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2012

Sana, B., Calista, M., & Lim, S. (2012). Protein Cage Assisted Metal-Protein Nanocomposite Synthesis: Optimization of Loading Conditions. International Conference on Nanotechnology - Research and Commercialization 2011, 1502, pp. 82-96.

<https://hdl.handle.net/10356/79886>

<https://doi.org/10.1063/1.4769136>

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Citation: [AIP Conf. Proc.](#) **1502**, 82 (2012); doi: 10.1063/1.4769136

View online: <http://dx.doi.org/10.1063/1.4769136>

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Protein Cage Assisted Metal-Protein Nanocomposite Synthesis: Optimization of Loading Conditions

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Abstract. Ferritin is an iron-storage protein in most living systems with a cage-like structure. It has inherent property to form metallic nanocore within its cavity. The metallic core formed within the *Archaeoglobus fulgidus* ferritin cavity is stabilized by modulating the protein structure by site directed mutagenesis. Encapsulation protocol of various metals within the engineered ferritin cage (AfFtn-AA) is optimized. Dense metallic cores are visualized using electron microscopy and the bound metal was quantified by ICP-spectrometry. The AfFtn-AA is loaded with up to about 350 cobalt, 2000 chromium, and as high as 7000 iron atoms, separately. The metal-protein nanocomposites formed by encapsulation of cobalt, chromium, and iron are studied. Magnetic resonance imaging of the agarose embedded nanocomposites shows brightening of T_1 -weighted images and signal loss of T_2 -weighted images with increasing concentration of the nanocomposites. Shortening of magnetic relaxation times in the presence of the nanocomposites confirm their ability to enhance magnetic relaxation rate and suggests that the nanocomposites have potential application as MRI contrast agent.

Keywords: *Archaeoglobus fulgidus*; Ferritin; MRI; Contrast agent, Protein cage.

PACS: 62.23.Pq; 87.85.jf; 87.14.E-

INTRODUCTION

The bottom-up approach of nanoparticle synthesis is inherent to the progress of nanotechnology. There are several traditional methods for nanoparticle synthesis but demand is increasing for synthesis of nanoparticles with more specific physical / chemical characteristics. Chemical methods for nanoparticle synthesis mostly need high temperature, extreme pH or adverse chemical treatments. Biological synthesis of nanoparticles provides an advantage over the chemical approach in the relatively milder synthesis conditions as well as excellent spatial control. For example, excess iron in a living system is stored within a caged protein template called ferritin as insoluble iron nanoparticles. Nanoparticle synthesis within protein cages appears to be a promising way to produce nanoparticles with narrow size distribution and reproducible physical / chemical properties. Viral capsids, virus-like particles, and some other protein cages can also be used for nanoparticle synthesis [1-2]. These protein cages are made-up with multiple identical subunits, which self-assemble to form a cage-like structure with a porous shell. Small particles and molecules can be encapsulated within a protein cage either by migrating through small pores or channels on the protein shell or by disassembling the protein cage followed by self-assembly surrounding the particles. Loading capacity of a protein cage and characteristics of the nanoparticles may depend on the synthesis protocol, which need to be optimized to get the nanocomposite with desired characteristics.

Ferritins are iron storage proteins in most living systems, it forms a cage-like structures with an internal cavity of

about 8 nm diameter. There is more than one pathway for iron nanoparticle synthesis within ferritin and the extent of each reaction depends on the amount of ferritin, iron, oxygen or other oxidising agent within the reaction system [3]. *In vivo* ferritin iron cores were reported to be magnetic and able to enhance contrast in MR imaging [4-5]. *In vitro* experiments showed that ferritin has inherent capability to form metal cores [6-7]. Gadolinium and iron-loaded ferritins are proven to have high magnetic relaxivity [8-9]. In addition, the ferritin cage itself can work as carrier of these nanoparticles due to high solubility, ease of chemical / genetic modifications and non-hazardous metabolic fate.

