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Engineered Protein Cages for Nanomaterial Synthesis

Ryan M. Kramer,[†] Chester Li,[‡] Daniel C. Carter,[‡] Morley O. Stone,[†] and Rajesh R. Naik*,[†]

Contribution from the Air Force Research Laboratory, Materials and Manufacturing Directorate, Biotechnology Group, Wright-Patterson Air Force Base, Dayton, Ohio 45433, and New Century Pharmaceuticals, 895 Martin Road, Hunstville, Alabama 35824

Received June 3, 2004; E-mail: Rajesh.Naik@wpafb.af.mil

Abstract: Self-assembled particles of genetically engineered human L subunit ferritin expressing a silver-binding peptide were used as nanocontainers for the synthesis of silver nanoparticles. The inner cavity of the self-assembled protein cage displays a dodecapeptide that is capable of reducing silver ions to metallic silver. This chimeric protein cage when incubated in the presence of silver nitrate exhibits the growth of a silver nanocrystal within its cavity. Our studies indicate that it is possible to design chimeric cages, using specific peptide templates, for the growth of other inorganic nanoparticles.

Introduction

The ability of biological systems to control the deposition and structure of inorganic materials has led to the development of biomimetic approaches for inorganic nanomaterial synthesis.[1−4] Size constrained reaction environments for the synthesis of nanoparticles have employed the use of protein cages such as cowpea chlorotic mottle virus (CCMV) capsids, ferritins, and ferritin-like proteins.[5−8] These supramolecular structures represent novel reaction vessels for the synthesis of inorganic materials with controlled dimensions. In addition, the proteinaceous shell provides a platform for surface modifications.[9−11] For example, exposed amino acid functional groups can be chemically modified for the attachment of a variety of ligands including peptides.

Ferritins, uniformly nanosized proteinaceous particles, are ubiquitously found in nature and serve as an iron storage protein.[12] In higher eukaryotes, ferritins are composed of 24 subunits and are self-assembled into a spherical cage with a molecular mass of 450 kDa. Ferritin has an outer diameter of 12 nm and an inner cavity diameter of 8 nm that stores iron in the form of microcrystalline ferric oxyhydroxide.[13] The subunits of ferritins employ two structurally similar but mechanistically different homologues, H and L types, which give the protein the ability to oxidize and nucleate iron within its core. The H-type subunit catalyzes the oxidation of Fe (II) to Fe (III) and is responsible for iron loading into ferritin, while the L-subunit lacks this activity, but promotes the nucleation inside the cage.[12] Apoferritin (lacking the iron oxide core) has been used in the mineralization of other metals such as cobalt, nickel, and palladium.[5,14−16] The nucleation and subsequent mineral growth from a variety of different precursor ions suggest that other metals may be deposited within the constrained core of ferritin. The hydrophilic protein channels that perforate the ferritin cage allows for the influx of metal ions and other small molecules into the inner cavity. In some cases, metal ions within the protein cage can then be reduced to form a metal nanoparticle by the addition of a chemical reducing agent.

The addition of novel functionalities to self-assembling protein cages such as CCMV, has been elegantly demonstrated by the Douglas group[7] and by members of our group using ferritin as a therapeutic platform.[10] In this study, we have functionalized ferritin protein cages through genetic engineering to impart specific functionalities for the constrained synthesis of silver nanoparticles. The dodecapeptide -Asn-Pro-Ser-Ser-Leu-Phe-Arg-Tyr-Leu-Pro-Ser-Asp- (AG4), previously identified by our group from a phage peptide display library, reduces

[†] Air Force Research Laboratory, Materials and Manufacturing Directorate, Biotechnology Group, Wright-Patterson Air Force Base.
[‡] New Century Pharmaceuticals.

