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Self-Assembly of Proteinaceous Shells Around Positively Charged Gold Nanomaterials Enhances Colloidal Stability in High Ionic Strength Buffers

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Abstract: The enzyme lumazine synthase (LS) has been engineered to self-assemble into hollow shell structures that encapsulate non-natural cargo proteins via complementary electrostatic interactions. Here we show that a negatively supercharged LS variant can also form organic-inorganic hybrids with gold nanomaterials. Simple mixing of LS pentamers with positively charged gold nanocrystals in aqueous buffer spontaneously affords protein-shelled gold cores. The procedure works well with differently sized and shaped gold nanocrystals, and the resulting shelled complexes exhibit dramatically enhanced colloidal stability over a wide range of pH (4.0–10.0) and at high ionic strength (up to 1 M NaCl). They are even stable over days upon dilution in buffer. Self-assembly of engineered LS shells in this way offers an easy and attractive alternative to commonly used ligand exchange methods for stabilizing inorganic nanomaterials.

Proteins that assemble into hollow spherical shells are widespread in nature. Among other roles, they serve as functional containers for iron storage (ferritins),^[1] nucleic acid delivery (viral capsids),^[2] and enzymatic cascade reactions (bacterial microcompartments).^[3] Studies showing that the neuronal messenger RNA arc can be encapsulated by its own translated protein and delivered to target cells^[4,5] has even highlighted the importance of protein shells in the mammalian brain.

Lumazine synthase from *Aquifex aeolicus* (AaLS) is a bacterial microcompartment that spontaneously forms a 16-nm-wide dodecahedron-like shell from 12 pentamers.^[6] We have engineered its structure for electrostatically-mediated cargo encapsulation.^[7] For instance, AaLS-13,^[8] an evolved variant that possesses a negatively supercharged luminal surface, reversibly encapsulates positively charged cargo proteins at rates approaching the diffusion limit.^[9,10] Cryo-electron microscopy (EM) analysis revealed that AaLS-13 self-assembles into a 39-nm

Figure 1. (A) Schematic representation of proteinaceous shell formation around a positively charged gold nanoparticle. (B) Illustration of the (11-mercaptoundecyl)-N,N,N-trimethylammonium (TMA) ligand on the surface of the gold nanomaterials. (C) Zeta potentials of the positively charged AuNP ($d = 22$ nm) with and without the AaLS-13 protein shell.

icosahedrally symmetric hollow sphere composed of 72 pentamers.^[11] Partially assembled AaLS-13 pentamers have also been used to create multilayered shell structures.^[12,13]

In the current study, we have examined the feasibility of assembling proteinaceous AaLS-13 shells around inorganic nanomaterials.^[14–20] Spherical gold nanoparticles (AuNPs) are useful for drug delivery,^[21] photocatalysis,^[22] as biological sensors^[23,24] and as conjugation platforms for oligonucleotides and biomolecules.^[25] Anisotropic gold nanorods (AuNRs), on the other hand, have advantages for dynamic photothermal therapy^[26,27] and quantum yield enhancement of nearby fluorophores.^[28,29] Nevertheless, poor water solubility generally limits the utility of such materials, and water-soluble synthetic ligands are usually attached to their surface to enhance stability. Here we show that positively charged gold nanomaterials of different sizes and shapes can be further stabilized simply by mixing with AaLS-13 pentamers (Figure 1A). The resulting shelled structures possess substantially enhanced colloidal stability in aqueous solution over a wide range of pH and NaCl concentrations.

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Figure 2. Negative-stain TEM images of (A) unshelled AuNPs ($d = 6\text{--}16\text{ nm}$) and (B) the corresponding AuNPs with the AaLS-13 protein-shell. Scale bars: 50 nm. (C, D) Magnified images of the selected particles from (A, B), respectively. Scale bars: 50 nm.

