful realization of a single-component, pure liquid phase of nanoscale objects in the absence of any solvent requires a broadening of the dimensional range covered by the attractive force field. Moreover, reducing the depth of the potential well should

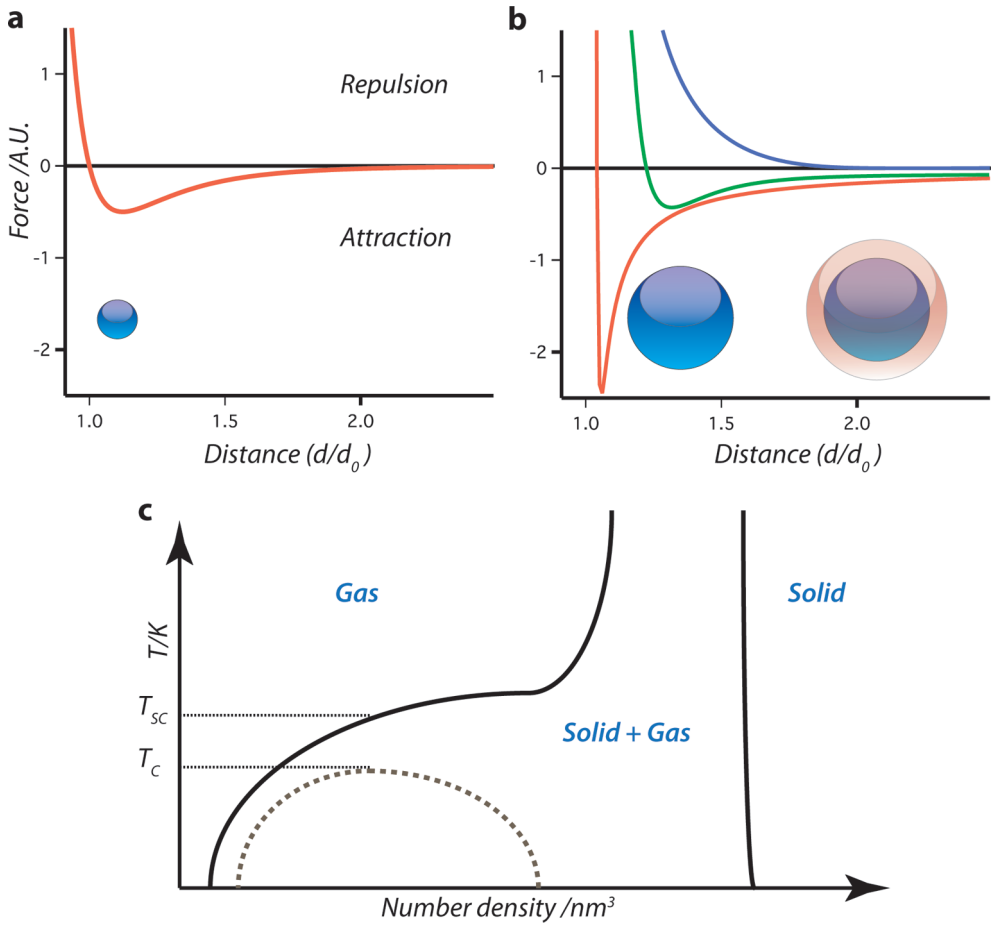

Figure 1. Dependence of phase on particle size. (a) Schematic of force in arbitrary units (AU) against normalized distance showing a shallow attractive well corresponding to a molecular-size particle. The normalized distance is given as the ratio of d/d_o, where d is the interparticle separation and d_o is the size of the particle. (b) As the particle size is increased to nanoscale dimensions, the attractive well deepens and narrows (red line, blue sphere). The introduction of a steric layer to the surface of a nanosize particle (sphere shown on right) can result in an increased repulsive potential (blue line), and as a consequence, the depth and range of the attractive well are decreased and increased, respectively (green line). (c) Plot showing temperature *versus* number density (number of particles/unit volume) phase diagram for a nanoscale object. The solid–fluid coexistence temperature (T_{SC}) is greater than the critical temperature (T_C), such that there is no stable liquid phase. The solid lines denote the phase boundaries, and the broken curve shows the metastable liquid–gas coexistence boundary.

