The effect of PEGylation of ferritin on the biomineralization of \( \text{Co}_3\text{O}_4 \) core

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Abstract: - The outer surface of horse spleen apoferritin (HsA Fr) was chemically modified by poly(ethylene glycol) (PEG) to suppress the bulk precipitation during the artificial biomineralization of cobalt oxide core \( \text{Co}_3\text{O}_4 \) inside the cavity. PEG \( M_w=2000 \) was successfully attached to the outer surface of HsA Fr. \( \text{Co}_3\text{O}_4 \) biomineralization which was carried out using obtained PEG modified HsA Fr showed significant suppression of bulk precipitation, compared to the native HsA Fr. The PEGylation was proven to be effective for suppression of bulk precipitation. The best condition for \( \text{Co}_3\text{O}_4 \) synthesis was also surveyed and the highest core formation ratio and the yield of the core formed ferritin reached over 80% and 50%, respectively.

Key-Words: - Ferritin, PEGylation, Biomineralization, Nanoparticle, \( \text{Co}_3\text{O}_4 \), Poly(ethylene glycol)

1 Introduction

Recently biological nanoparticle (NP) synthesis has been attracting researchers’ attention for the potential that the homogenous NPs can be produced inside its cavity and outer surface of the protein can be used to deliver NP to the desired position via specific interaction. There are a lot of cage-shaped proteins and, among them, the most intensively studied for biomineralization is the apoferritin.

Apoferritin is a major cellular iron-storage protein and the native apoferritin consists of 24 polypeptide subunits. Figure 1 shows a schematic drawing of apoferritin with core looking down along four fold axis. There are two types of subunits, the light chain subunit (L-subunit) and the heavy chain subunit (H-subunit), and the ferroxidase center in a H-subunit oxidizes ferrous iron to ferric iron. The inner and outer diameters of the protein shell are about 7 nm and 12 nm respectively. The cavity and outside bulk solution are connected with narrow hydrophilic channels along the three-fold axis responsible for the transit positive iron ions. This inner cavity stores about 4000 iron atoms as the ferrihydrite \( \text{in vivo} \)1,2. The preceding works indicated that the electrostatic potential profile enhanced positive iron ion introduction into apoferritin cavity.

Using apoferritin as a spatially restricted chemical reaction chamber in aqueous condition, many kinds of inorganic NPs were synthesized by selecting the appropriate conditions \( \text{in vitro} \)3-11 including our \( \text{Co}_3\text{O}_4 \) NP synthesis.12 The obtained \( \text{Co}_3\text{O}_4 \) NPs have a narrow size distribution, because the biomineralization of NP cores were limited by the apoferritin protein shell. However, the \( \text{Co}_3\text{O}_4 \) synthesis always accompanied with much bulk precipitation which caused the low yield of ferritin with \( \text{Co}_3\text{O}_4 \) core (Co-ferritin). Some method preventing bulk precipitation was needed.

Fig.1 Schematic drawing of apoferritin molecule. (a) Molecule viewed down a four-fold axis, and (b) cross section including two three-fold channels.
PEGylation is widely used to modify the protein surface which can change and control the protein outer surface characteristics depending on the purposes. We employ this PEGylation of ferritin surface to prevent bulk precipitation.

In this paper, we chemically modified the outer surface of the horse spleen apoferitin (HsAFr) with poly(ethylene glycol) (PEG) and the effect of the PEGylation of ferritin on the biomineralization of Co$_3$O$_4$ was studied and the yield of the Co-ferritin and the deterrence of bulk precipitation is discussed.

2 Experimental

2.1 PEGylation of ferritin molecule

The horse spleen apoferitin (HsAFr) was purchased from SIGMA-Aldrich and purified by gel filtration. General procedure for chemical modification of ferritin with PEG derivatives was conducted following our previous work. The chemical modification of the ferritin exterior surface was achieved by the reaction with MeO-PEG-NHS to generate the amide ester of the PEG derivatives exposed on the exterior surface. Horse spleen ferritin (6 mg, 13 nmol) was incubated with about 800 mol excess of the PEG derivatives per ferritin subunit at 4°C in 30 mL phosphate buffer (100 mM, pH 8.2). After overnight, the sample was concentrated using centrifugal ultra filtration membranes (Apollo 7 mL QMWL 150 kDa, Orbital Biosciences) with a 150 kDa $M_w$ cutoff. After concentration, several times larger volume of 150 mM NaCl was added and concentrated again. This process was repeated more than twenty times.

The purity of products was confirmed by analytical size exclusion chromatography (SEC). The stoichiometry of the ferritin derivatization was measured by time of flight mass spectrometry (MALDI-TOF/MS, Voyager-DE™ STR, Applied Biosystems).

2.2 Biomineralization of Co$_3$O$_4$ ferritin core

A 0.5 mg/mL (1 µM) apoferitin in the solution of 100 mM HEPES pH 8.3, 37.5 mM Na$_2$SO$_4$ was prepared. Ammonium cobalt sulfate (ACS) was added to create a final concentration of 2 to 9 mM, followed by the addition of H$_2$O$_2$ with a half-stoichiometric concentration of ACS. The solution was stirred and left about 20 min at R.T. and then was left overnight at 50°C in the temperature control aluminum box. The solution after Co$_3$O$_4$ NP synthesis was centrifuged at low speed to remove the precipitates. The protein concentration of the supernatant was measured by the Bradford protein assay method. The core formation was observed by the transmission electron microscopy (TEM) with negative staining with 1% aurothioglucose, which is proven not to stain the inside the cavity. The core formation ratio (CFR) was calculated by dividing the number of core-containing apoferitins by the number of all apoferitin in TEM images and used for the evaluation of core formation efficiency. The yield of the ferritin ratio (YFR) was calculated by dividing the products of the protein concentration in the supernatant after Co$_3$O$_4$ core formation and the CFR by the initial apoferitin concentration.