We recently reported the preparation of cationic gold nanocrystals derivatized with short (1–2.5 nm), positively charged organic ligands.^[30] For example, (11-mercaptoundecyl)-N,N,N-trimethylammonium bromide (TMABr) (Figure 1B) was found to stabilize 22-nm AuNPs in water. Consistent with a positive surface charge, 22-nm TMABr-capped AuNPs have a zeta potential of +20 mV (Figure 1C). When these particles were mixed with negatively charged AaLS-13 pentamers, the zeta potential dropped to -25 mV (Figure 1C), suggesting formation of a specific complex. The presence of some free pentamer may alter the measured value of zeta potential due to the variation in the solution ionic strength. It acts as a highly charged ion that impacts the diffusion layer around the nanocrystals, adjusting the Debye screening length, but not altering the sign of the potential at the slipping plane. This measurement thus confirms that the entire ensemble of NPs had acquired negative surface charge. Coexistence of cationic and anionic highly charged large species would lead to their fast aggregation, followed either by precipitation of aggregates or, in some cases, formation of organized halo structures as reported elsewhere.^[30–32] Negative-stain EM images (Figure 2,3) confirmed that the resulting structures consisted of a core NP coated with a uniform, ~4-nm thick proteinaceous shell as expected for a single layer of AaLS-13 pentamers. Because the particles did not change color, shelling appears not to alter their colloidal properties.

The dominant interaction force between AaLS-13 and the AuNPs must be electrostatic as seen for encapsulation of the proteinaceous cargo.^[9,10,12] To clarify this point, we tested the ability of AaLS-13 pentamers to shell a 20-nm AuNP capped with 11-mercaptoundecanoic acid (MUDA), an analogous anionic ligand of TMABr that has a terminal carboxylate moiety in place of TMABr's quaternary ammonium group. As expected, given that the AuNPs and the protein are both negatively charged, AaLS-13 did not protect the NPs, and the color of the mixture immediately turned blue when they were mixed in Tris-HCl buffer (pH 7.8) containing 0.2 M NaCl. The same results were obtained in control experiments without the protein pentamers and indicate rapid aggregation of the AuNPs in both cases. These results confirm that positive charge of the AuNPs is important for shell formation.

Figure 3. Negative-stain TEM images of (A) unshelled AuNPs ($d = 22\text{ nm}$) and (B) the corresponding shelled AuNPs. Scale bars: 50 nm. (C) Absorption spectra of the unshelled AuNPs ($d = 22\text{ nm}$) in H_2O (1), and the corresponding unshelled (2) and protein-shelled (3) AuNPs incubated in PBS for up to 24 h. (D) Photographs of 1, 2, and 3.

Similar experiments were carried out with mixtures of 6 to 16-nm TMABr-capped AuNPs in Tris-HCl buffer (pH 7.8) containing 0.2 M NaCl. Regardless of the particle diameter, complexes were obtained in which the AuNPs were encapsulated within ~4-nm thick proteinaceous shells (Figure 2B). These structures, which had diameters ranging from 14 to 24 nm, are smaller than the native AaLS-13 cage (39 nm). Thus, in contrast to many viral proteins that only form shelled structures comparable in size to their native capsid,^[14,16–18] AaLS-13 can apparently adapt to the templating NP and assemble into quasi-spherical structures of variable dimensions. The spontaneous radius of curvature of the AaLS-13 pentamers, as well as malleable interactions between adjacent subunits,^[11] may account for the formation of such shells. Although icosahedrally symmetric assemblies are possible, nonicosahedral arrangements of pentamers covering the NPs is perhaps more likely.^[13,33] In control experiments with bovine serum albumin, which also has a negatively charged surface at neutral pH, proteinaceous layers around the TMABr-capped AuNPs were not observed (Figure S1 in the supporting information).

Because AaLS pentamer fragments spontaneously self-assemble into capsids and higher order aggregates,^[13] dynamic light scattering (DLS) could not be used to monitor the colloidal stability of the shelled AuNPs over time. Instead, we used absorption spectroscopy for this purpose. According to Mie's scattering theory, metallic NPs absorb strongly at a specific wavelength due to resonance excitation of a surface plasmon.^[34]

Figure 4. (A, C) Absorption spectra and (B, D) photographs of the protein-shelled gold nanoparticles ($d = 22$ nm) in biological buffers at various pHs (A, C: 4.0–10.0) or in aqueous solution containing increasing amounts of NaCl (B, D: 0–1 M). The spectra and photos were taken 2 h and 1.5 h after mixing, respectively.