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silver ions to metallic silver.[17] This peptide sequence was fused to the C-terminus of L chain ferritin. The AG4 peptide is displayed on the surface of the inner cavity of the self-assembled ferritin cage and growth of silver nanoparticles can be achieved within the confines of the chimeric protein cage. It has been shown that ferritin can be genetically engineered to display both peptides and proteins on its inner and outer core by fusing the polypeptides to the C- and N-termini, respectively.[10] For example, N-terminal ferritin fusions with small peptides have been used to demonstrate that high titer antibodies could be achieved by immunizing animals with the chimeric human peptides without the use of an adjuvant.[18] Here, we demonstrate that nanoparticles can be biomimetically synthesized by fusing specific peptides to the C-terminus of the L subunit of ferritin as a template for controlling the inorganic nucleation, growth and eventual size distribution during nanoparticle formation.

Experimental Section

Creation of the Light Chain Ferritin-AG4 Fusion Protein (LCF-AG4). The design of the fusion construct is shown in Figure 1. The human ferritin L subunit (Genebank ID: M11147) was cloned into the *Nde*I- *Bam*HI sites of pET11b expression vector (Novagen, San Diego, CA) using a PCR based method. A short DNA oligomer (Integrated DNA Technologies, Coralville, IA) encoding for the AG4 sequence was ligated to the C-terminus of ferritin light chain gene via a glycine linker into the corresponding restriction sites of pET11b-LCF using engineered restriction enzyme sites. The resulting plasmid pET11b-LCF-AG4 was sequenced to confirm the coding sequence of the L subunit-AG4 fusion (Supplemental Figure S1).

Expression of LCF-AG4. The pET11b-LCF-AG4 plasmid was transformed into *Escherichia coli* BL21 (DE3) cells. For expression, an overnight culture was diluted (1:50) into fresh LB medium containing ampicillin (100 μg/mL). Expression was induced by the addition of 1 mM IPTG when cells reached an optical density at 600 nm of ~0.6. The cells were grown for 5 h at 37 °C. Cells were harvested and resuspended in 50 mL Buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl) and stored frozen. Protein was extracted by incubating the thawed cell suspension in 5 mg lysozyme and ultrasonicated for 15 s. The lysed crude cell suspension was centrifuged at 5000 rpm for 30 min and the supernatant was transferred to a fresh tube and heat treated at 70 °C for 15 min. The sample was centrifuged to remove denatured protein. The LCF or LCF-AG4 protein was isolated by anion exchange chromatography (MonoQ Amersham Pharmacia) and eluted using a linear 0.1−0.8 M NaCl gradient. The eluted fractions were monitored at 280 nm (for protein) and 420 nm (for silver). The fractions were also analyzed using SDS-PAGE and fractions containing the ferritin-fusion were collected and concentrated for gel filtration chromatography (Superdex 200) and eluted using Buffer B (50 mM Tris-Cl pH 8.0, 100 mM NaCl). For in vivo mineralization experiments, aqueous silver nitrate was added to the growth medium (final concentration of 5 mM) 1 h after induction of the cells with Isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for 6−16 h at 25 °C. For the in vitro mineralization experiments, the cells were grown in absence of silver nitrate and purified as described above. 10 mg/mL of purified recombinant LCF-AG4 fusion, LCF or AG4 peptide was incubated in the presence of 0.4 mM $AgNO_3$ at 37 °C for 3 h in either 50mM Tris-Cl pH 8.0 or in 50mM phosphate buffer pH 7.5. The process was repeated four times to maximize silver loading and centrifuged after each step. Following the final loading step, the solution was dialyzed against Buffer B to remove excess silver nitrate free in solution. The solutions were subsequently centrifuged at 14 000 rpm for 10 min to remove any bulk silver precipitate. Recombinant wild-type LCF was also expressed and purified as a control.

