Supramolecular Assemblies for Transporting Proteins Across an Immiscible Solvent Interface

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S *Supporting Information*

ABSTRACT: Polymeric supramolecular assemblies that can effectively transport proteins across an incompatible solvent interface are described. We show that electrostatics and ligand–protein interactions can be used to selectively transport proteins from an aqueous phase to organic phase. These transported proteins have been shown to maintain their tertiary structure and function. This approach opens up new possibilities for application of supramolecular assemblies in sensing, diagnostics and catalysis.

Figure 1. Schematic representation of reverse micelle driven protein transportation.

Transporting molecules across incompatible interfaces is a significant challenge, especially for macromolecules. A striking example of an interfacial barrier is the cellular membrane, where an organized presentation of hydrophilic and hydrophobic functional groups provides a formidable barrier for molecular transport.[1] Whereas small ions or molecules can cross the membrane through ion channels or passive diffusion, globular proteins with large hydrophilic surfaces offer no easy access.[2,3] Nonetheless, cells do transport proteins when necessary for intercellular communication, often using nanoscopic vesicular compartments called exosomes.[4,5] Inspired by these cell-derived vesicles, we became interested in exploring the possibility of transporting proteins into a nanoscopic compartment across a solvent interface. Indeed, small molecule surfactants mostly and occasionally polymers have been explored as a means of loading proteins into reverse micelles.[6−9] Loading selectivity with such systems is limited or nonexistent, even in an electrostatics context, presumably due to the low stability of small molecule based micelles.[10] Though transporting proteins across interfaces has many implications, selective transport, while retaining structure and function, could be transformative in applications such as sensing, delivery, and diagnostics. Supramolecular assemblies have shown great potential in these areas[11−13] and supramolecular protein transport would be an important advance. Here we report a simple polymeric platform that selectively transports water-soluble proteins from an aqueous phase to the water-pool of reverse micelles in an apolar organic phase based on complementary electrostatic interactions or specific ligand-protein binding interactions (Figure 1).

For the initial proof-of-concept, we synthesized a polystyrene-based amphiphilic anionic random copolymer **P1** (M_n = 11 kDa, Đ = 1.09) (Figure 2a) and a cationic polymer, **P2**, (Figure 2b) using nitroxide-mediated polymerization (see SI for
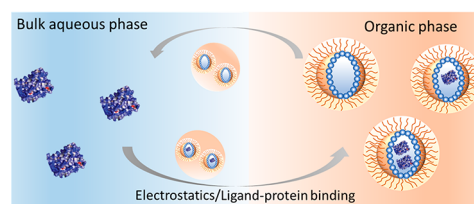
details). By distributing these polymers in toluene along with two equivalents of water per carboxylate or quaternary ammonium moiety, assemblies with a fairly homogeneous size distribution of 50 nm for **P1** and 37 nm for **P2** were observed, as discerned by both dynamic light scattering (DLS) (Figure 2c) and transmission electron microscopy (TEM) (Figure 2d,e, S1).

The key premise for the work here is that these self-assembled structures would bind to complementarily charged proteins in the aqueous phase, and ferry them across the interface to the interior of the reverse micelles in toluene. To test this possibility, porcine liver esterase (plE, MW = 16.8 kDa), a negatively charged enzyme at pH 8.0 (pI = 5.3), was used as the model protein. Upon equilibrating a plE solution with cationic **P2** (1 mg/mL) reverse micelles, presence of proteins in both phases was detected using matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Figure 3a,b shows the MS of the aqueous and organic phases, respectively, before equilibration. After equilibration, we were gratified to observe a peak corresponding to plE in the organic phase (Figure 3c), suggesting that plE was successfully transported into interior of the reverse micelles.

To quantify the extent of protein that was encapsulated within the reverse micelles, we analyzed the organic phase for proteins using the bicinchoninic acid (BCA) assay. This analysis showed that 1 mg of polymer is capable of transporting and binding to 0.05 mg of plE, an equivalent of 5 wt % loading capacity. This estimate is consistent with the corresponding SDS-PAGE analysis of the liberated proteins (Figures S6 and S7). This capacity compares more favorably than that with liposomes.[14] A more compelling analysis is to identify whether the extracted enzyme remains active in the reverse micelle. To investigate this possibility, we designed an amphiphilic
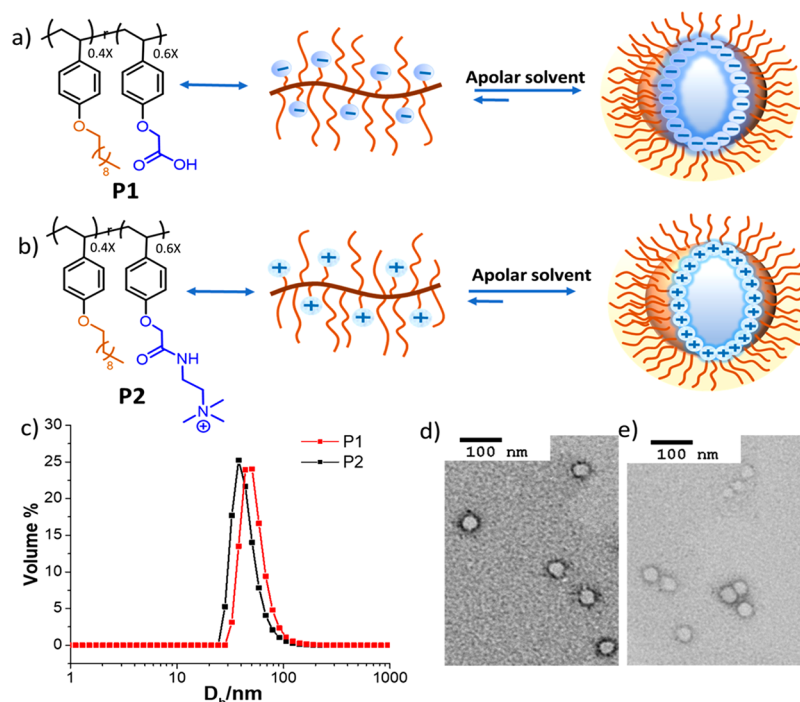
Figure 2. Structural features of polymeric reverse micelles. Molecular structure of polymer **P1** (M_n = 11 kDa, Đ = 1.09) (a) and **P2** (b) (M_n = 12 kDa, Đ = 1.15), (c) DLS profile of **P1** and **P2** in toluene, TEM of **P1** (d) and **P2** (e).
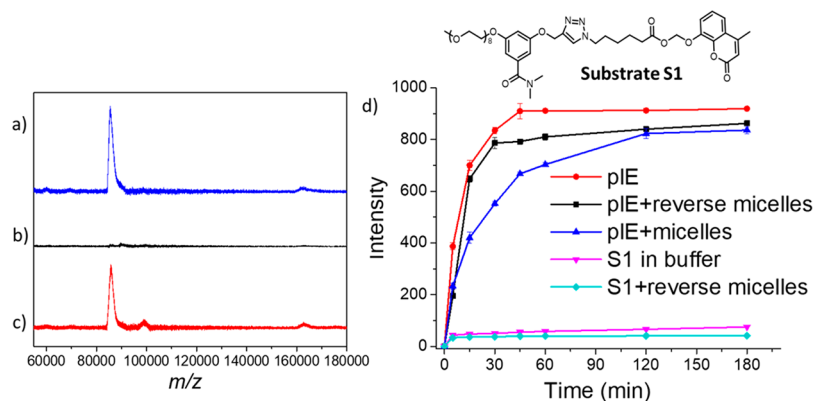

Figure 3. MALDI-MS analysis of (a) aqueous phase before equilibration, (b) organic phase before equilibration, and (c) organic phase after equilibration. (d) Activity of esterase (based on substrate cleavage) inside reverse micelles compared with esterase activity in bulk aqueous phase.

coumarin-based profluorophore **S1** as a substrate for plE. The alkylated ether of this substrate is nonfluorescent. When the ester bond of **S1** is cleaved by the enzyme, the resultant hemiacetal rapidly degrades to generate umbelliferone, a fluorescent coumarin molecule (see SI for details).

The substrate itself was quite stable in PBS buffer as well as after equilibration with toluene solution containing **P2**. In the presence of plE, however, a rapid hydrolysis of **S1** to generate the fluorescent umbelliferone was observed (Figure 3d). We then analyzed the possibility of this reaction in toluene in the presence of reverse micelles loaded with plE. Interestingly, the hydrolysis rate was found to be quite similar to that of the free enzyme, suggesting that the plE activity is maintained inside the reverse micelles. As another control experiment, we mixed plE and **P2** in aqueous phase and found that the activity of the enzyme was slightly lower, suggesting that interactions between **P2** and plE have little effect on plE activity.

When considering the pathway by which these polymers could transport proteins across the interface, we equilibrated the reverse micelle assemblies of polymers **P1** and **P2** with water. UV−visible absorption spectra of both phases indicate that these polymers fully remain in the apolar phase (Figure S8). Though these polymers were initially assembled as micelles in the aqueous phase and equilibrated with toluene (Figure S9), the exclusive presence of these polymers in the aqueous phase shows that these assemblies are kinetically trapped in the solvent that they are initially assembled. Overall, these results suggest protein molecules can exchange between phases, but only remain in the apolar phase if they have favorable interactions with the reverse micelle interior.

Following these observations, we were interested in exploring the applicability of this approach to other nonenzymatic proteins. Green fluorescent protein (GFP) was chosen, not only because it can be readily monitored using fluorescence, but also because the fluorescence itself is a good indicator of
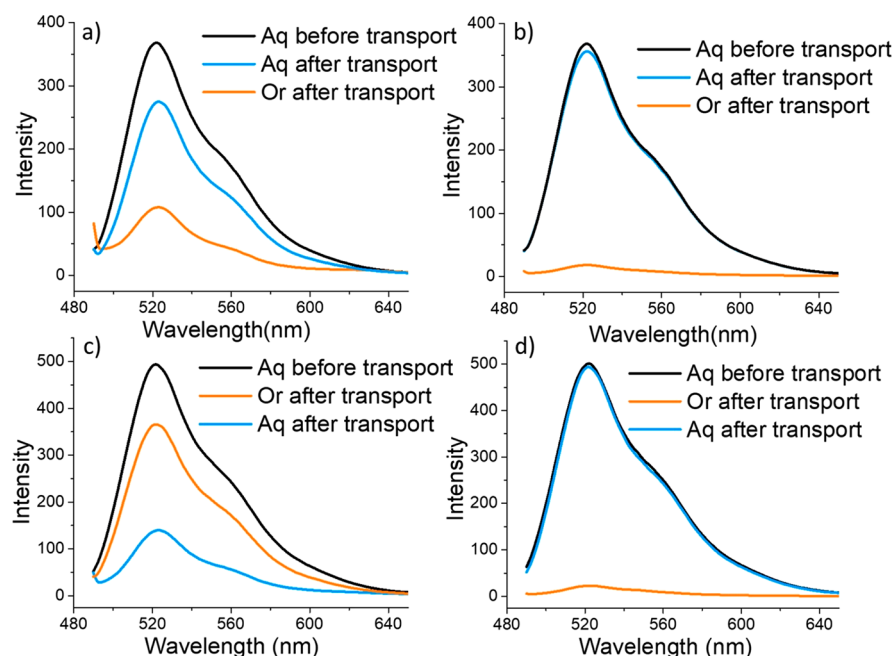
Figure 4. Emission spectrum of GFP before and after transport, (a) GFP (−7) transport by **P2**, (b) GFP (−7) transported by **P1**, (c) GFP (+15) transported by **P1**, (d) GFP (+15) transported by **P2**.
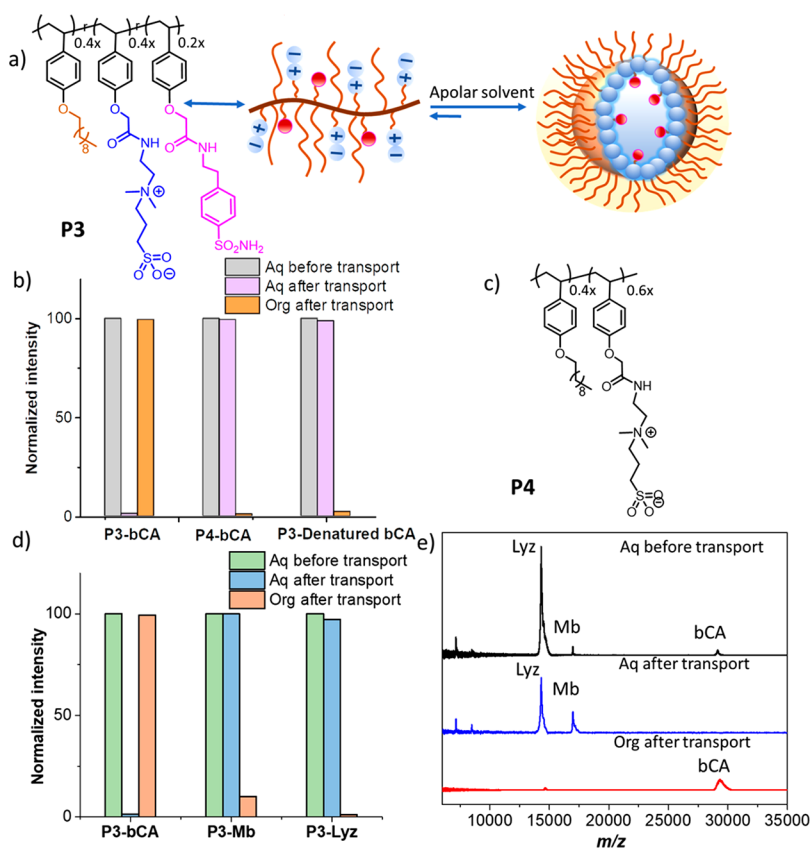
Figure 5. (a) Molecular structure of P3, (b) fluorescence change of aqueous/organic phase using P3 or P4 for bCA transport, (c) molecular structure of P4, (d) fluorescence change of aqueous/organic phase of P3 to transport bCA, Myb or Lyz, (e) MALDI-MS analysis of protein mixture before and after transportation.

