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Thermally Induced Reversible Self-Assembly of Apoferritin - Block Copolymer Complexes

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Protein cages are interesting building blocks for functional supramolecular assemblies. Here we describe a multi-responsive system composed of apoferritin and thermoresponsive block copolymers complexed through electrostatic interactions. The polymers are linear chains with cationic and thermo-responsive blocks, and both diblock and triblock copolymers are studied. The apoferritin can be reversibly assembled and disassembled in aqueous solutions by altering the temperature and electrolyte concentration of the solutions. The control over the conditions is straightforward and all the components can be recovered, offering a potential alternative for systems requiring chemical or genetic modification of proteins.

Nanoscience has greatly advanced in terms of producing nanoscale objects and harnessing their unique properties. Lately, the focus has moved from individual components into larger assemblies, which exhibit novel functions and customized properties.^[1] Nature produces such structures by self-assembly, a process where components spontaneously arrange into larger systems. This repeats hierarchically, and can extend from nanoscale to macroscopic proportions.^[2] Self-assembly is most commonly carried out by non-covalent forces, as these relatively weak interactions allow the molecules to attach to and detach from each other in a dynamic manner, enabling them to reach the most favorable orientation.^[3] Self-assembly can be triggered by inducing an environmental stimulus, for example change in temperature or pH.^[4] Many polymers are known for their responsiveness and have, due to their modifiability, been studied for several biomedical applications, for example drug delivery,^[5-7] tissue

engineering,^[8] molecular adsorption^[9] and bioelectronics,^[10] but also in materials science, for example as self-healing materials^[11] and in soft robotics.^[12] By utilizing copolymers, multifunctional materials can also be designed.^[13]

Protein cages and other virus-like particles have emerged as an interesting platform in materials chemistry. They are hollow protein structures with uniform size and distinct functionalities.^[14] Ferritins are a widely studied subgroup of protein cages. They are abundant in living organisms, including higher mammals.^[15] Ferritins are spherical iron storage containers, most of which consists of 24 subunits, with an outer diameter of 12 nm and an inner cavity of 8 nm.^[16] Their ability to nucleate and store inorganic materials has led to their usage as scaffolds for 2D^[17] and 3D^[18] oriented inorganic nanostructures. Ferritin cage can also be further modified to enable the design of stimuli-responsive assemblies. Polymers can impart the systems with properties untypical for highly ordered structures, like self-healing^[19] and thermo-responsiveness. Covalently modified thermo-responsive ferritin has been reported,^[20] but the modification requires extra synthesis steps and is irreversible.

Electrostatic self-assembly is an appealing choice to design environmentally responsive systems, as it is straightforward to employ and can be reversed by increasing the electrolyte concentration, which screens the Coulombic interactions between the components.^[21]

Examples of such systems include for example protein cage directed binary nanoparticle superlattices.^[22]

Here we present an attempt to produce multi-responsive materials by complexing apoferritin (ferritin with no cargo in the inner cavity, aFT) with synthetic block copolymers consisting of cationized poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA) and thermo-responsive poly[di(ethylene glycol) methyl ether methacrylate] (PDEGMA) (**Figure 1**). The system is sensitive to both temperature and concentration of small electrolytes in the solution.

PDEGMA displays lower critical solution temperature (LCST) type behavior, meaning it is hydrophilic at low temperatures, but turns hydrophobic with increasing temperature. The used

polymers were linear diblock or triblock chains (sequence: PDMAEMA-PDEGMA-PDMAEMA).