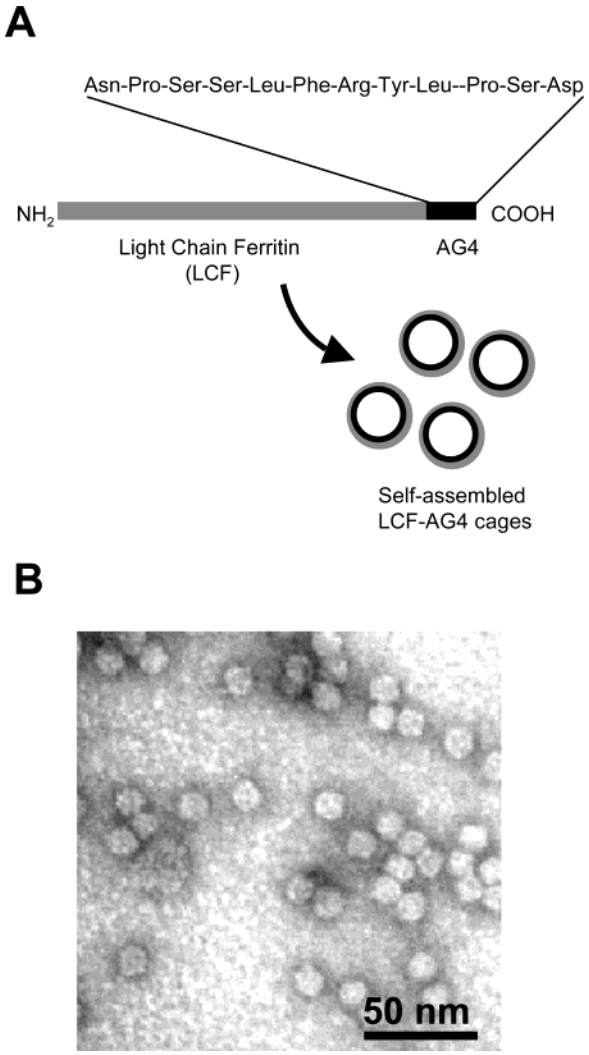

Figure 1. Creation of the LCF-AG4 chimeric protein cages. (A) Schematic illustration of the L subunit ferritin-AG4 (LCF-AG4) fusion and the self-assembled protein cage. The AG4 peptide sequence is fused to the C-terminal of LCF. (B) TEM micrograph of LCF-AG4 protein cages negatively stained with uranyl acetate to show the presence of fully assembled protein cages.

Transmission Electron Microscopy. Micrographs were obtained using a Philips EM208 operating at 200 kV with Noran Voyager energy-dispersive X-ray analysis system. Low voltage transmission electron microscopy (LVTEM) analysis was performed using as Delong Instruments transmission electron microscope at an operating voltage of 5 kV. Some samples were negatively stained with uranyl acetate.

Results and Discussion

We have previously demonstrated the synthesis of silver nanoparticles using specific peptides identified from a phage peptide display library.[17,19] These peptides are capable of nucleating and controlling the growth of nanoparticles in vitro. For example, the AG4 peptide, with the amino acid sequence -Asn-Pro-Ser-Ser-Leu-Phe-Arg-Tyr-Leu-Pro-Ser-Asp-, was demonstrated to specifically nucleate and control the growth of silver nanoparticles. The AG4 peptide is capable of synthesizing silver

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nanoparticles by reducing silver ions to metallic silver at neutral pH and at ambient temperature.[17,19−21] The silver nanoparticles synthesized using the AG4 peptide exhibit polyhedral morphologies (hexagons, spheres, triangles) with an average diameter of 102 ± 28 nm.[17,19] We assumed that by displaying the AG4 peptide within a constrained environment like a protein cage, the reduction of the silver ions to silver would be confined within the cavity of the cage. On the basis of the crystal structure of L-chain ferritin, addition of amino acids to the carboxy-termini should result in the display of the foreign sequences on the interior of the assembled protein cage. The human L chain ferritin (LCF) protein was genetically modified by fusing the 12 amino acid sequence coding for the AG4 peptide to the C-terminal end of the human L chain ferritin (Figure 1A). The LCF-AG4 gene fusion product was recombinantly expressed and subsequently purified from bacterial cells using a combination of ion exchange and gel filtration chromatographies. The purified LCF-AG4 fusion protein was capable of self-assembling to spherical cages similar to native L chain ferritin. The TEM micrograph revealed the presence of fully assembled LCF-AG4 particles with an average diameter of 12 ± 1 nm (Figure 1B). The purified LCF-AG4 protein eluted from size exclusion gel chromatography exhibited a retention factor consistent with the recombinant ferritin and the electrophoretic mobility of LCF-AG4 was also compared to native ferritin on a native polyacrylamide gel. Therefore, the additional 12 amino acids fused to the C-terminus of L chain ferritin did not have any deleterious effects on the overall architecture of the protein cage. On the basis of the size and morphology of the chimeric protein cage, we estimate that the LCF-AG4 cage is composed of the expected 24 subunits self-assembled into the prototypical ferritin cage with a hollow internal cavity.