whether the protein maintains its tertiary structure. Wild-type GFP (pI 6.2) has a net charge of −7 at pH 7.4, we treated this GFP (−7) solution with **P2** reverse micelle solution in toluene. We were gratified to find that the emission spectrum of the

organic phase clearly showed the presence of GFP (Figure 4a). MALDI-MS peak with a m/z of 28 432 Da further confirmed the presence of GFP in the organic phase (Figure S10). However, there was no discernible change in the emission

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intensity of the aqueous phase (Figure 4b) using the anionic reverse micelle **P1**, suggesting that this transport is indeed due to electrostatic complementarity.

To further test this idea, we utilized the so-called supercharged GFP (+15).[15] Indeed, the anionic polymeric reverse micelle from **P1** is able to transport the protein across the interface, whereas the cationic reverse micelle from **P2** does not affect the protein in the aqueous phase (Figure 4c,d). The results from these studies show that (*i*) transport of proteins across the interface is due to electrostatic complementarity, not due to spurious differences in inherent binding abilities of **P1** and **P2**; (*ii*) the tertiary structure of the proteins can be preserved upon transport across the interface as indicated by the emission spectrum before and after equilibration; (*iii*) at similar polymer and protein concentrations, the extent of protein extraction in GFP (+15) is considerably higher than GFP (−7), showing that binding affinity can influence the extent of proteins transported across the interface.

Though electrostatic complementarity can be utilized to simplify protein mixtures and enable identification of the presence of specific proteins, this ability will be greatly enhanced if proteins can be transported across an interface in response to a specific ligand−protein interaction. To investigate this possibility, we used bovine carbonic anhydrase (bCA) as the model protein, because aryl sulfonamides are well-established as small molecule ligands for this protein.[16] The design hypothesis here is that if this ligand was installed in the polymeric reverse micelles, it should be able to selectively transport bCA to the organic phase due to specific binding.