All polymers were synthesized using atomic transfer radical polymerization (ATRP) reactions, which is a well-studied method for producing linear polymers with narrow polydispersity.^[23] The blocks were polymerized by synthesizing first a homopolymer, which was purified and used as a macroinitiator for the diblock copolymer. The triblock copolymer was similarly initiated from purified diblock copolymer. The length of the blocks and their ratio to one another is crucial for obtaining both electrostatically self-assembling and multi-responsive system. The cationic block must be long enough to have sufficient charge density for strong binding between the polymer and aFT, but not too long to cause aggregation regardless of the state of the thermo-responsive block. The LCST point of the polymer depends on the length of the PDEGMA block, which also needs to be long enough to induce sufficient hydrophobicity to aFT to induce the self-assembly. Additionally, the triblock copolymer was designed to study the effect of having cationic block at both ends of the chains, potentially enabling bridging between aFT cages. To meet the demands on both block types, we aimed for PDMAEMA block length of 10 kDa (in triblock 10 kDa at both ends of the chains) and mass ratio of PDMAEMA : PDEGMA to be 1 : 4 (in triblock PDMAEMA : PDEGMA : PDMAEMA mass ratio of 1 : 4 : 1). Nuclear magnetic resonance (NMR) spectroscopy and gel permeation chromatography (GPC) were used to characterize the synthesized polymers and quantitative ¹H NMR confirmed the diblock copolymer to have 10.6 kDa PDMAEMA block and 40.9 kDa PDEGMA block, giving mass ratio of PDMAEMA : PDEGMA to be 1.00 : 3.86. Triblock copolymer had an additional PDMAEMA block of 10.1 kDa, giving PDMAEMA : PDEGMA : PDMAEMA mass ratio of 1.00 : 3.86 : 0.95. Detailed synthesis processes and polymer characterization are described in the supporting information (**Figures S1 and S2**).

The multi-responsive behavior of the complexes was studied by dynamic light scattering (DLS) as a function of temperature and electrolyte concentration. To study the assembly process, diblock and triblock copolymers were titrated into aFT solution in various sodium chloride (NaCl) concentrations (**Figure 2a** for diblock and **2d** for triblock). The initial concentration of aFT was 100 mg L^{-1} and NaCl concentration ranged from 0 to 350 mM in increments of 50 mM. Both copolymers were titrated into these solutions at 25 and 50 °C up to a concentration of 90 mg L^{-1} . These temperatures were chosen to observe the system well below and above the LCST, which was determined to be roughly at 33 °C for the polymers (**Figure S4**). Without NaCl, both samples aggregated heavily at the higher temperature as assemblies with hydrodynamic diameters of 2–4 μm were observed. At 50 mM NaCl, the triblock sample formed aggregates of up to 6 μm at 50 °C. Then, up to 150 mM NaCl, both polymers aggregated at 25 °C to large assemblies of 1–2 μm , as the screening effect from NaCl is not strong enough inhibit the assembly process and the thermo-responsive blocks do not interfere with it. At 50 °C, the structures were in the size range of 300–800 nm, suggesting more compact assemblies. At the higher temperature, most systems also had the trend of shrinking assemblies with increasing polymer concentration, likely due to more efficient obscuring of the surface charges of aFT. From 200 mM NaCl upwards, only minimal amount of assemblies were formed at 25 °C and free aFT could be clearly observed at roughly 12 nm. Assemblies were still formed at 50 °C (**Figure 2b** for diblock and **Figure 2e** for triblock copolymer) and they presented the previous trend of shrinking with increasing polymer concentration up to 250 mM NaCl. From 300 mM NaCl up, assemblies formed at 50 °C at low polymer concentrations, but increasing the amount of polymer disassembled the structures back to free aFT roughly at polymer concentration of 60 mg L^{-1} for diblock and 70 mg L^{-1} for triblock copolymer. The maximum polymer concentration at which the assemblies formed decreased with increasing NaCl concentration, suggesting that the electrostatic

interactions between the cationic blocks and aFT became effectively screened at these NaCl concentrations.

