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Anisotropic nanocrystal arrays organized on protein lattices formed by recombinant clathrin fragments

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Abstract

Recombinant clathrin protein fragments form assemblies that template gold nanocrystals in an array across the latticed surface. The nanocrystals exhibit unusual anisotropic morphologies with long range ordering, both of which are dependent upon the presence of a hexahistidine tag on the clathrin heavy chain fragments.

The fabrication of inorganic nanocrystals with anisotropic morphologies can lead to remarkably distinct physicochemical properties compared to analogous bulk materials, establishing desirable optical, electronic, magnetic and mechanical properties useful for devices and sensors.^{1–4} However, the controlled assembly of anisotropic nanocrystals to create patterned arrays has not yet been fully explored. Although current methods for ordering anisotropic nanoparticles include electrified jetting,⁵ microcontact printing,⁶ emulsion drying⁷ and selective deposition,⁸ these approaches can require costly reagents or specialized instrumentation. In this study, we explore the potential of biologically inspired methods^{9–11} to enable the formation and patterned array of anisotropic nanocrystals.

Nature can produce organized, nanometer-scale supramolecular architectures incorporating inorganic materials under ambient conditions.^{12–13} In addition to the exquisite self-assembling proteins present in viruses,¹⁴ plants,¹⁵ filaments,¹⁶ and microorganisms¹⁷, natural proteins from organisms such as diatoms,¹⁸ sponges,¹⁹ and sea urchins²⁰ as well as peptides selected from phage and cell displays^{21–35} are able to control the biomineralization and crystallization of inorganic materials. We seek to couple the ability for supramolecular self-assembly with the growth of inorganic materials using recombinant clathrin proteins as a platform for the fabrication of anisotropic nanocrystals in an ordered array.

Clathrin proteins play a critical role in forming coats on intracellular transport vesicles during endocytosis and organelle biogenesis.³⁶ During vesicle coat formation, clathrin heavy

chains in a triskelion configuration self-assemble into polyhedral cages ranging in size from 80–200 nm that feature a pentagonal/hexagonal lattice pattern.³⁷ Recently, full length clathrin triskelia, comprising both the heavy and light chain clathrin subunits, have been used to synthesize organic/inorganic hybrid nanoparticles of defined composition and size. Mann and coworkers have employed the clathrin surface for condensation of preformed aminopropyl-functionalized magnesium phyllosilicate and aminopropyl-functionalized silica, along with template-directed co-precipitation of CdS quantum dots.³⁸ The Heilshorn group has exploited the clathrin cage interior for templation of Au, Ti and Co metal nanoparticles.³⁹ The potential biomedical applications of these assemblies are becoming evident, as Vitaliano *et al.* have covalently modified clathrin assemblies with gadolinium chelators for magnetic resonance imaging.⁴⁰ In these reports, the nanoparticle synthesis is constrained by the yields of clathrin that can be purified from porcine³⁸, bovine³⁹ and rat⁴⁰ tissues, limiting the ability to modify the protein cage and to develop new materials synthesis protocols.

Previously, we have characterized recombinant clathrin heavy chain protein fragments that can reproduce features of native clathrin upon pH-triggered self-assembly *in vitro*.⁴¹ Described here as “Hub-His₆”, the protein comprises the C-terminal 1074–1675 residue fragment of clathrin heavy chain along with an N-terminal hexahistidine tag (Fig. 1a). Hub-His₆ proteins have the capacity to trimerize into a truncated triskelion, and these triskelia can further polymerize into two varieties: open cage-like structures and extended, branched assemblies.⁴¹ Both these structures are comprised of irregular lattices of hexagonal and pentagonal faces, which are more visible in the cage-like assemblies.^{41–42} The N-terminal hexahistidine tag facilitates protein purification with additional potential to template the deposition of metallic chemical species, such as gold.⁴³ Because the recombinant system enables reliable and potentially modifiable production of self-assembling clathrin proteins, we explore Hub-His₆ as a scaffold for materials synthesis. Here, we report the use of the self-assembling recombinant clathrin Hub-His₆ to simultaneously control the anisotropic growth and organization of gold nanocrystals into a unique organic/inorganic hybrid structure. Along with characterizing the cage-like assemblies by transmission electron microscopy (TEM), dynamic and static light scattering (DLS, LS), we analyze the templated gold nanocrystals as well as their arrangements using high resolution-transmission electron microscopy (HR-TEM), scanning transmission electron microscopy (STEM) and energy dispersive x-ray spectroscopy (EDS).