Archaeoglobus fulgidus ferritin (AfFtn) has unique self-assembly property. In low ionic strength buffer it stays as dimeric species and the subunits self-assemble to form 24-meric cage in high ionic strength solutions [10-11]. In the presence of divalent metal ions AfFtn dimers self-assemble to form a 24-meric protein cage with simultaneous synthesis of metallic nanoparticles within its cavity. These metal-ferritin nanoparticles have potential application in medical imaging but their physical properties and performance may depend on the synthesis conditions. Current communication reports the optimization of cobalt, chromium and iron nanoparticle synthesis within a mutant *A. fulgidus* ferritin (hereafter would be referred to as AfFtn-AA) and screening of the metal-ferritin nanocomposite for development as MRI contrast agent. Potential application of the iron bound mutant *A. fulgidus* ferritin has been reported elsewhere [9].

MATERIALS AND METHODS

Mutation, Overexpression, and Purification

The *A. fulgidus* ferritin (AfFtn) gene was cloned in between NdeI and BamHI restriction sites of pET-11a expression vector (Novagen). The Lys150 and Arg151 were mutated to alanine residue by PCR-based site-directed mutagenesis using primers containing the mutated sequence and the wild-type gene as a template (Stratagene). The resulting AfFtn-AA gene was located within the pET-11a vector at the position identical to the wild-type gene. The recombinant construct was stored in *E. coli* strain DH5 α .

E. coli BL21(DE3)CodonPlus-RIL (Stratagene) competent cells were transformed with the AfFtn-AA/pET-11a construct and positive clones were screened by antibiotic (ampicillin + chloramphenicol) selection. For over-expression, LB broth media was inoculated with overnight culture of a single colony and the gene expression was induced with 1 mM IPTG when the absorbance (A_{600}) reached 0.6. Cells were harvested by centrifugation 4 hours post induction and the cell pellets were stored at -80°C.

Harvested cells were disrupted at high pressure (15,000 psi) within a French pressure cell (Thermo Electron Corporation) after resuspending them in 25 mM HEPES (pH 7.5) containing 50 mM NaCl (Buffer A). The insoluble fraction was removed by centrifugation at 150,000 $\times g$ at 4 °C for 1 hour. The soluble fraction was thermally purified by heating at 90 °C for 10 minutes followed by removal of denatured proteins by centrifugation at 150,000 $\times g$ at 4 °C for 1 hour.

AfFtn-AA was purified to homogeneity by hydrophobic interaction chromatography on HiPrep 16/10 Phenyl FF (high sub) column using ÄKTA-Explorer FPLC system (GE Healthcare). The column was pre-equilibrated with the

binding buffer B (buffer A + 500 mM ammonium sulphate). The bound protein was eluted by a single step gradient with the buffer A. Purified proteins were concentrated by ultrafiltration through 10 kDa MWCO membrane and buffer-exchanged to obtain the final protein preparation of ~5 mg/ml AfFtn-AA in Buffer A. Protein yield was 10-20 mg/l culture. Homogeneity of the final preparation was confirmed using SDS-PAGE. Protein concentration was measured by bicinchoninic acid method following the manufacturer's protocol and using bovine serum albumin as standard (BCA protein assay kit, Thermo Scientific).

Mineralization

AfFtn-AA was mineralized with different metal ions i.e. Co, Cr, and Fe. $\text{Co}(\text{NO}_3)_2$ and $\text{Cr}_2(\text{SO}_4)_3$ solutions were prepared in water and, FeSO_4 was dissolved in 0.1 % HCl. Encapsulation of cobalt and chromium was optimized using different mineralization protocols described below. Iron binding to AfFtn-AA was done following Protocol 3 that was optimized previously [9]. Unbound metals from all mineralized samples were removed by 100 kD MWCO centrifugal filter device (Millipore, Billerica, MA, USA) followed by desalting through PD-10 column and the encapsulated metals within the ferritin cavity were quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Protocol 1: Metal was added step-wise to 0.05 mg/ml AfFtn-AA solution in 100 mM HEPES buffer (pH 8.5) containing 100 mM NaCl to final concentration of 0.1 mM. The interval between each of the 10-step addition was 5 min. A final concentration of 0.03 % H_2O_2 was added to the mixture over a period of 2 hr with 15 min interval between additions. The solutions were subsequently incubated overnight at 4 °C [12]. AfFtn-AA with aliquots

of cobalt rapidly formed a homogeneous olive-green solution.