An absorption peak around 520 nm accounts for the characteristic pink color of colloidal solutions of the small (<33 nm) AuNPs studied here. When these NPs aggregate, electromagnetic multipole interactions between the particles alters their optical properties and the solutions turn blue.^[35,36] Although 22-nm TMABr-capped AuNPs are stable in water, the pink particles immediately turned blue in PBS (<2 sec) (Figure 3C,D), indicating aggregation,^[34] and precipitation was observed upon standing for extended periods. Formation of a protective shell upon incubation with AaLS-13 pentamers (Figure 3B) prevented this color change. Absorption spectra of the shelled AuNPs showed a plasmon resonance at 555 nm, which is red-shifted by 30 nm relative to the plasmon resonance of the unshelled AuNPs, owing to an increase in refractive index upon shelling,^[15,17] but ca. 100-nm blue-shifted relative to aggregated particles (Figure 3C). The plasmon band of the shelled AuNPs did not change over 24 h, demonstrating the protective effects of the protein shell (Figure 3C,D). The gradual decrease in absorption of the shelled NPs without accompanying changes in the shape of the absorption band indicates that the NPs slowly settle over days but retain their integrity. Such behavior is expected for large and heavy nanocrystals, because steric and electrostatic repulsion between the NPs is no longer sufficient to outweigh the gravity.^[30]

The benefits of shelling are not restricted to PBS. The AaLS-13-AuNP complexes were stable for several days in various biological buffers (acetate pH 4.0 and pH 5.0, MES pH 6.0, MOPS pH 7.0, Tris-HCl pH 8.0, and CHES pH 9.0, 10.0) and in aqueous solutions containing up to 500 mM NaCl (Figure 4). In 1 M NaCl, the samples were stable for several hours, but some aggregation was observed after 1 day. These results show that the shelled AuNPs are sufficiently stable for practical applications in biologically relevant environments.

In addition to studying spherical gold nanomaterials, we tested whether our shelling procedure could be applied to rod-shaped nanomaterials. TMABr-capped AuNRs (13 x 50 nm) were synthesized as previously reported^[30] and incubated with AaLS-

Figure 5. Negative-stain TEM images of (A) unshelled AuNRs (13 x 50 nm) and (B) the corresponding shelled AuNRs. Scale bars: 50 nm. (C) Absorption spectra of unshelled AuNRs (1) in H₂O, and the corresponding unshelled (2) and protein-shelled (3) AuNRs incubated in PBS for up to 24 h. (D) Photographs of solutions containing 1, 2, and 3.

13 pentamer fragments. As for the spherical particles, negative-stain TEM images show that a single layer (~ 4 nm) of the AaLS-13 protein formed around the AuNRs (Figure 5B). The AuNRs exhibited characteristic plasmon bands at 800 and 510 nm in water (Figure 5C; 1), which correspond to longitudinal and transverse resonances, respectively.^[37] After protein shelling, the former broadened somewhat and the latter was red-shifted to 520 nm. However, these spectral features did not change over the course of the experiment, indicating that the shelled complexes were stable in buffer solution for at least 24 hrs (Figure 5C; 3). In contrast, substantial broadening of the plasmon bands and a decrease in intensity were observed for the unshelled AuNRs. The latter quickly aggregated and gradually precipitated from solution (Figure 5C,D; 2).

How many AaLS-13 pentamer fragments are needed to shell the gold nanomaterials? To answer this question, the number of AaLS-13 pentamers required to stabilize the 22-nm TMABr-capped AuNPs was determined by varying the amount of AaLS-13 while keeping the AuNP concentration constant. Judging by the color of the sample solutions as well as their absorption spectra, a 300 to 400-fold molar excess of AaLS-13 monomer was sufficient to stabilize the 22-nm AuNPs (Figure 6). A 200- and 100-fold excess afforded only partial stabilization, whereas a 50-fold molar excess resulted in immediate color change, signaling aggregation of the particles. Since quasi-spherical 29 and 39 nm AaLS structures are known to contain 180 and 360 monomers, respectively,^[11] ca. 200 monomers, corresponding to 40 pentamers, are needed to construct a single-layer protein shell of ~ 4 -nm thickness and ~ 30 -nm diameter around the 22-nm AuNP core. The greater protection provided by