Hub-His₆ was produced through recombinant bacterial expression and purified via cobalt affinity resin, followed by size exclusion chromatography.⁴¹ As a negative control, the C-terminal sequence lacking the histidine tag was generated by thrombin protease treatment of purified Hub-His₆, using the cleavage site present between the hexahistidine tag and clathrin heavy chain fragment (producing “Hub-NoTag”) (Fig. 1a). SDS-PAGE of the purified proteins displayed discrete bands at 68 kDa and 66 kDa, indicating the presence of Hub-His₆ and Hub-NoTag, respectively (ESI, Fig. S1[†]). Following purification, the Hub-His₆ and Hub-NoTag proteins were induced to assemble into latticed structures in 10 mM HEPES buffered solution by adjusting the pH from 7.9 to 6.8. The clathrin structures could be subsequently disassembled by increasing the pH to 9.0 (Fig. 2).

LS monitored at 320 nm, revealed that both Hub-His₆ and Hub-NoTag displayed similar assembly and disassembly behaviour, consistent with our previous studies of recombinant clathrin proteins (Fig. 2a,b).⁴¹ Although Hub-His₆ and Hub-NoTag can form both cage-like assemblies and extended, branched lattices (ESI, Fig. S2 and S3[†]), our interest is in nanostructures that most resemble native clathrin assemblies, which are typically 80–200 nm

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cages. TEM images revealed the presence of irregular cage-like assemblies with diameters of 99.4 ± 46.9 nm and 122.4 ± 70.2 nm for Hub-His₆ and Hub-NoTag, respectively, which was confirmed via DLS (Fig. 2c,d and ESI, Fig. S4 and Table S1[†]). Presumably, these structures of limited size arise from self-assembly of the Hub fragments, allowing closure. Cage-like structures formed from Hub-His₆ and Hub-NoTag proteins were reliably reproduced from several different sample preparations (ESI, Fig. S3[†]).

The Hub-His₆ and Hub-NoTag assemblies were used to template gold nanocrystals (Fig. 1b). Both samples at 0.1 mg/mL were incubated with (CH₃)₃PAuCl in H₂O and triggered for simultaneous protein assembly and gold nucleation via addition of HEPES buffer, pH 6.5.^{44–45} After incubation for 2.5 hours at room temperature, the final gold-templated assemblies were assessed via DLS and TEM. The overall sizes of the gold-templated Hub-His₆ and Hub-NoTag structures demonstrated diameters of 155.9 ± 47.3 nm and 191.2 ± 57.6 nm, respectively by TEM, exhibiting a slight increase in size over untemplated parent cages (ESI, Table S1[†]). To quantify the extent of gold nanoparticle formation, gold-templated Hub-His₆ and Hub-NoTag were counted across a 240 μ m $\times$ 240 μ m of grid space for both uranyl acetate-stained and unstained conditions using TEM. The Hub-His₆ possessed templated gold nanoparticles for 100% of the assemblies, while gold crystallization was present only in 5% of Hub-NoTag assemblies (ESI, Table S2[†]). This indicated that the presence of a hexahistidine tag can significantly enhance the capability of the recombinant clathrin assemblies to template formation of gold nanocrystals. Hub-His₆ demonstrated organized plate-like structures of dense gold crystals that were absent in the case of Hub-NoTag, indicating that the histidine tag serves to specifically nucleate gold in a defined configuration (Fig. 3a,b).

Further analysis using HR-TEM revealed that the gold nanocrystals present on the Hub-His₆ assemblies had dimensions of 11.8 ± 2.7 nm $\times$ 3.4 ± 1.1 nm and an average aspect ratio of 3.5 (Fig. 3a and ESI, Fig. S5[†]). Notably, the nanocrystals were oriented in a single direction with plate-like morphologies extending across the cage-like assembly (Fig. 3a). In contrast, the Hub-NoTag assemblies possessed a diminished number of spherical nanocrystals with an average diameter of 2.5 ± 0.6 nm that lacked an organized pattern (Fig. 3b and ESI, Fig. S5[†]). Fourier transforms of the HR-TEM images revealed crystal lattices with a d-spacing of 0.26 nm for both Hub-His₆ and Hub-NoTag assemblies, corresponding to the (111) facet of FCC gold crystals (Fig. 3c,d and ESI, Table S3[†]).⁴⁶

To characterize the metal templation on Hub-His₆ and Hub-NoTag protein assemblies, dark-field imaging via STEM was performed on gold-templated lattices for both samples (Fig. 3e,f). Consistent with TEM analysis, the Hub-His₆ assemblies demonstrated dense gold deposition, while the Hub-NoTag assemblies displayed significantly less gold (Fig. 3e,f).