For incorporation of silver into the cavity of LCF-AG4, two different methods were used. The in vivo method involves growing cells actively expressing the protein cages in silver nitrate, while the in vitro method involves incubating purified protein cages in silver nitrate. For the in vivo method, *E. coli* cells actively expressing the protein cages were grown in the presence of 5 mM silver nitrate. This was done in order to determine whether the actively expressed protein could incorporate inorganic metal in vivo and limit crystal growth to the confines of the ferritin cage. The addition of silver nitrate (5 mM) to growing control *E. coli* cells (lacking LCF-AG4) exhibited a 50% reduction in cell viability after 6 h of growth. In contrast, cells expressing LCF-AG4 exhibited a 30% reduction in cell viability (Supplemental Figure S2). Cells expressing LCF-AG4 cages were more resistant to the bacteriocidal activity of the added silver than cells lacking LCF-AG4. The sequestration of silver ions and changing the redox state of the metal ion from Ag^+ to Ag° by LCF-AG4 may be responsible for increased silver resistance.

The LCF-AG4 protein cages were purified from cell lysates using ion exchange and gel filtration chromatographies. The gel filtration eluted fractions were monitored at 280 nm, for absorption due to the presence of protein, and at 420 nm, the characteristic surface plasmon resonance absorption for silver nanoparticles (Figure 2A). Noble metal nanoparticles exhibit a strong UV−vis absorption band, also known as surface plasmon resonance, that results from the coupling of the incident photon frequency with the conduction electrons of the nanoparticle. The absorption profiles of the gel filtration fractions indicated that proteinaceous material coeluted with material producing surface plasmon resonance characteristic of silver nanoparticles. Although the absorption spectra indicates that the fractions rich in protein also exhibit the characteristic surface plasmon resonance signature of silver nanoparticles, confirmation that silver deposition occurred within the cavity of the protein cage was obtained by TEM analysis. The purified unstained fraction that exhibited protein (280 nm) and surface plasmon absorption (420 nm) was spotted onto TEM grids and imaged using transmission electron microscopy operating at low voltage (LVTEM). LVTEM allows for visualization of proteinaceous material without the need for heavy metal staining. The TEM micrograph shown in Figure 2B reveals the presence of electron dense spherical particles with an average diameter of 7 ± 1 nm, while the electron lucent areas surrounding the electron dense core regions are indicative of a surrounding proteinaceous shell. Empty or partially filled cores were also observed. While the in vivo loading method can be used to deposit silver within the confines of the LCF-AG4 cage, we also explored an in vitro deposition method.