Protocol 2: A final concentration of 0.5 mM metal was added to 0.5 mg/ml AfFtn-AA solution in 100 mM HEPES buffer (pH 7.5) containing 50 mM NaCl. The mixture was treated by a thermal cycling program comprising 50 cycles of 2.5 min periods at 55 °C followed by 12 min periods at 4 °C. Total cycle time was 12.5 hr [10].

Protocol 3: Metal solution was added in 5 steps within 0.2 mg/ml AfFtn-AA solution in 100 mM HEPES (pH 7.5) containing 50 mM NaCl to 1 mM final concentration. The mixture was incubated for 1 hour at room temperature followed by overnight incubation at 4 °C [13].

Protocol 4: AfFtn-AA solution of 0.2 mg/ml was prepared in 5 ml 100 mM HEPES, pH was adjusted to 8.6 using NH₄OH solution. Metal solution was prepared in water containing dissolved CO₂. Aliquots of metal solution were added in 5 steps until the final concentration reached 1 mM. The mixture was incubated at room temperature for 3 hr followed by overnight incubation at 4 °C [14].

Protocol 5: Aliquots of metal solution were added in 0.5 mg/ml AfFtn-AA in 100 mM HEPES buffer pH 8.3 in 10 steps with 5-min interval between each step to reach a final concentration of 2 mM. A final concentration of 0.03 % H₂O₂ was added to the mixture over a period of 2 hr with 15-min interval between each addition. The solutions were incubated overnight at 50 °C. AfFtn-AA with aliquots of cobalt rapidly formed a homogeneous olive-green solution [14].

Self-Assembly Study

Self-assembly of AfFtn-AA upon mineralization was studied by comparing the hydrodynamic diameters of the samples before and after mineralization. The hydrodynamic diameter was measured with dynamic light scattering

(DLS; Zetasizer Nano ZS; Malvern Instruments, UK). Samples were equilibrated for 5 minutes at 25 °C.

The self assembly of mineralized AfFtn-AA was further confirmed by comparing its molecular mass with that of the unmineralized protein. Molecular mass of both species were determined by comparing their elution volume through a Size Exclusion Chromatography column (Superdex 200 10/300 GL column; GE Healthcare, USA) with the elution volume of some standard proteins of known molecular weight. The mobile phase of the chromatographic experiment was 25 mM HEPES at pH 7.5.

Electron Microscopy

Mineralized AfFtn-AA samples (0.1 mg/ml in 10 mM HEPES buffer, pH 7.5) were applied on 200 square-mesh carbon-coated copper grids and allowed to adhere for 1 minute followed by air drying for 10 minutes. Samples were negatively stained with 1% uranyl acetate for 1 minute, excess stains were soaked with blotting paper and the grids were air dried for at least 16 hours. Samples were observed in a JEOL JEM-1400 Transmission Electron Microscope (Jeol, Tokyo, Japan) at an accelerating voltage of 100 kV and images were acquired at different magnifications.

Magnetization Study

For magnetic characterization, mineralized AfFtn-AA samples were buffer exchanged with water and lyophilized. Magnetization of lyophilized specimens were examined in a 1.5 Tesla 7300 series vibrating sample magnetometer (VSM, Lakeshore) under magnetic field strength of up to 1.2 T at room temperature.

