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Synthesis and Characterization of Ferritin–Polymer Hybrid

Yoshitsugi Fukushige, Masahiro Muraoka and Ichiro Yamashita*
CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan
Fax: 81-743-72-6196, e-mail: fukusige@ms.naist.jp
* Advanced Technology Research Laboratories, Matsushita Electric Industrial Co., Ltd.,
3-4 Hikaridai, Seika, Kyoto 619-0237, Japan
Fax: 81-774-98-2515, e-mail: yamashita.ichiro@jp.panasonic.com

The ferritin protein was modified using the succinimidyl ester of poly(ethylene glycol) derivatives, for which the quantitative reactions were monitored by measuring the absorbance of the fluorescent derivatives of PEG and observing the retention time of the hybrid materials during size exclusion chromatography. The resultant hybrid products were characterized by transmission electron microscopy in order to observe the spherical shapes of the ferritin cages incorporating iron oxide nanoparticles. The dynamic light scattering data showed an enlargement of the spherical size of the ferritin cage and the diameter for the reacted hybrid materials with a molecular weight 5000 of the poly(ethylene glycol) derivatives is greater than the poly(ethylene glycol) with a molecular weight 2000, suggesting that the polymer chains were attached to the exterior of the ferritin cage.
Key words: ferritin, poly(ethylene glycol), chemical modification, protein hybrid, nanotechnology

1. INTRODUCTION

The hybridization of metal and semiconductor nanoparticles with biomolecules has been of recent interest in nanobiotechnology.[1–3] Biomolecules, such as proteins and DNA, provide various kinds of unique properties and exhibit nanoscale structures with various dimensions making them attractive candidates for constructing the nano–structured materials. As one of the conventional synthetic strategies by utilizing biomolecules, cage–shaped proteins[4–12] and spherical viruses[13] as bio–supramolecular templates have been utilized to prepare well–defined nanoparticles by biomineralization. In order to produce hybrid nanomaterials of biomolecules with nanoparticles for future devices such as nanoelectronics devices and bio–medical sensors, it is important that the biomolecules are integrated into 2D and 3D ordered structures on the surfaces.[14–16] For example, biotinylated ferritin was assembled by controlled aggregation using streptavidin connectors.[17] A protein cage was also used for the 2D array formation on a Au surface by means of asymmetric chemical immobilization.[18] The viruses attached with oligonucleotides led to a DNA–directed assembly.[19] These assembly systems of proteins have been derived from the chemical modification of the protein surfaces by appropriate reagents, which is potent for the aggregation by the supramolecular interactions. We now describe the chemical modification of the exterior surface of the cage–shaped protein, ferritin, with poly(ethylene glycol) derivatives (PEGs). We have quantified the degree of chemical modification by analyzing the fluorescein–derived PEG coupled to the lysine residues (amino groups). Ferritin is a cage–shaped protein with a 12 nm external diameter and consists of 24 identical polypeptide subunits that form a spherical shell. It has a central hollow cavity with a 7 nm internal diameter in which iron is stored in the form of iron oxyhydroxide ferrihydrite. Apoferritin can be prepared by the removal of the nanoparticle cores, allowing non–native nanoparticle cores for the purpose of exploring nanotechnology applications. Ferritin is thus an attractive biomolecule that can be modified for hybridization with a functional polymer and organic molecule.

2. EXPERIMENTAL SECTION

2.1 Materials

Horse spleen ferritin was obtained as 0.15 M NaCl aqueous solutions from Sigma–Aldrich Co., which was purified by size exclusion chromatography (SEC) before using the chemical modification (Fig.1). The methoxy poly(ethylene glycol) succinimidyl esters (MeO–PEG–NHS) with M_w = 2000 and M_w = 5000 were received from the NOF Co. The fluorescein poly(ethylene glycol) succinimidyl esters (Fluo–PEG–NHS) with M_w = 3400 and M_w = 5000 were purchased from Nektar Co. Milli-Q deionized water (18 MΩ) was used for preparing all the solutions.