3 Result and Discussion

3.1 PEGylation of HsAFr

To estimate the degree of PEGylation of HsAFrs, MALDI-TOF/MS measurement was carried out. Figure 2 shows the results of ferritins PEGylated by PEG2000 along with the native ferritin. Since MALDI-TOF/MS technique detects the mass of ferritin subunits which are intact or covalently attached by PEG molecule, PEGylated subunits should be detected easily by weight change. HsAFr showed a single peak around 20 kDa, which corresponds to the apoferitin subunit weight before PEGylation. After PEGylation, there are two peaks appeared. One corresponds to the subunit with one PEG molecule and the other to that with two PEG molecules. There was also a very low peak corresponding original subunit. This result indicated that almost all subunits have one or two covalently
attached PEG molecules, i.e. each HsAFr has around 24-48 PEG molecules.

The PEGylation of HsAFr was also confirmed by the size exclusion chromatography analysis using HPLC and gel filtration column (G4000SWxLPEEK, TOSOH). Figure 3 shows the elution volume profile of unmodified and PEGylated HsAFrs. The elution volume of unmodified HsAFr, the molecular weight of which is about 450 kDa, is 10.3 mL. On the other hand, the modified ferritin showed smaller elution volume, 10.0 mL which showed PEGylated HsAFr became larger than unmodified HsAFr. Taking together with the MALDI-TOF/MS measurement result, HsAFr were successfully PEGylated and 24-48 PEG molecules were added to one HsAFr molecule.

3.2 Biomineralization of Co$_3$O$_4$

The biomineralization of Co$_3$O$_4$ was carried out using the solution of 100 mM HEPES pH 8.3, 37.5 mM Na$_2$SO$_4$ and ACS with a concentration from 2 mM to 9 mM.

Figure 4(a) shows the states of reaction solutions with 5 mM ACS 20 min after addition of H$_2$O$_2$. Upon addition of H$_2$O$_2$, the color of reaction solutions changed from light pink to thin brown, accompanying the oxidation of divalent cobalt ion.

Figure 4(b) shows the reaction solutions after 16 hours from the addition of H$_2$O$_2$. The solutions were centrifuged at slow speed and the precipitates were settled down as a pellet. From left to right, picture shows with HsAFr, HsAFr PEGylated by PEG2000 (HsAFr-2000) and without protein. The picture clearly shows that the reaction solution without protein produced heavy precipitates and some particles were adhered on the tube wall. The supernatant itself was clear, which indicates that all cobalt ions turned into insoluble precipitation. The solution with HsAFr was the same as that without protein but did not show any adhesive precipitates. The pellet was soft and bulky.

On the other hand, the solution with PEGylated HsAFr shows little precipitation. The color changed from thin brown to dark brown and clear supernatant was retained, which indicated that the solution contained cobalt ion or cobalt nanoparticles dispersed in the solution. The precipitation was suppressed greatly by the PEGylation of HsAFr.

The Figure 4(b) inset is the TEM image of HsAFr-2000, stained with aurothioglucose which does not stain the cavity. It was seen that cores were surrounded by protein shell which looked like white ring. This indicates that PEGylated HsAFr can synthesize Co$_3$O$_4$ core efficiently even under the condition that native HsAFr co-precipitated.

The core formation ratios (CFR) were calculated from TEM images and protein concentration of the supernatant was measured. Based on the CFR and protein concentration, yield of the ferritin ratios (YFR) were calculated. Figure 5 shows the dependence of CFR, YFR, and protein concentration on the initial
ACS concentration. In the case of unmodified HsAFr, the protein concentration was low and the YFR was affected by the low protein concentration very much and never exceed 40%. On the other hand, in the case of HsAFr-2000, the CFR increased up to around 6 mM ACS concentration and became plateau. The amount of protein concentration in the supernatant slowly and monotonously decreased but much higher protein concentration was retained than HsAFr. This is consistent with the suppression of bulk precipitation by PEGylation. The YFR was improved very much compared with HsAFr. The YFR reached around 50% between 3 mM and 5 mM ACS concentration. CFR is greatly improved and reached 80% at 6 mM ACS concentration. These data indicated that PEGylation suppresses the bulk precipitation considerably and resulted in the high Co$_3$O$_4$ core formation and high yield of Co$_3$O$_4$ ferritin.

4 Conclusion

The PEGylation of HsAFr was conducted by using the chemical reaction of MeO-PEG-NHS with Lys amino-acids at the outer surface of HsAFr protein shell. PEGylated HsAFr, which displayed PEG2000 molecules at the surface, suppressed the bulk precipitation dramatically during the artificial biomineralization of Co$_3$O$_4$ in the HsAFr cavity. The core formation ratio reached around 80% and the high yield of Co$_3$O$_4$ homogenous nanoparticles was achieved. It was clearly demonstrated that this new approach for high yield of homogenous Co$_3$O$_4$ nanoparticle biomineralization utilizing PEGylated ferritin is very effect and promising.

References:


