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Controlling gold nanoparticle seeded growth in thermophilic ferritin protein templates

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ABSTRACT

Ferritin protein cages provide templates for inorganic nanoparticle synthesis in more environmentally-friendly conditions. Thermophilic ferritin from *Archaeoglobus fulgidus* (AfFtn) has been shown to encapsulate pre-formed 6-nm gold nanoparticles (AuNPs) and template their further growth within its 8-nm cavity. In this study, we explore whether using a gold complex with electrostatic complementarity to the anionic ferritin cavity can promote efficient seeded nanoparticle growth. We also compare wt AfFtn and a closed pore mutant AfFtn to explore whether the ferritin pores influence final AuNP size.

1. Introduction

Ferritins are hollow, multimeric proteins whose native function is the oxidation, mineralization, and storage of iron [1]. Because of their unique structure, ferritins can be used as mini-reactors to prepare inorganic nanoparticles (NPs) by reducing metal salts inside the protein cavity. In addition to iron, ferritins naturally bind other metals [2–6] and can also be redesigned to enhance metal ion binding [7,8]. Ferritin-templated NP synthesis can occur in room-temperature aqueous conditions, which is significantly more environmentally friendly compared to other conventional NP synthesis methods, avoiding the need for organic solvents or high temperatures. The resulting NPs are more biocompatible than bare particles, with enhanced aqueous stability, even in high ionic strength solutions [9]. Furthermore, protein-constrained growth offers potential advantages in controlling nanoparticle shape and size and can result in monodisperse samples. In 1991, Meldrum et al. presented the first examples of non-native inorganic NP synthesis inside ferritin, producing MnO_2, FeS, and uranyl oxyhydroxide cores [10]. Since that time, metallic cores of widely varying composition have been synthesized using ferritin, including semiconducting quantum dots [11–13], metal oxide NPs [14], noble metal NPs [8,15–18], and magnetic NPs [19–22]. This wide range of protein-assisted reactions motivates the study of model ferritin systems, where the composition and presentation of polypeptides is well defined compared to many biologically controlled biomineralization reactions, e.g., the formation of shells and bone.

While ferritin provides an excellent nanoreactor for the formation of inorganic NPs, it has been challenging to restrict particle growth to the interior of the protein cage. Many amino acids such as histidine and cysteine can bind metal ions, generating multiple nucleation sites on the interior and exterior ferritin surfaces. Zhang et al. demonstrated ferritin-aided AuNP growth on the surface of horse spleen apoferritin (HSAF) via reduction of $HAuCl_4^-$ with either $NaBH_4$ or 3-(*N*-morpholino)propanesulfonic acid (MOPS) [17]. Without HSAF present, synthesized AuNPs were unstable, highlighting the improved passivation by ferritin. However, TEM showed the AuNPs resided mostly on the exterior protein surface, with average diameters of 3.6 ± 1.2 and 15.4 ± 4.5 nm for $NaBH_4$ and MOPS, respectively.

Ideally, NP formation should occur exclusively inside the ferritin cage, taking advantage of complete protein-NP templating and coating. Towards this aim, Butts et al. mutated human H ferritin (HuHFtn) to encourage interior particle growth by removing all histidines and cysteines (four per monomer) present on the outer protein surface [8]. A second protein mutant added four interior-surface cysteine residues per monomer to promote NP growth in the central cavity. It was found that all eight exterior and interior mutations were necessary to form stable Au and Ag NPs.