also facilitate the melting phase transition, although this would be ineffective without extending the range of the force field. Surprisingly, these simple concepts appear not to have been widely appreciated by the nanoscience community and, to the best of our knowledge, have not been demonstrated experimentally until recently. Significantly, Giannelis and co-workers showed that the highly attractive van der Waals forces between inorganic nanoparticles can be overcome by introducing an organic steric layer to the surface of the nanoscale objects.[15] This process involves grafting a charged organic corona to the nanoparticle surface, followed by electrostatically induced assembly of an oppositely charged polymer–surfactant canopy layer. By careful selection of the inorganic and organic components, anhydrous powders of the surface-functionalized hybrid nanoparticles are thermally transformed into fluids comprising high concentrations of discrete inorganic nanoparticles. Although there are many investigations based on the preparation of multicomponent blends comprising dispersions of nanoparticles in polymer melts,[16–18] the studies by Gianellis and colleagues highlight the possibility of preparing solvent-free nanoparticle liquids using discrete components in the absence of unbound polymer–surfactant molecules.

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Liquid Proteins. Inspired by the work on nanoparticle liquids, we realized that developing similar methodologies

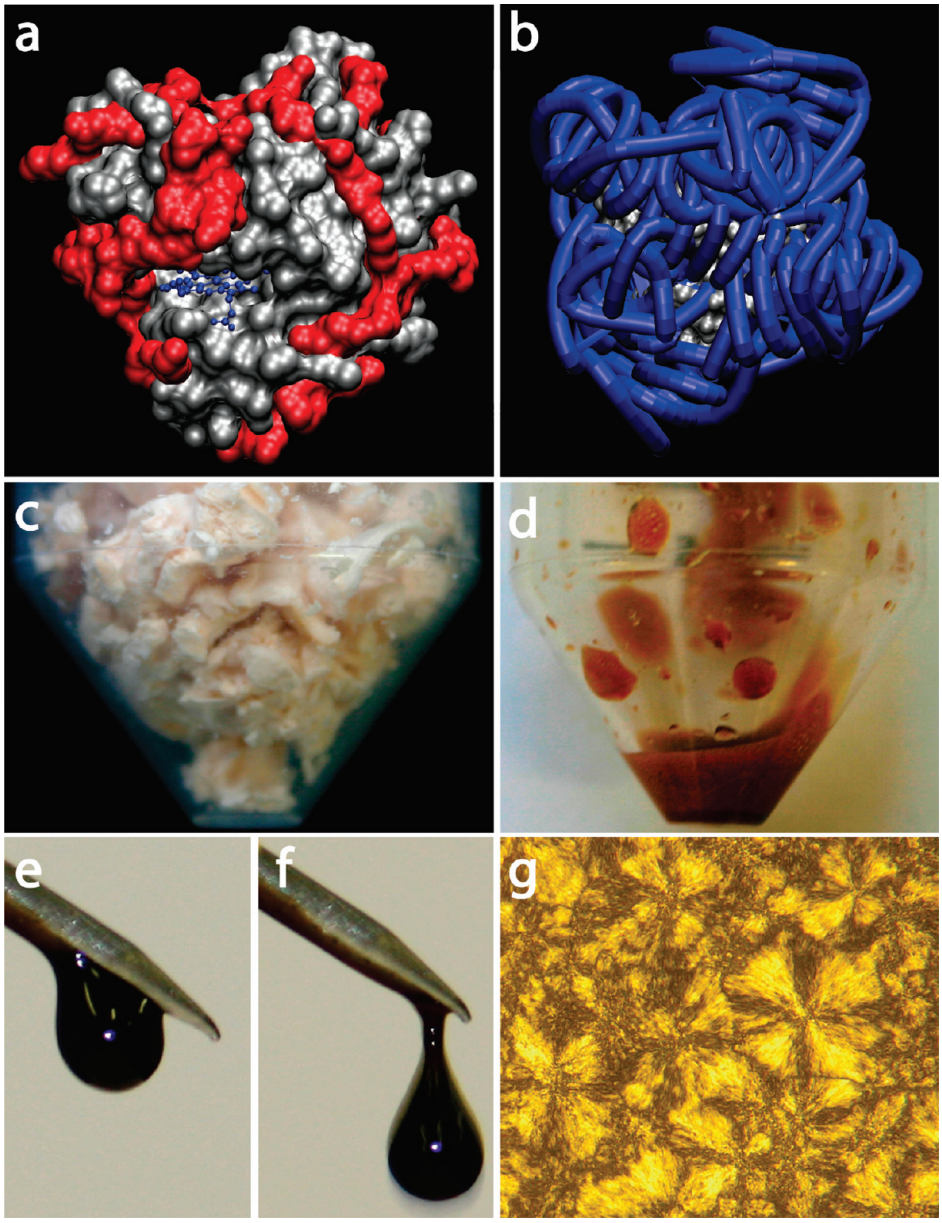

Figure 2. Formation of solvent-free liquid proteins. (a) Molecular graphic showing the solvent-accessible surface-positive charge residues (red) of myoglobin after cationization. (b) Model showing surface profile of a cationized myoglobin molecule after electrostatic assembly of a polymer–surfactant coronal layer (blue). (c) Pink waxy solid produced by lyophilization of a ferritin/polymer–surfactant aqueous conjugate. (d) Anhydrous nanoconjugate shown in (c) after heating to 50 °C and formation of a solventless liquid protein phase. (e,f) Gravity-induced flow of a solventless liquid myoglobin droplet at 60 °C and atmospheric pressure after 5 and 10 s (e and f, respectively). (g) Polarized light microscopy image of a solventless ferritin melt at 32 °C showing optical birefringence (Maltese cross patterns) corresponding to a thermotropic liquid crystalline phase.

using globular proteins would provide a unique opportunity to prepare biomolecule-based nanoconstructs in the form of highly concentrated solventless melts with unprecedented properties. A key aspect of our current strategy is to exploit the high-fidelity architecture of the protein as a stereospecific nanoscale platform for the site-directed attachment of a polymer–surfactant corona layer to produce hybrid nano-objects with well-defined, fixed compositions. The latter is of critical importance as our goal is to prepare pure phases in the form of solventless liquids that comprise single, integrated nanoconstructs rather than stabilized dispersions of surface-modified biomolecules suspended in an excess of unbound polymer–surfactant. We therefore use an experimental protocol with three fundamental steps:[11,12] (i) cationization *via* covalent coupling of *N*,*N*′-dimethyl-1,3-propanediamine to acidic amino acid side chains on the surface of globular proteins such as ferritin and myoglobin to increase the positive surface charge density (Figure 2a); (ii) electrostatically induced grafting in water of anionic polymer–surfactant molecules to the positively charged groups on the protein surface to produce a single component charge-neutral stoichiometric construct (Figure 2b); and

(iii) lyophilization of the resultant aqueous single-component constructs to yield a waxy solid (Figure 2c), which when gently heated just above room temperature melts to produce a solvent-free protein liquid (Figure 2d–f). The water content in the liquid protein is very low indeed, typically less than 0.1%. This corresponds to only four water molecules per myoglobin/polymer–surfactant nanoconstruct, which is far less than what is required for a solvation shell.

Although the size and structure of ferritin and myoglobin are very different, in both cases, highly viscous solventless liquids with protein concentrations of 725 or 440 mg mL^{-1}, respectively, are produced using a polymer–surfactant comprising a sulfonate or carboxylate headgroup, a minimum of 11 poly(ethylene glycol) (PEG) monomer units in the central region, and a linear C_{12} alkyl chain or nonylphenyl hydrophobic tail. Significantly, the corresponding grafting densities are predetermined by the number of native and engineered cationic residues on the surface of the globular protein. For example, 264 polymer–surfactant chains are electrostatically bound to each cationized ferritin molecule (11 organic chains per polypeptide subunit), corresponding to a coronal layer thickness of *ca.* 1 nm and a grafting density of around 1 chain per 7 nm^2. As a consequence, the protein–protein equilibrium separation distance is increased and the depth of the attractive well in the potential energy profile reduced. Specifically, the intermolecular interactions are modulated *via* two mechanisms: steric repulsion between the surface-modified biomolecules resulting from the entropically driven osmotic force associated with compression of the polymer surfactant chains and a reduction in the van der Waals attractive force as a result of the similarities in the refractive indices between the protein and the surfactant.[19] Hence, when thermal energy is supplied, it becomes possible to overcome the positional rigidity associated with the solid phase and achieve the expansion in volume and correlated motions required for melting. Moreover, as the nanoconstructs remain intact after melting—that is, there is a conspicuous absence of free, unbound polymer–surfactant molecules—the liquid proteins may be in part analogous to a melt of star polymers, where thermally induced fluctuations are manifested by retraction and expansion of the polymer chains from the branch-point rather than *via* reptation,[20] which involves the lateral movement of individual, free (unbound) polymer chains.

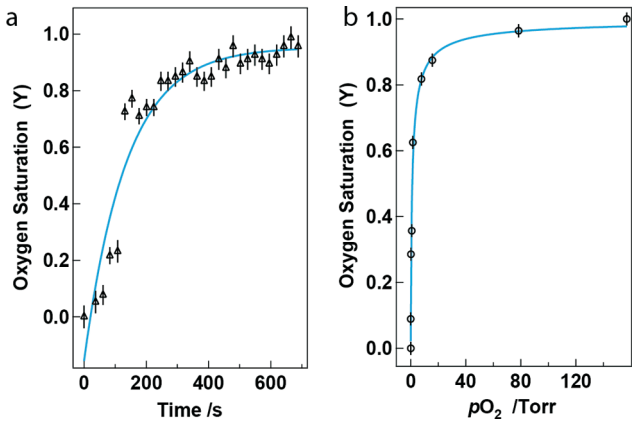

Figure 3. Dioxygen binding in solvent-free deoxy-myoglobin liquids. (a) Plot showing the rate of dioxygen binding at 37 °C. Data points (triangles) originate from Guassian fits to the Soret band and were fitted using an exponential decay function (blue line). (b) Equilibrium dioxygen association curve showing the fraction of bound dioxygen as a function of the partial pressure of oxygen (pO_2) at 37 °C. The solid line results from fitting using the Hill equation.

Many of the general properties associated with solventless liquids of myoglobin and ferritin are similar; for example, they are highly viscous liquids (20 Pa·s at 32 °C for ferritin), have respective melting temperatures of 23.5 and 30 °C, exhibit considerably enhanced decomposition temperatures (408 and 405 °C) compared with the native proteins (315 °C) or surfactant alone (380 °C), and redissolve in aqueous or organic solvents. However, the ferritin melt shows unexpected viscoelastic and optical behavior. Specifically, polarized light microscopy images of the liquid ferritin exhibit temperature-dependent Maltese cross birefringence patterns that persist up to 37 °C (Figure 2g) and which are concomitant with an endothermic phase transition observed by differential scanning calorimetry. Significantly, rheological studies on the optically anisotropic ferritin melt show an abrupt shear thinning to a limiting shear viscosity at 32 °C, which transforms to a Newtonian fluid at 50 °C. In addition, temperature-dependent wide- and small-angle X-ray scattering experiments undertaken at 32 °C reveal Bragg diffraction peaks at repeat distances characteristic of polymer–surfactant PEG chain–chain (0.38 nm) and alkyl tail–tail (0.46 nm) interactions, as well as a reflection at 13 nm corresponding to the repeat distance between protein molecules. The reflections are absent at 40 °C, confirming the presence of a thermotropic liquid crystalline phase in the ferritin melt between 30 and 37 °C. These results are intriguing as ferritin is a spherical nanoparticle with a highly symmetric arrangement of 24 polypeptide subunits and, as such, is not expected to exhibit anisotropic behavior. However, the architecture consists of two types of subunits that are distributed heterogeneously within the nanoscale structure. As the subunit types have different surface charges, they provide different numbers of attachment points for assembly of the coronal layer, and

as a consequence, the hybrid nanoconstruct has an anisotropic distribution of polymer–surfactant molecules that facilitates liquid crystalline ordering.