2.2 Methods

A preparative SEC was performed using a recycling preparative HPLC system (LC–908W–G10, Japan Analytical Industry Co., Ltd.) equipped with a JAIGEL-GS710 SEC column (Japan Analytical Industry Co., Ltd.). An analytical SEC was performed with a UV (observed at 280 nm) /fluorescence (excitation at 495 nm, observed at 510 nm) dual detector HPLC system (UV2070 and FP2025 spectrophotometers, JASCO), using a TSK gel α4000 column (Tosoh) and 0.1 M potassium phosphate buffer (pH 7.4) as the eluent. The stoichiometry of the ferritin derivatization was determined by real–time observation using UV–VIS absorbance spectroscopy measured with a UV2070 spectrophotometer on an analytical SEC. High–resolution transmission electron micrographs

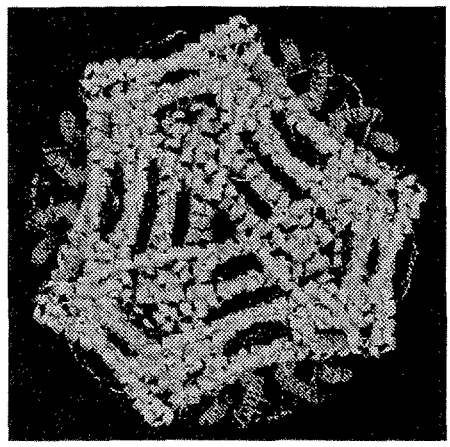

Fig.1 Ribbon diagram of ferritin looking down the 3-fold channel.

(HRTEM) were obtained using a JEM-2200FS (JEOL) transmission electron microscope. Samples were prepared by placing 4 μL aliquots of the modified ferritin solution on a carbon-coated copper TEM grid (200 mesh, Okenshoji Co., Ltd.) for 1 min, which had been pretreated by glow-discharge to render the carbon surface hydrophilic, and then stained with 4 μL of 2% phosphotungstic acid (Alfa Aesar). Dynamic light scattering (DLS) measurements were made using a DynaPro Molecular Sizing Instument (Proterion Co.).

2.3 General Procedure for Chemical Modification of Ferritin with PEG Derivatives

The chemical modification of the ferritin exterior surface was achieved by the reaction with MeO-PEG-NHS or Fluo-PEG-NHS to generate the amide ester of the PEG derivatives exposed on the exterior surface. Horse spleen ferritin (1 mg, 2.1 nmol) was incubated with 20-1000 mol excess of the PEG derivatives per ferritin subunit at 4 °C in 5mL phosphate buffer (100 mM, pH 8.2). After 48 h, the sample was concentrated using centrifugal ultrafiltration membranes (Apollo 7 mL QMWL 150 kDa, Orbital Biosciences) with a 150 kDa M_w cutoff, and the derivatized ferritin (MeO-PEG-fer or Fluo-PEG-fer) was purified by SEC which was performed on a recycling preparative HPLC system. The products were confirmed by analytical SEC. The stoichiometry of the ferritin derivatization was determined by UV-VIS absorbance spectroscopy. The fluorescein concentrations were obtained by measurement of the absorbance at 492 nm compared with known quantities of ferritin from the absorbance at 280 nm.

3. RESULTS AND DISCUSSION

3.1 Preparation of Fluo-PEG-fer and stoichiometry of ferritin derivatization

Various concentrations of Fluo-PEG-NHS were used for the chemical modification of ferritin due to the pursuit of the different degrees of modification. When a 600 mol excess or less of fluorophores (<30 μmol) per ferritin subunit was used for the chemical modification of the ferritin molecules, the modified ferritins bore the PEG polymer chains with an increasing loading giving rise to a stronger absorbance at 492 nm and a maximum of about 24 fluorophores could be attached to the ferritin cage. However, an 800 mol excess or more of fluorophores (40 and 50 μmol) per ferritin subunit has led to no further change in the amounts attached to the ferritin cage as shown in Fig. 2(a). Similar results have been also observed with regard to the extent of the chemical modification by analyzing the retention time of SEC for the modified ferritin. As shown in Fig. 2(b), the samples of Fluo-PEG-fer prepared with various concentrations of the PEG derivatives eluted on the SEC as one peak at retention time shorter than the native ferritin, indicating an increase in the effective size of the particle. Whereas in a previous report,[14] the average