Overall, the behavior of the diblock and triblock copolymers was similar: the only variables were the sizes of the assemblies and the exact polymer and NaCl concentrations at which the systems underwent changes. The titration data suggests there is no significant binding between multiple aFT cages *via* single polymer chains at NaCl concentrations of 200 mM and above, and even the triblock polymer chains are coordinated mostly to individual aFT cages. With effective cross-linking of aFT by the polymers, it would be expected that assemblies are formed also at low temperatures, as the distances between individual aFT cages would be limited by the length of the polymer chains. Additionally, the system appeared more sensitive to NaCl concentration at 25 °C than at 50 °C. This is most likely because at the lower temperature electrostatic interactions mainly determine the behavior of the system, but at the higher the solubility of the polymers contributes to the behavior, pushing the systems towards assemblies. For clarity, the DLS data in only selected NaCl concentrations is shown in **Figure 2** and the numerical data at a single polymer concentration in **Table 1**. The complete DLS data is presented in the supporting information (**Figure S3**). The self-assembly was found to be a reversible process, which could be repeated several times with one sample by repeating heating and cooling of the solution (**Figure 2c** for diblock and **2f** for triblock).

The formed complexes were imaged with transmission electron microscope (TEM). As control over the temperature of the sample is impossible during the imaging, samples of pure aFT and aFT complexes with both diblock and triblock copolymer were placed on TEM grids and dried at 25 and 50 °C (**Figure 3** and **Figure S5**). Without polymer complexation, aFT is spread relatively evenly on the grid and, as expected, shows no change with temperature. In the polymer containing samples prepared at the lower temperature, aFT is distributed evenly similarly to free aFT, but a lot of individual aFT cages are associated with each other in a linear manner, especially in the samples prepared with diblock copolymer. The samples

prepared at the higher temperature show larger and more numerous complexes, but isolated aFT cages are sometimes observable. The complexes are several hundred nanometers in size, which is smaller than predicted by DLS, but they likely shrink when drying, as the polymers contract strongly when the solvent is removed. The assemblies are also not uniform in shape and lack crystalline order. At 50 °C, the complexes do not significantly differ whether they contain the diblock or triblock copolymer. The electrolyte concentration of the solution is low, which leads to strong electrostatic interactions between aFT and the polymers. This limits the mobility of the molecules and creates kinetically trapped structures with amorphous morphology.^[24,25]

The polymer chains are not visible in the TEM images, and therefore the nature of their binding with aFT cannot be directly confirmed. As the associated aFT cages show close interactions at 25 °C and short linear chains of several cages, it is most likely that the polymers are bound to the surface of aFT by the cationic block and the thermo-responsive block stretches outwards from the protein surface. This hypothesis is also supported by the fact that the DLS data shows one sharp population of sizes at the lower temperature indicating there are only one type of particles present in the solution. At 50 °C, it is unclear whether the polymers remain attached to aFT or if they form polymer micelles with hydrophobic core and cationic shell, which then act as intermediary particles for aFT assembly. However, the micelle formation is less likely as the polymers are already associated with aFT as the temperature is increased, and polymer micelles are not seen in either DLS or TEM analysis. For a future note, in addition to linear polymers, the system would be fascinating to form using aFT and star-shaped block copolymers. The effect of the end-group block would supposedly increase in the system, making the system more sensitive to either temperature or electrolyte concentration by design.

In conclusion, we have prepared multi-responsive protein systems based on electrostatic interactions with synthetic block copolymers consisting of both cationic and environmentally

sensitive part. aFT used as the protein could be assembled into supramolecular assemblies in aqueous media by switching the temperature of the solution. The interactions between aFT and the polymers is based on electrostatic interaction, which is why the system is sensitive to the electrolyte concentration of the solution. Different behaviors of the systems were observed depending on the amount of small molecular weight salt present in the solutions, but the behavior of diblock and triblock copolymers differed little. In optimal electrolyte concentrations, the system could self-assemble into μm scale complexes and reversibly disassemble back to individual aFT cages by adjusting the temperature of the solution. By altering the composition of the polymers, we believe it is possible to adjust the LCST temperature of the system or add alternative environmental triggers without covalent modification of aFT.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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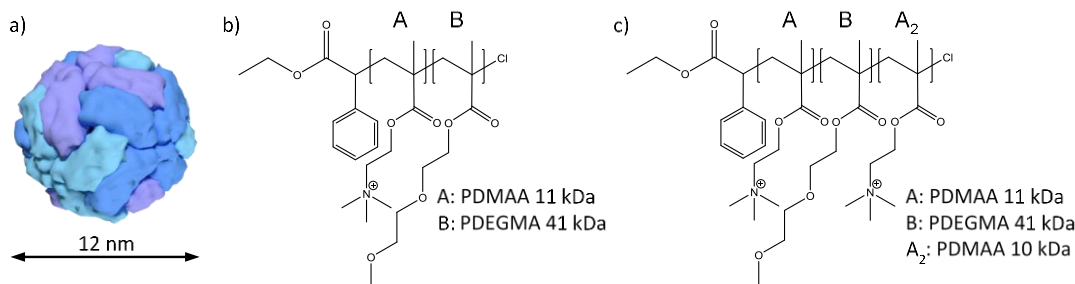