Figure 6. (A) Absorption spectra and (B) a photograph of the gold nanoparticles ($d = 22$ nm) in the presence of various concentrations of AaLS-13. Ratios of AaLS-13 monomer to AuNP were adjusted to 500:1, 400:1, 300:1, 200:1, 100:1 and 50:1, respectively, while the NP concentration was kept constant. The photo and spectra were recorded 1 and 2 h after mixing, respectively.

higher molar excesses suggests that some unbound AaLS-13 pentamers may be needed during shell formation. Nonetheless, the shelled AuNP complexes exhibit stability over days upon dilution in buffer or electrolyte solutions containing no pentamers, indicating that, once formed, the pentamer shell does not easily dissociate from the AuNP. In addition, pellets of the shelled AuNPs after centrifugation could be redissolved in water or buffer although it is not the case for the aggregated NPs.

In summary, we have demonstrated that negatively charged AaLS-13 pentamer fragments can form proteinaceous shells around positively charged gold nanomaterials. We found that self-assembly of the AaLS-13 protein is easy and scalable, with shell dimensions dictated by the size and shape of the AuNP core. Even rod-shaped gold nanomaterials can be coated simply by mixing the components in aqueous buffer. The AaLS-13 shelling procedure thus offers an attractive alternative to commonly used ligand exchange methods for enhancing the colloidal stability of relatively large (>20 nm) nanocrystals in aqueous buffer. Further engineering could also extend the utility of this system. Introduction of targeting tags to control the fate of the encapsulated nanomaterials in complex biological fluids would be particularly interesting in this context.

Experimental Section

Preparation of the gold nanocrystals with the positively charged ligand. (i) AuNPs (11 ± 5 nm): The synthesis of the 11 ± 5 nm AuNPs capped by both oleylamine and trioctylphosphine oxide (TOPO) was performed according to a previously published procedure.^[38] The resulting AuNPs were then converted to TMABr-capped AuNPs by a ligand exchange reaction.^[30] (ii) AuNPs (22 nm): The 22 nm AuNPs capped by hexadecyltrimethylammonium chloride (CTAC) were synthesized following a published procedure.^[39] The resulting AuNPs were converted to TMABr-capped AuNPs by a round-trip ligand exchange reaction (from hydrophilic CTAC to hydrophobic 1-dodecanethiol (DDT) and then back to hydrophilic TMABr) as described before.^[30,40] (iii) AuNRs (13×50 nm): The AuNRs capped by hexadecyltrimethyl-ammonium bromide (CTAB) were synthesized according to a published procedure.^[41] The resulting AuNRs

were converted to TMABr-capped AuNRs by the round-trip ligand exchange reaction.^[30,40]

Preparation of the gold nanocrystals with the negatively charged ligand. The synthesis of the 20 nm AuNPs capped by MUDA was performed according to a previously published procedure.^[42]

Preparation of the AaLS-13 pentamers. The AaLS-13 protein was produced with a C-terminal His₆-tag in *E. coli* strain BL21-gold (DE3) and purified by Ni-NTA affinity chromatography as previously described.^[8,13] The AaLS-13 pentamer fraction was isolated by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-400 column (GE Healthcare Life Science) eluted with 20 mM Tris-HCl buffer (pH 8) containing 0.2 M NaCl.^[13]

Preparation of the protein-shelled gold nanomaterials. In a typical shelling reaction, small amounts of the TMABr-capped gold nanocrystals in H₂O were added to the AaLS-13 pentamer solution in 20 mM Tris-HCl buffer (pH 8) with 0.2 M NaCl ("shelling solution"). For AuNPs of 6–16 nm in diameter, a 50:1 ratio of AaLS-13 monomer (2 μ M final monomer concentration) to AuNP (40 nM final concentration) was used, whereas a 500:1 or 1000:1 ratio of AaLS-13 monomer (5 μ M final monomer concentration) to AuNP (5 or 10 nM final concentration) was used for the 22-nm AuNPs. For the AuNRs (13×50 nm), a 2000:1 ratio of AaLS-13 monomer (20 μ M final monomer concentration) to AuNR (10 nM final concentration) was used to produce the shelled materials. The mixed solutions were left standing or shaken at 400 rpm for 3–16 h at room temperature.