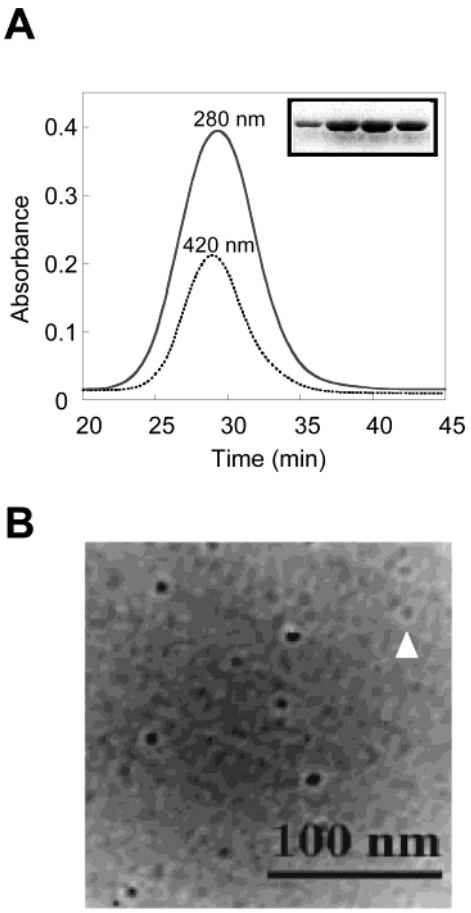

Figure 2. In vivo loading of LCF-AG4 protein cages with silver. (A) Gel Filtration chromatography elution profile of the in vivo mineralized LCF-AG4 protein cages at 280 (solid line) and 420 nm (dashed line). Eluted fractions (25−30 min) separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) stained for protein using Coomassie Blue (*inset*) (B) Low voltage TEM micrograph of LCF-AG4 particles. The electron dense regions (mineralized silver) surrounded by an electron lucent protein shell. An empty LCF-AG4 cage (arrow).

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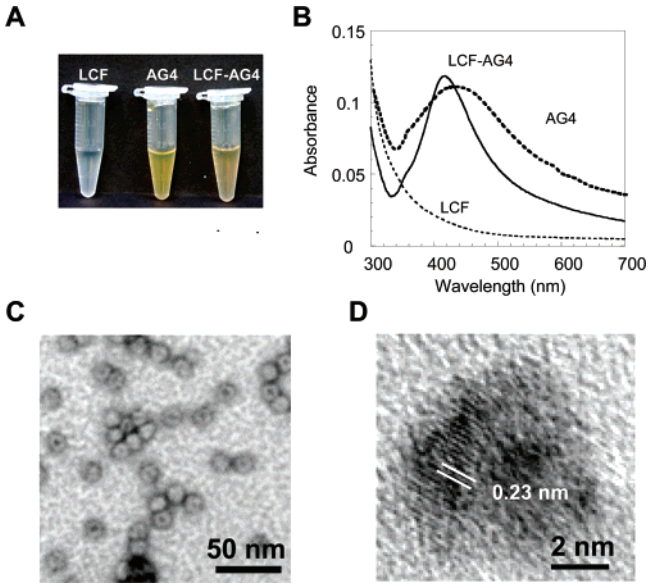

Figure 3. In vitro mineralization of LCF-AG4 protein cages. (A) Incubation of 10 μg/mL native ferritin, AG4 peptide or LCF-AG4 protein cages with 0.4 mM silver nitrate at room temperature for 24 h resulted in the formation of a colored solution. (B) UV−vis spectra of the solutions shown in panel A. (C) TEM micrograph of negatively stained (with uranyl acetate) sample of LCF-AG4 shows discrete particles containing electron dense cores. (D) High-resolution TEM micrograph of a single silver nanoparticle present within the LCF-AG4 cavity showing lattice fringes.

For the in vitro silver deposition method, purified empty LCF-AG4 protein cages were incubated in the presence of 0.4 mM $AgNO_3$ and the reaction was allowed to proceed for several hours at 37 °C. The solutions containing either LCF-AG4 or the AG4 peptide turned reddish in color, while the control LCF sample remained colorless (Figure 3A). The samples were then analyzed using UV−vis spectroscopy. Both the LCF-AG4 protein cages and AG4 peptide exhibited characteristic surface plasmon resonance (Figure 3B). The incubation of the AG4 peptide by itself in silver nitrate resulted in the formation of silver particles and exhibited broad absorbance with a peak absorption wavelength of 430 nm with a full width at half-maximum (fwhm) of 210 nm. In comparison, the silver nanoparticles obtained using the LCF-AG4 protein cages displayed a characteristic surface plasmon resonance peak at 410 nm with a fwhm of 93 nm. The control native ferritin lacking the AG4 silver precipitating sequence exhibited no absorption peak in the silver surface plasmon resonance region. The narrowing of the absorption peak and differences in the UV-absorption profile is most likely due to the differences in the sizes and distribution of the silver nanoparticle.[22] Clearly, the nature of absorption profile is indicative of a tighter size distribution of the silver nanoparticles in the LCF-AG4 sample compared to the AG4 sample.