Figure 1. a) Schematic presentation of aFT protein cage and chemical structures of b) diblock and c) triblock copolymers with the molecular masses of the blocks.

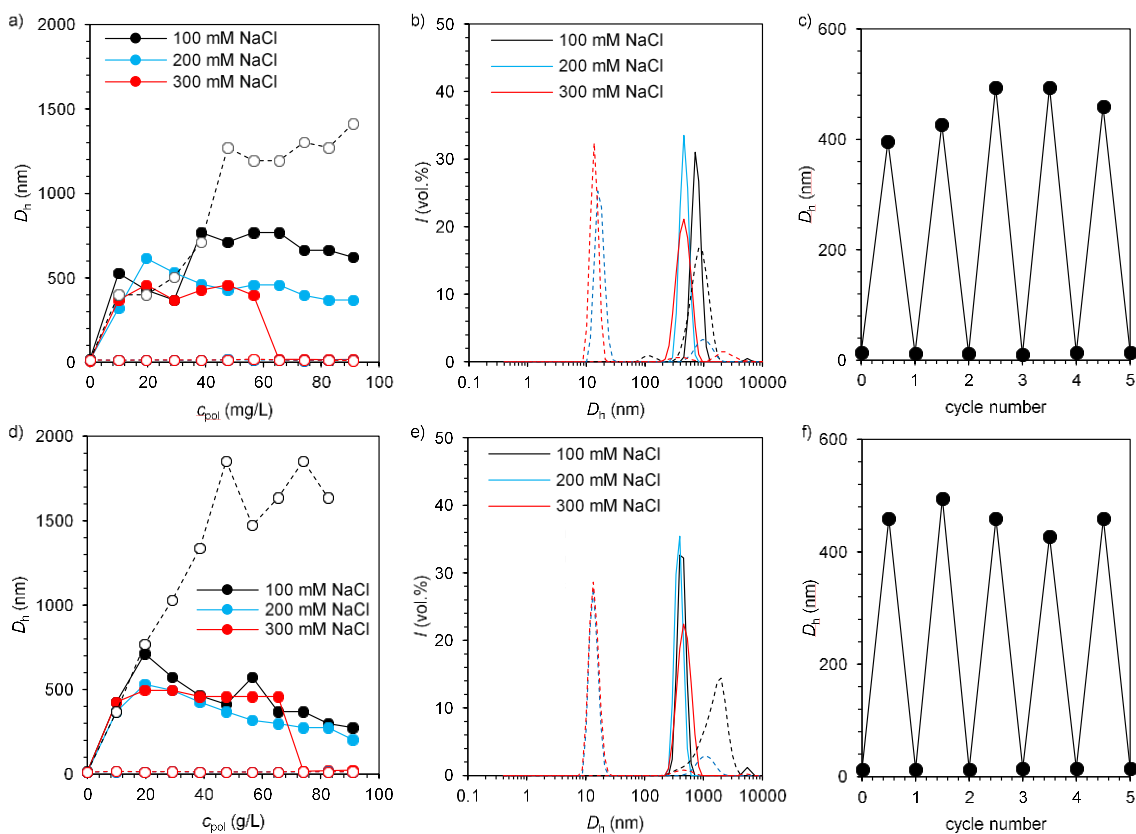


Figure 2. DLS characterization of aFT - polymer complexes at 25 °C (dashed lines) and 50 °C (full lines). a) Change in hydrodynamic diameter of the assemblies when diblock copolymer was titrated into 100 mg L⁻¹ aFT solution containing 100, 200 or 300 mM NaCl. b) Volume proportions of assemblies from panel a) at polymer concentration 50 mg L⁻¹. c) Change in hydrodynamic diameter of assemblies of 50 mg L⁻¹ diblock copolymer and 100 mg L⁻¹ aFT at 200 mM NaCl when temperature was cycled between 25 and 50 °C. Figures 2d–f are identical to panel 2a–c, respectively, except that triblock copolymer was used instead of the diblock copolymer.