Imaging and Relaxation Time

MRI samples were prepared by fixing different concentrations of mineralized AfFtn-AA within 0.8 % agarose matrix in 24-well tissue culture plates. Samples were imaged in a 3.0 T whole-body scanner using a dedicated knee coil. MRI was performed at Radlink Diagnostic Imaging and Singapore General Hospital. For the measurement of longitudinal relaxation times (T_1), T_1 -weighted images were acquired with inversion recovery spin echo sequence ($T_R = 2800$ ms $T_E = 8.5$ ms, $T_1 = 30, 50, 100, 200, 400, 500, 600, 800, 1000, 1200, 1800, 2000$ and 2500 ms, matrix = 512×512 , slice thickness = 3.0 mm, number of averages = 1 , field of view = 27 cm). For the measurement of transverse relaxation times (T_2), T_2 -weighted images were acquired with a spin-echo sequence with different echo times ($T_R = 1000$ ms $T_E = 10, 15, 20, 40, 60, 80, 100, 150, 200$ ms, matrix = 512×512 , slice thickness = 3.0 mm, number of averages = 1 , field of view = 27 cm). The T_1 and T_2 values for each sample were calculated from mean signal intensity of MR images by using MATLAB (The Mathworks, Natick, MA, USA). For the calculation of T_1 values, intensity data of T_1 -weighted images were plotted against inversion time (T_1) and fitted to the inversion-recovery signal equation $[A - B \cdot \exp(-t/T_1)]$ where $I = \text{Intensity}$, $t = T_1$, A , B and C are constant] using cftool-fitting in the customized equation. T_2 values were calculated by fitting the intensities of T_2 -weighted images to a mono-exponential decay curve $[A \cdot \exp(-t/T_2) + C]$ where $I = \text{Intensity}$, $t = T_E$, A , B and C are constant] using the same method.

RESULTS AND DISCUSSION

Protein purification

The purification of AfFtn-AA involves heat treatment. Since AfFtn-AA is highly thermostable protein, most of the contaminating *E. coli* proteins are precipitated by heat treatment. It was further purified to homogeneity by hydrophobic interaction chromatography; the elution profile is shown in Figure 1. The AfFtn-AA was bound in the presence of 500 mM ammonium sulphate in binding buffer and eluted as a single peak in the elution buffer. Purity of the pooled fraction after each purification step was analyzed on SDS-PAGE. The purified AfFtn-AA migrated as a single band of ~20 kDa molecular mass in SDS-PAGE under reducing conditions, suggesting that the purified AfFtn-AA was homogeneous (Figure 2).

FIGURE 1. Elution profile of AfFtn-AA purification through hydrophobic interaction column. Pure AfFtn-AA was pooled from elution fractions B4-B6.

FIGURE 2. SDS-PAGE of AfFtn-AA at different purification steps. Lane 1: Cell lysate of *E. coli* BL21(DE3)C+RIL (negative control), Lane 2: Cell lysate *E. coli* culture overproducing AfFtn-AA, Lane 3: Molecular weight marker, Lane 4: Thermally purified AfFtn-AA, Lane 5 & 6: purified AfFtn-AA.

Self-Assembly Study

Self-assembly study was done to understand the cage formation upon addition of different metal ions. Effect of mineralization on the hydrodynamic diameter of AfFtn-AA was observed in dynamic light scattering (DLS). Upon mineralization of AfFtn-AA with any of the three metals the peak was shifted towards larger hydrodynamic diameter, which suggest self-assembly of the AfFtn-AA to form the protein cage. Hydrodynamic diameter of apo-AfFtn-AA was ~8 nm and after mineralization that changed to ~12 nm, which agree with size of the protein cage. Change of molecular size is also observed from their elution profile in size exclusion chromatography (data not

shown). The molecular size of each species was determined from the elution volume, which confirmed that mineralization of dimeric AfFtn-AA (molecular mass ~40 kDa) resulted in the formation of 480-kDa protein cage by self-assembly of 12 dimers.

FIGURE 3. Size distribution of AfFtn-AA and its mineralized forms as revealed by dynamic light scattering (DLS) technique.

Metal Binding

The amount of metal encapsulated in each protein cage was estimated from the ratio of molar concentration of the metal and AfFtn-AA 24-mer. Analysis of different mineralized AfFtn-AA showed that maximum encapsulation of different metals was achieved using different protocols. Maximum loading of cobalt (334 Co/24-mer) was possible following Protocol 5 while Protocol 4 was optimum for loading of chromium (2000 Cr/24-mer). However, some differences exist using similar protocols. In a previous report of encapsulating about 2000 cobalt in each 24-meric cage of horse spleen ferritin has been reported following Protocol 1 [12], while in another study reported encapsulation of 800 – 1500 cobalt within the cage of a horse spleen ferritin [15]. Maximum iron loading was possible following Protocol 3 and AfFtn-AA

could encapsulate about 7000 Fe/24-mer. Metallic cores within the protein cage were visualized in transmission electron micrograph (TEM) of negatively stained mineralized ferritin samples while TEM without prior staining showed dense metallic core but not the protein cage (Figure 4).

FIGURE 4. Electron micrographs of (A) cobalt- and (B) chromium-loaded AfFtn-AA stained with uranyl acetate and (C) iron-loaded AfFtn-AA without staining. Scale bar is 50 nm.

Magnetization and Relaxation Time

Magnetization at variable field strength was studied to understand the magnetic property of the (metal)AfFtn-AA nanocomposites. Typical hysteresis loops on VSM confirm paramagnetic property of the nanocomposites (data not shown). Magnetizations of (Co)AfFtn-AA and (Cr)AfFtn-AA and (Fe)AfFtn-AA nanocomposites are reported to be 50.4 memu/g, 45.2 memu/g and 434.5 memu/g, respectively. Enhanced brightness of T_1 -weighted images and signal loss of T_2 -weighted images were observed in the MR images with increasing concentration of mineralized AfFtn-AA (Figure 5). The relative visual signal of MR images at 3.0 T, demonstrated increased contrast at long TEs. Fitness of mean signal intensity data of (Co)AfFtn-AA T_1 -weighted images and T_2 -weighted images with inversion-recovery curve and mono exponential decay curve is shown in Figures 6A and 6B, respectively. T_1 and

T_2 values obtained from 0.8 % agarose matrix and that with various mineralized AfFtn-AA are shown in Table 1.

FIGURE 5. Brightening of T_1 - weighted MR images and signal loss of T_2 -weighted MR images of agarose embedded (Fe)AfFtn-AA nanocomposite.

FIGURE 6. Data fittings for calculation of T_1 and T_2 values of (Co)AfFtn-AA (at different protein concentration) from inversion recovery curve (A) and mono exponential decay curve (B), respectively.

TABLE 1. T_1 and T_2 values of metal loaded AfFtn-AA at 1 μ M protein concentration in 0.8% agarose matrix.

Metal	Metal ion encapsulated per AfFtn-AA	Concentration of the metal in matrix (mM)	T_1 (ms)	T_2 (ms)
No metal	--	--	1428	105
Iron	7000	7.0	969	33
Cobalt	350	0.35	1315	79
Chromium	2000	2.0	1176	82

All three (metal)AfFtn-AA nanocomposites showed visible shortening of T_1 and T_2 values. Decreased relaxation time of iron loaded AfFtn-AA is likely due to the encapsulation of a large number of Fe atoms in the cavity and better magnetization of the iron complex. Cobalt showed shortening of T_2 value with much smaller amount loaded within the AfFtn-AA cage and loading of larger amount of cobalt within the AfFtn-AA cage may significantly enhance the T_2 -shortening property. However, chromium showed better T_1 -shortening property. Enhanced T_1 and T_2 relaxations by cobalt and chromium complexes have been reported previously [16-17]. Parks *et al.* [16] reported magnetic relaxations of polymer coated cobalt nanoparticle but there is no previous report on the shortening of T_1 and T_2 relaxation time by cobalt- or chromium-based ferritin nanocore. Iron-loaded AfFtn-AA is more efficient in shortening the T_2 values than the T_1 values, which agrees with previous reports on the development of iron-based T_2 contrast agents [18].

CONCLUSION

Engineered *Archaeoglobus fulgidus* ferritin can form metallic core with cobalt, chromium, and iron with maximum loading capacity of 350, 2000 and 7000 metal ions per 24-meric protein cage, respectively. Protocol for maximum metal encapsulation varies from one metal to another. All three metallic cores can shorten magnetic relaxation time during MR imaging with iron being the more efficient one compared to cobalt and chromium. The shortening of both T_1 and T_2 values at higher concentration of iron-containing nanocomposite indicates its potential application as dual contrast agent. This is the first report on the shortening of relaxation time by cobalt- and chromium-based ferritin nanocore.

ACKNOWLEDGEMENT

We are thankful to Dr. Kenneth Sheah at Radlink Diagnostic Imaging and Prof. Chueh Loo Poh at NTU Bioengineering for the access, technical discussion and data analysis on MR imaging. This work is supported by the Singapore Ministry of Education Academic Research Fund Tier 1 (RG33/07).

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