For this purpose, we designed a zwitterionic amphiphilic polymer **P3** (Figure 5a), containing 40% decyl chain as the hydrophobic moiety, 40% zwitterionic sulphobetain group as the hydrophilic moiety and 20% benzenesulfonamide as the ligand moiety. **P3** forms a similar-sized assembly in apolar solvents (Figure S3). When **P3** was equilibrated with tetramethylrhodamine-5-isothiocyanate (TRITC)-labeled bCA, we observed a strong emission peak in organic phase, indicating the transportation of TRITC−bCA conjugates. Concurrently, there is a dramatic decrease in the fluorescence intensity in the aqueous phase, indicating bCA is successfully transported across the interface.

To investigate whether this is driven by the ligand-protein binding, we designed a structurally similar amphiphilic polymer, **P4**, which forms reverse micelles but lacks the sulfonamide functional group. No change is observed in the emission spectrum of both organic and aqueous phases, when using **P4** as the transporter for TRITC−bCA (Figure 5b). To further test whether the specific ligand-protein interaction is responsible for the observed transport across the interface, we designed another control experiment where the structure of the protein was disrupted with acetonitrile and heat. The denatured bCA should not be able to bind the sulfonamide ligands and thus would not be transported into the organic phase. Indeed, no fluorescence changes in the aqueous or organic phase are observed, showing that no bCA was transported into the organic phase (Figure 5b). These results confirm that transportation occurs only when bCA's native structure is maintained in such a way to preserve its ability to bind the sulfonamide ligand. Overall, these results suggest that specific ligand-protein interactions can be utilized to bind and transport proteins across the solvent interface.

Next, to test the ligand−protein binding based selectivity associated with this process, we performed another set of control experiments using myoglobin (Mb) and fluorescently labeled lysozyme (Lyz). Because benzenesulfonamide ligands have little to no binding affinity to these proteins, we predicted that Mb and Lyz would remain in the aqueous phase. Indeed, no discernible fluorescence change was observed in both aqueous and organic phases for these two proteins, suggesting that the ligand attached reverse micelles are specific for the target protein bCA (Figure 5d). These experiments were initially done separately due to the possible bleeding of fluorescence emission. Selective transport from a mixture of these proteins by **P3** was tested using MS. We were gratified to find that only bCA is transported to the organic phase, whereas Mb and Lyz remained in the aqueous phase as indicated by the mass spectra before and after equilibration (Figure 5e). These data strongly support the idea that ligand-attached reverse micelle systems are specific for target proteins.

In conclusion, we have demonstrated a set of supramolecular assemblies, based on amphiphilic polymers, that can transport proteins across a solvent interface. We have shown here that (*i*) simple electrostatic complementarity in polymeric reverse micelle systems can transport proteins from bulk aqueous phase into the interior of a reverse micelle assembly in the apolar organic phase; (*ii*) the activity of the transported proteins is retained in the process; (*iii*) the efficiency of protein binding is dependent on the charge density presented on the protein surface; (*iv*) the kinetically trapped nature of the assemblies suggest that the polymers do not ferry the proteins, but instead transport likely occurs during the solvent exchange within the interior of the assembly, when these assemblies transiently find themselves at the interface during equilibration, as illustrated in Figure 1; (*v*) specific ligand−receptor interactions can be used to selectively extract proteins from the aqueous phase. Overall, the most gratifying finding here is that whole proteins can be moved across a solvent interface into the interior of a supramolecular assembly, even though the resident location of the assembly is in an incompatible solvent for the protein. The preliminary findings here have implications in many areas, especially in sensing, diagnostics, and catalysis. For example, these systems can be further developed to detect biomarkers in more complex mixtures of proteins.[17−20] Similarly, facile incorporation of active proteins in organic solvents could facilitate enzyme-based catalysis for a broader range of organic substrates.[21−25] These constitute examples of future directions for this research in our own laboratories.

■ ASSOCIATED CONTENT

⒮ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b13245.

> Materials and methods, syntheses and characterization of polymers, and supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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