Absorption spectroscopy. Absorption spectra were measured on a Jasco V-670 UV-VIS-NIR Spectrophotometer.

Zeta potential. Zeta potentials were measured using Zetasizer Nano-ZS (Malvern Instruments). The zeta potentials of the 22-nm TMABr-capped AuNPs (0.7 nM final concentration) with and without the AaLS-13 protein shell (0.35 μ M final monomer concentration) were recorded in H₂O.

TEM. To evaluate crystal size, images of the gold nanocrystals were recorded using a JEOL JEM 2200 fs microscope operated at 200 kV. TEM grids were first immersed in toluene for 20 s to remove formvar protection, followed by hydrophilization via a short plasma treatment prior to deposition of the nanocrystals in 5 μ L of H₂O. The grids were dried in vacuo for 3 h prior to the TEM measurements. To visualize the gold nanocrystals with proteinaceous shells, negative-stain EM images were recorded with a Morgagni 268 (FEI) microscope operated at 100 kV. Typically, 4 μ L of the "shelling solution" described above was deposited onto glow-discharged copper grids with carbon support films. The grids were washed with 20 mM Tris-HCl buffer (pH 8) containing 0.2 M NaCl and stained with 2% uranyl acetate prior to TEM analysis.

Stability in PBS. PBS (pH 7.4) containing 1 mM potassium phosphate, 3 mM sodium phosphate and 155 mM NaCl was purchased from ThermoFisher Scientific. A 50 μ L aliquot of the "shelling solution" described above was diluted in 1 mL PBS (final concentrations of the AaLS-13 monomer and the AuNP concentrations were 250 nM and 250 pM, respectively; final concentrations of the AaLS-13 monomer and the AuNRs were 1 μ M and 500 pM, respectively). Absorption spectra were measured immediately after dilution (~ 3 min) or after incubating the solutions for 1.5, 6 and 24 h at room temperature. Notice that the associated photographs in Figure 3 and Figure 5 contain a more concentrated solution of the gold nanocrystals in PBS than that measured by UV-vis absorbance. Briefly, 50 μ L of the "shelling solution" was diluted in 400 μ L of PBS (final concentrations of the AaLS-13 monomer and the AuNPs were 560 nM and 560 pM, respectively; final concentrations of the AaLS-13 monomer and the AuNRs were 2.2 μ M and 1.1 nM, respectively). Unshelled AuNPs and AuNRs at corresponding concentrations were used

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as reference, in both H₂O and PBS, for the absorbance measurements and photographs.

Stability in different pH and in different NaCl concentrations. A 50 μ L aliquot of the "shelling solution" described above was diluted in 1 mL with the desired buffer pH or NaCl concentration (final concentrations of the AaLS-13 monomer and AuNPs were 250 nM and 250 μ M, respectively; final concentrations of the AaLS-13 monomer and the AuNRs were 1 μ M and 500 μ M, respectively). Specifically, 20 mM acetate buffer (pH 4.0, 5.0), MES buffer (pH 6.0), MOPS buffer (pH 7.0), Tris-HCl buffer (pH 8.0), and CHES buffer (pH 9.0, 10.0) were used. Photographs of the solutions were taken after 2-h incubation at room temperature. Absorption spectra were measured after additional 30 min incubation.

Ratio experiment. A calculated volume of the stock AaLS-13 pentamer (93 μ M monomer concentration) was added to a 20 mM Tris-HCl buffer (pH 8) containing 200 mM NaCl, bringing the total volume to 190 μ L. To this solution, 10 μ L of the AuNPs in H₂O (approx. 6 mg/mL, 90 nM) were added, bringing the final AuNPs concentration to 4.5 nM. The resulting shelling solutions, where the ratios between the AaLS-13 monomer and the AuNPs were 500:1, 400:1, 300:1, 200:1, 100:1, 50:1, respectively, were left to incubate at room temperature. The color change was readily apparent immediately after the addition of the AuNPs, although the photographs were taken after 1 h incubation. An absorption spectrum was measured at each ratio by diluting the sample (200 μ L) to 1 mL with the same buffer solution (final concentrations of the AaLS-13 monomer and the AuNPs were 0–450 nM and 0.9 nM, respectively) after additional 1-h incubation.

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