Conformation of silver loading within the protein cages was obtained by TEM analysis. The TEM images revealed the presence of intact protein shells with electron dense cores (Figure 3C). The spherical particles within the cavity of LCF-AG4 had an average diameter of 5 nm. Energy-dispersive X-ray analysis of the protein sample confirmed the presence of silver. The particles appeared homogeneous and high-resolution TEM (HRTEM) revealing the monocrystalline nature of the silver nanoparticles with lattice spacings of 0.23 nm, analogous to the (111) facet of *fcc* silver crystal (Figure 3D).

Although several empty protein cages can be observed in the TEM micrographs, we estimate the efficiency of the loading (ratio of empty protein cages to cages containing electron dense cores) to be approximately 40% for the in vivo and 60% for the in vitro loading method. Optimization of the reaction conditions is currently being studied using a variety of buffers, additives, and reaction conditions for the production of large quantities of organic−inorganic composite nanostructures. Recently, we fused a 12 amino acid sequence (CO2 peptide), previously identified as a template in the synthesis of magnetic CoPt nanoparticles,[19] to LCF for the synthesis of magnetic nanoparticles within the core of LCF protein cages.[26] Preliminary results demonstrate the formation of CoPt nanoparticles within the internal cavity of the LCF-CO2 protein cage (Supplemental Figure S3) and characterization of the CoPt nanoparticles is currently in progress. Although CoPt nanoparticles have been previously grown within ferritin cores,[6] the introduction of specific peptide sequences, such as the CO2 peptide, could impart control over the nucleation and crystal phase resulting in the synthesis of CoPt nanoparticles with enhanced magnetic properties.

Together, our results suggest that ferritin cages can be genetically engineered to selectively deposit materials within its constrained internal cavity and can be potentially used in the creation of unique nanoreaction containers for other inorganic materials. Both the in vivo and in vitro methods resulted in the deposition of silver within the cavity of the LCF-AG4 cage. However, in vivo methods may not be a suitable option for some metals or metal alloys.

Conclusions

We have shown here that chimeric ferritin cages displaying a silver nucleating peptide in its internal cavity can be used to nucleate and control the growth of nanoparticles within its core. Other peptide fusions possessing the ability to sequester and deposit other metals can be generated similar to the method described here. These novel ferritin-based chimeric inorganic particles will have broad application in a number of nanomaterial based disciplines, including many potentially important applications in medical imaging and radiotherapy.

There are a number of advantages in the use of ferritin as a protein cage for constrained nanomaterials. For example, the ease and rapidity of recombinant expression in bacteria to produce large quantities of the material, the relatively straightforward purification procedures, and particle size distribution and stability. Peptides that have the ability to bind and precipitate a number of inorganic materials have been identified through phage display techniques. Ferritin and ferritin-like cages can be genetically engineered to impart new functionalities by introducing peptide sequences on the interior surface or exterior surface of the protein cage. For instance, peptide sequences with

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the ability to produce nanometer-sized particles of CdS, ZnS, SiO_2, CoPt, and other metals have already been identified.[19,23−25] The display of specific peptide templates within a protein cage as described here can be used to create controlled nanoenvironments that are suitable for the generation of monodispersed inorganic nanoparticles with similar composition and crystallographic properties. It is likely that heat treatment or annealing of the as prepared nanoparticles may be required to impart ordering to the nanocrystals; however the presence of a protein coat can be used as protective shell to prevent sintering and oxidation of the nanocrystals thus greatly reducing nanoparticle agglomeration.

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Supporting Information Available: The amino acid sequence of the LCF-AG4 fusion, cell viability in the medium containing 5 mM silver nitrate, and the formation of CoPt nanoparticles within the LCF-CO2 protein cage. This material is available free of charge via the Internet at http://pubs.acs.org.

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