Differences in gold templation of Hub-His₆ and of Hub-NoTag were further established by EDS. While both EDS spectra showed the presence of M α and L α Au peaks at 2.1 and 9.7 keV, respectively, there was substantially weaker gold detection in the EDS spectra for Hub-NoTag (Fig. 3e,f).

Conclusions

While protein assemblies, including virus nanoparticles and other protein cages^{30, 47–50} have been engineered to crystallize a variety of inorganic materials, the resulting particles are generally isotropic and spherical in nature or coat the entire surface in a continuous layer. Recently, superlattice structures generated from anisotropic nanocrystals have been engineered via selective functionalization with DNA leading to 3D assemblies.⁵¹ In that case, premade rhombic dodecahedral particles were chemically modified with DNA, which

enabled the self-assembly into a defined body-centered cubic lattice. Our studies represent the first demonstration of anisotropic templation of inorganic nanocrystals with long-range order via recombinant protein assemblies. Through this approach, well-defined nanocrystals are formed concomitant with protein lattice assembly, eliminating the need to separately fabricate the particles and conduct subsequent chemical functionalization.

The formation of anisotropic nanocrystals typically involves a two-step, seed-mediated growth process.⁵² Previous investigations have described a fast initial gold nucleation stage, demonstrating that gold seed particles can be formed within the first minute of reduction.^{52–53} A slower subsequent growth step can then propagate the nanoparticle formation within a distinct environment.⁵² For example, the presence of surface-interacting peptides,^{53–54} proteins⁵⁵ or cellular structures,⁵⁶ can modify the morphologies of metal nanoparticles by preferentially interacting with one region of the nanoparticle, restricting nanoparticle growth and leading to anisotropic forms.

In our system, the presence of the hexahistidine tag on the hub is necessary for producing anisotropic gold nanocrystals on clathrin assemblies. Although Ueno *et al.* have employed a hexahistidine tag to grow isotropic gold nanoparticles on a protein assembly,⁴³ our clathrin system presents unique conditions for anisotropic, plate-like, nanocrystal growth. The oligohistidine tags presented on the clathrin hub may allow for rapid nucleation of gold nanoparticles. As Au atoms continue to associate at the nucleation site, the surrounding protein matrix may modulate particle growth, leading to the generation of shape-controlled nanocrystals on the clathrin assembly.⁵⁷ The exact role of the recombinant clathrin scaffold in conjunction with the hexahistidine tags and how the clathrin assemblies lead to anisotropic gold nanocrystal growth remain subjects for further investigation.

In summary, Hub-His₆ assemblies demonstrated organized plate-like structures of highly dense gold nanocrystals that were absent in the case of Hub-NoTag, indicating that the presence of the oligohistidine tag serves to produce gold in a defined morphology. Although the presence of hexahistidine tags on recombinant proteins has been reported to template gold nanoparticles,⁴³ the hexahistidine tag in the context of these clathrin cage-like assemblies facilitates: (1) anisotropic growth of gold nanocrystals and (2) uniform patterning of the nanocrystals across the protein surface. The advantage of our system arises from the potential to genetically program various inorganic substrate-recognizing peptide sequences into the self-assembling clathrin heavy chain protein.²² Further modifications are possible in a recombinant system, including the addition of clathrin light chain subunit proteins capable of binding to the clathrin heavy chain.^{58–59}

Experimental section

General

Cobalt resin was purchased from Talon. Imidazole, tris base, dithiothreitol (DTT), Isopropyl β -D-1-thiogalactopyranoside (IPTG), HEPES were purchased from Fisher Scientific. (CH₃)₃PAuCl, Thrombin CleanCleave™ Kit and uranyl acetate were purchased from Sigma Aldrich. QUANTIFOIL holey carbon-coated 300 mesh grids were purchased from Ted Pella. Carbon/formvar-supported copper grids were purchased from Pacific Grid Tech. BL21 cells were obtained from Novagen.

Protein Expression and Purification

pET15b vector containing an N-terminally Hub-His₆ protein was produced in the Brodsky laboratory at UCSF. The above plasmid was transformed into BL21 cells, and the Hub-His₆ protein was expressed as described previously.⁴¹ The resulting purified Hub-His₆ in 10 mM

HEPES (pH 7.9) buffer was assessed by 12% SDS-PAGE. The protein was either used directly for templation experiments or further treated for His₆-tag cleavage.

Thrombin cleavage of hexahistidine-tag

The Hub-His₆ protein was treated with Sigma Thrombin CleanCleave™ Kit, as per manufacturer's instructions. Briefly, 0.63 mg/mL of Hub-His₆ protein was incubated with thrombin-conjugated agarose resin for 2 hours while gently rotating in 4 °C. The solution was then subjected to centrifugation at 4 °C to recover the cleaved protein from the supernatant. Pure Hub-His₆ and Hub-NoTag protein were assessed on a 14% SDS-PAGE.