Another approach to ensuring particle growth within the cavity is not through changes to ferritin itself, but in removing excess Au ions through desalting before reduction. Fan et al. demonstrated a two-step AuNP formation specifically inside HSAF [23]. They first generated small “seed” particles by incubating HSAF with $HAuCl_4$, desalting to

Abbreviations: AuNP, gold nanoparticle; AfFtn, thermophilic ferritin from *Archaeoglobus fulgidus*; HSAF, horse spleen apoferritin; TEM, transmission electron microscopy; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HuHFtn, human H ferritin; BSPP, bis-(*p*-sulfonatophenyl)phenylphosphine; BSA, bovine serum albumin; AfFtn-AA, closed-pore AfFtn with two alanine mutations; IPTG, isopropyl β-D-1-thiogalactopyranoside; SEC, size exclusion chromatography; rt, room temperature

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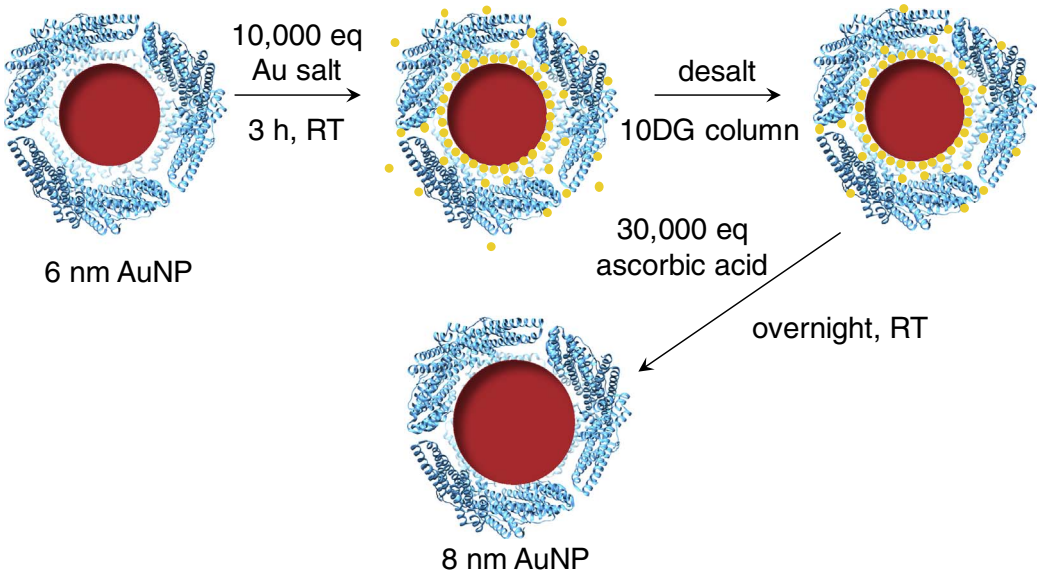

Fig. 1. Templated seeded growth scheme for AuNPs encapsulated within AfFtn. After addition of gold ions, the sample is desalted to remove excess reagent and favor growth inside the cavity. Ascorbic acid reduces gold ions on the surface of the AuNP, growing the AuNP to fill the protein cavity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

remove excess ions, and reducing the remaining ions with $NaBH_4$. Additional particle growth was achieved by adding $HAuCl_4$ along with mild reducing agent ascorbic acid. The AuNPs were found to reside exclusively within the protein cavity, and were mostly single crystalline with average diameter of 6.3 ± 0.8 nm.

Our recent investigations of protein-templated inorganic nanoparticle chemistry have involved thermophilic ferritin from *Archaeoglobus fulgidus* (AfFtn) [24], which has unusually large (4.5-nm diameter) triangular pores that span the protein shell. AfFtn exhibits reversible, ionic-strength-mediated protein assembly, a feature that we have previously exploited for the quantitative encapsulation of 6-nm AuNPs coated in bis-(*p*-sulfonatophenyl)phenylphosphine (BSPP) [9,25,26]. We demonstrated a hybrid technique of encapsulating a 6-nm seed AuNP and performing in situ growth in the AfFtn cavity as shown in Fig. 1 [9]. Addition of gold ions and reducing agent promoted AuNP growth. Briefly, $HAuCl_4$ was added to AfFtn and incubated at rt for 3 h. The solution was desalted using a sizing column to remove excess $AuCl_4^-$ and AfFtn surface-bound $AuCl_4^-$. Mild reducing agent ascorbic acid was added to reduce gold ions interacting with the AuNP surface. This process led to small, incremental growth. Two cycles led to AuNPs with an average diameter of 8.2 ± 1.9 nm, filling the ferritin inner cavity. Control experiments with AuNPs without protein present led to rapid precipitation, demonstrating the importance of the protein template in generating monodisperse, stable particles.

Moving beyond simply producing particles within ferritin, we were interested in exploring the role of two parameters that have often been ignored in protein cage-templated AuNP synthesis: the gold complex used and the porosity of the cage.

AuNPs are most commonly prepared in ferritins using $HAuCl_4$ or $AuCl_3$ as the gold salt. Intrigued by the need for two rounds of seeded growth reaction in order to grow the AuNP to fill the AfFtn cavity, we decided to explore the role of the charge of the gold salt. Because the interior of AfFtn is negatively charged [25], we hypothesized that electrostatic repulsion was preventing $HAuCl_4$ from entering the cavity in concentrations needed for significant particle growth. Shown in Fig. 2, we used three gold salts with different charges in solution: $HAuCl_4$, $AuCl_3$, and Au(ethylenediamine)$_2Cl_3$. $HAuCl_4$ deprotonates in aqueous solution to form a variety of anionic species in the form of $[AuCl_{4-n}(OH)_n]^-$, where n = 0–4, depending on pH, chloride concentration, and oxidizing conditions [27]. $AuCl_3$, which exists as Au_2Cl_6 in crystalline form [28], is initially neutral in solution. Electrospray ionization of aqueous gold(III) chloride produces a large variety of mononuclear clusters in the gas phase evidenced by mass spectrometry, e.g., $[AuCl_2]^+(H_2O)_n$ (n = 0–4), as well as dinuclear $[Au_2Cl_{5-x}OH_x]^+(H_2O)_n$ (x = 0–1) species [29]. However, on the short timescale of our seeded growth reaction, the dimeric neutral Au_2Cl_6 species likely predominates in aqueous buffer, pH 7.6. Finally, Au(en)$_2Cl_3$ loses its chloride counterions in water and has a + 3 charge [30].

We also compared the templating capabilities of AfFtn with bovine serum albumin (BSA), which does not form an ordered cage around the AuNP and can directly adsorb to the AuNP surface [31]. Previous studies using BSA to promote particle growth led to polydisperse Ag, Au, or Ag/Au alloy NPs [32–34]. We therefore expected that BSA in seeded growth reactions would be unable to control AuNP growth to the same extent as AfFtn. Finally, we compared wt AfFtn templating with a double mutant AfFtn-AA (K150A/R151A) [35] lacking the characteristic large pores, to determine whether the pores can facilitate molecular diffusion processes required for seeded nanoparticle growth.

2. Experimental procedures

2.1. Protein expression and purification

Thermophilic ferritin protein was expressed and purified as previously published [26]. The pAF0834 plasmid containing AfFtn was obtained from Eric Johnson (California Institute of Technology) and the plasmid containing AfFtn-AA (K150A/R151A) was purchased from DNA 2.0 (now ATUM). Plasmids were transformed into BL21-CodonPlus(DE3)-RP cells and grown overnight at 30 °C in LB medium. Cells were transferred to 1 L terrific broth and grown at 37 °C until $OD_{600} \sim 0.8$. Protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 h. Cells were resuspended in low-salt buffer (20 mM NaCl, 20 mM phos, pH 7.6), and lysis was done through a combination of lysozyme (~1 mg/mL final concentration) and sonication on ice (amplitude of 30, 1 s on, 1 s off, 15 min total process time). Cellular debris were removed by centrifugation (6 krpm, 30 min, 4 °C) and the supernatant was heat shocked for 10 min at 80 °C. Precipitated proteins were removed by centrifugation (9 krpm, 60 min, 4 °C), and the supernatant was buffer exchanged to 2 mM EDTA, 2 M NaCl, 20 mM phos, pH 7.6. Protein was further purified by size exclusion chromatography using a

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