A New Phase for Protein Nanoscience?

A potential drawback of using proteins as the basis for single-molecule nanoconstructs is that, unlike conventional inorganic nanoparticles, the functional properties are extremely sensitive to structural perturbations, which can be readily induced by relatively small changes in temperature, ionic strength, *etc*. Moreover, the advent of solventless liquid proteins raises intriguing questions concerning the accepted paradigm that water molecules are essential for establishing and maintaining protein folding and biological function. In this regard, a range of spectroscopic studies undertaken at 25 °C indicates that myoglobin molecules in the solventless liquid phase retain their near-native nanoarchitecture with minimal perturbation of their α-helical secondary structure.[12] Moreover, the prosthetic heme group of the metallocenter remains intact in the solventless liquid phase and remarkably can undergo changes in coordination number and redox state analogous to those typical of the native protein in water. For example, solvent-free liquids of met-[Fe^{III}]myoglobin or deoxy-[Fe^{II}]myoglobin show UV–vis spectra corresponding to a six-coordinate low-spin center with a strongly bound water molecule or a five-coordinate heme center with no water ligand, respectively. Amazingly, even though highly viscous and solvent-free, the deoxy-myoglobin melts were able to bind dioxygen or carbon monoxide molecules reversibly when exposed to a dry atmosphere of the gases. Although it takes around 5 min for the dioxygen molecules to bind under equilibrium conditions due to the high viscosity, the binding curve shows classical behavior with regard to the oxygen-binding affinity and an absence of cooperativity (Figure 3). Indeed, the curves are almost identical to those obtained for deoxy-myoglobin under physiological aqueous conditions, indicating that the structure and function of the protein is maintained in the absence of water.

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These remarkable observations are currently being followed up by a more detailed analysis of the equilibrium structures of solvent-free liquid myoglobin at various temperatures.[21] Thermal denaturation experiments show that unfolding is indeed possible in the solvent-free liquid state but that the half-denaturation temperature (160 °C) is around 90 °C higher than that for aqueous cationized myoglobin. This implies that the solvent-free environment markedly stabilizes the globular nanoconstruct. We attribute this primarily to the favorable enthalpic contributions associated with dehydration and the corresponding decrease in dielectric constant of the protein interior. This gives rise to increases in the electrostatic, hydrogen bonding, and van der Waals forces within the interior of the globular nanostructure that will curtail thermally induced unfolding of the polypeptide chain. Moreover, the restriction in conformational freedom imposed by the high volume fraction of protein molecules in the melt and the lack of translational mobility of the conjugated polymer–surfactant molecules could also contribute toward the observed thermal stability.

To our great surprise, cooling the liquid myoglobin from temperatures as high as 100 °C results in almost complete refolding of the polypeptide chain. How can this occur since in the absence of a water solvation shell the hydrophobic effect is essentially invalid? We are currently attempting to elucidate the factors that could give rise to this unprecedented observation. One possibility is that the electrostatically grafted polymer–surfactant molecules present a low dielectric corona to the surface of the protein, and although this could provide a driving force to unfolding through exposure of the hydrophobic side chains, such effects might be offset at lower temperatures by a multitude of unfavorable interactions with the exposed polar peptide backbone. The balance of these competing interactions would then explain the refolding capability of the surface-modified myoglobin nanoconstructs in the absence of water.

CONCLUSIONS AND PROSPECTS

The above results indicate that solventless liquid proteins not only represent a novel phase of nanoscale matter but also offer new directions in protein nanochemistry. The realization that globular proteins can access the liquid state by simply modifying their intermolecular force fields, and that biomolecules present in solvent-free melts unfold, refold, and retain functional activity, challenge the existing paradigm on the role of hydration and the proposed sensitivity of protein nanoscale objects to aqueous environments. Significantly, these new

types of liquids represent a class of hybrid nano-objects for which the physical state can be controlled by integrative coupling of synthetic and biological components, thus paving the way to the synthetic construction and design of novel hybrid bionanomaterials with smart functions and advanced properties.

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