Hub-His₆ and Hub-NoTag assembly

Assembly into spherical, cage-like structures was triggered by lowering the pH using 10% volume of assembly buffer (1.5 M HEPES, pH 6.5), as described by Liu *et al.*⁴¹ Briefly, 100 µL of 0.1 mg/mL either Hub-His₆ or Hub-NoTag protein was treated with 10 µL of assembly buffer, and was immediately monitored by static light scattering at 320 nm on an Agilent Cary 100 spectrophotometer.

Gold metal templation using Hub-His₆ and Hub-NoTag

Approximately, 200 µL of 0.1 mg/mL of Hub-His₆ or Hub-NoTag protein in 10 mM HEPES (pH 7.9) were mixed with 100 µL of saturated (CH₃)₃PAuCl in water.⁶⁰ Simultaneous initiation of protein assembly and gold metal reduction was triggered by the addition of 30 µL of 1.5 M HEPES buffer (pH 6.5), and the samples were subjected to shaking for 2.5 hours at room temperature. The samples were assessed by DLS electron microscopy.

Dynamic Light Scattering

Malvern disposable 50 polystyrene cuvettes were utilized to measure sample particle sizes at 90° scattering using the Mark-Houwink parameters on a Malvern Zetasizer Nano 90 instrument. The logarithm of G1 correlation function was used to fit the data to a polynomial curve in order to derive the cumulants analysis using equation (1). This analysis was used to calculate the mean size value with a polydispersity index number indicated by the width parameter of the curve. In case of the Hub-NoTag sample, computation of average clathrin structure sizes only include peaks encompassing native clathrin size-range (80–200 nm). Other peaks representing non-clathrin cage-like extended branched-lattices were not included in this analysis. Standard deviations were obtained from DLS measurements taken with multiple protein assembly samples.

$$\ln[G1] = a + bt + ct^2 + dt^3 + et^4 + \dots \quad (1)$$

Electron Microscopy

For each untemplated and gold-templated Hub-His₆ or Hub-NoTag assembly sample, 7 µL were transferred onto 300 mesh, carbon/formvar-supported copper grids.⁴¹ The grids either were left unstained or were stained with 0.5% uranyl acetate. TEM images were obtained on a Philips CM-12 operating at 120 kV with an in-built Gatan digital camera (NYU Skirball Center) and on a JEOL 2100F operating at 200 kV with Tietz CCD camera (New York Structural Biology Center). Additional HR-TEM and EDS images were obtained with a Philips CM-20 operating at 200 kV with a Gatan CCD camera (LMSI, Stevens Institute of Technology).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Templation strategy through a peptide tag on recombinant clathrin fragments. (a) Sequence of Hub-His₆ denoting the hexahistidine (green), thrombin cleavage site (underlined) and clathrin heavy chain residues 1074–1675 (black). (b) Illustration of Hub-His₆ and Hub-NoTag following thrombin cleavage; Hub-His₆ and Hub-NoTag subsequently undergo assembly into cage-like structures with concomitant formation of gold nanocrystals (AuNCs).

Fig. 2.

Cage-like assemblies of Hub-His₆ and Hub-NoTag. pH-dependent assembly and disassembly of (a) Hub-His₆ and (b) Hub-NoTag, monitored by light scattering at 320 nm. Each protein preparation (0.1 mg/mL, 100 μ L) was assembled by the addition of 10 μ L of 1.5 M HEPES buffer (pH 6.5) at the respective times (as indicated by the white arrows); disassembly was triggered with the addition of 11 μ L of 1.0 M Tris buffer (pH 9.0) at the respective times (black arrows). Stained TEM images of (c) Hub-His₆ and (d) Hub-NoTag were obtained. Scale bar represents 200 nm.

Fig. 3. Gold nanocrystal templation by recombinant clathrin fragment protein assemblies. HR-TEM image of (a) Hub-His₆ showing anisotropic nanocrystals patterned in an oriented array. Inset: histogram of the distribution of gold nanocrystal aspect ratios (average aspect ratio = 3.5). (b) Hub-NoTag exhibiting spherical gold particles (average aspect ratio = 1.0). HR-TEM images of (c) Hub-His₆ and (d) Hub-NoTag revealing crystalline gold particle lattice fringes with insets illustrating that the Fourier transform confirm FCC geometry of gold crystals. EDS of (e) Hub-His₆ and (f) Hub-NoTag confirming presence of gold with insets demonstrating dark-field STEM images; the full-scale of the y-axis on both EDS spectra is normalized to 300 counts.