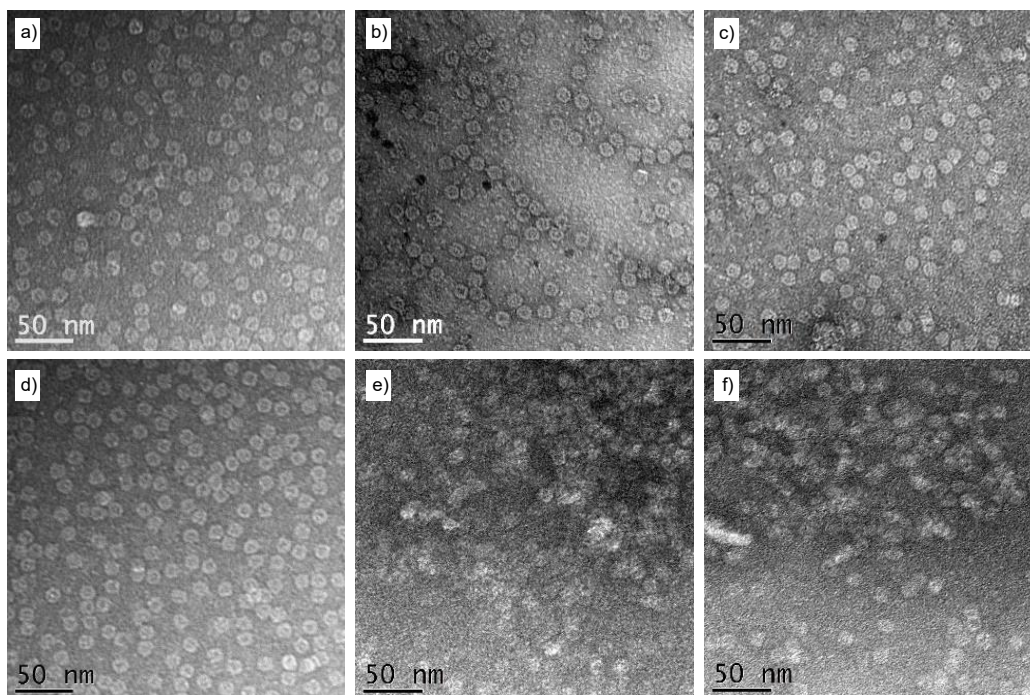


Figure 3. Negatively stained TEM images of a) pure aFT, b) diblock copolymer – aFT complex and c) triblock copolymer – aFT complex, all prepared at 25 °C. Samples d–f) are identical samples to a–c), respectively, but the samples were prepared at 50 °C. The polymer to protein ratio in all of the polymer containing samples was 1:2.

Table 1. Hydrodynamic diameters of the aFT-polymer complexes at 100 mg L⁻¹ aFT and 50 mg L⁻¹ in varying temperature and NaCl concentration.

Polymer	100 mM NaCl, 25 °C	100 mM NaCl, 50 °C	200 mM NaCl, 25 °C	200 mM NaCl, 50 °C	300 mM NaCl, 25 °C	300 mM NaCl, 50 °C
Diblock	1272 nm	712 nm	16 nm	427 nm	14 nm	459 nm
Triblock	1854 nm	413 nm	14 nm	369 nm	14 nm	459 nm