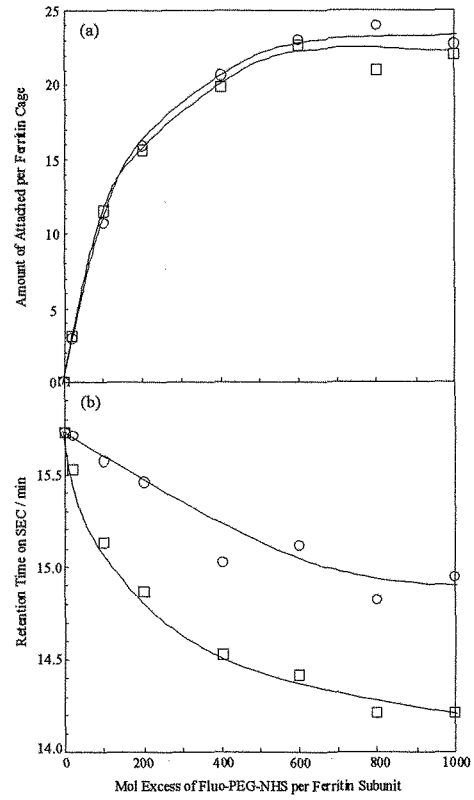

Fig.2 Plots showing (a) the amount of the covalently attached Fluo-PEG-NHS to the ferritin and (b) retention time of Fluo-PEG-fer eluted on SEC. o denotes the sample reacted with PEG MW3400 and □ denotes the sample reacted with PEG MW5000.

number of biotinylated groups attached to the exterior surface of the ferritin cage was determined as 72 per the cage; in our experiments it was observed that 24 PEG derivatives per the ferritin cage were modified on the exterior surface, presumably due to the steric hindrance of the PEG polymer chains and then symmetric modification of the 24 polymer chains on each of the 24 subunits of the ferritin molecules. In addition, it is obvious that the change in the retention time on SEC for the product is consistent with that for the amount calculated from the absorbance of fluorescein on PEG derivatives, implying that the limit of the chemical immobilization of the PEG derivatives on the exterior surface of the ferritin molecules can be signified by measuring the retention time on SEC for the modified ferritin. Therefore, it is possible that a maximum chemical modification of the PEG derivatives on the ferritin molecules is achieved by using from a 600 to

800 mol excess of the PEG derivatives per ferritin subunits.

3.2 Preparation of MeO–PEG–fer

Commercially available PEG (M_w = 2000 and 5000) samples, capped at one end with a NHS ester and at the other with a MeO group, were mixed with ferritin at various molar ratios to accomplish attachment on the exterior surface of the ferritin protein. Fig. 3 shows the retention time of MeO–PEG–fer prepared with various concentrations of MeO–PEG–NHS on SEC. The retention time on SEC for MeO–PEG–fer was unchanged using from a 600 to 800 mol excess of the PEG derivatives, which lead to a maximum modification on the exterior of the ferritin cage. These results for MeO–PEG–fer represent a change similar to that for Fluo–PEG–fer described above. Therefore, it is assumed that the degree of chemical modification of the MeO–PEG derivative on the ferritin cage can be estimated as 24, which was calculated using the fluorecein derivative of PEG attached to the ferritin cage.

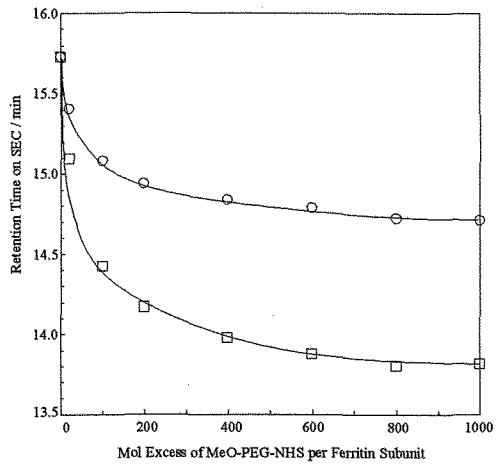

Fig.3 Plots showing the retention time of MeO-PEG-fer eluted on SEC. ○ denotes the sample reacted with PEG MW2000 and □ denotes the sample reacted with PEG MW5000.

3.3 Characterization of ferritin–PEG hybrids by TEM

The products of the chemical modification were characterized by TEM. The characterization was performed by comparing the ferritin–PEG hybrids with the native ferritin to confirm the structure of the ferritin cage. The purified, native ferritins encapsulating iron oxide nanoparticles were imaged by TEM, in which the nanoparticles were surrounded by the protein cages, as shown in Fig.4(a). On the other hand, the chemically modified ferritin with MeO–PEG having molecular weights of 2000 (Fig.4(b)) and 5000 (Fig.4(c)) also showed similar pictures and the nanoparticles were surrounded by the protein cages, indicating that the shape of the protein cage remains unaltered after chemical modification.

3.4 Characterization of ferritin–PEG hybrids by DLS

Since it is considered that the ferritin–PEG hybrids swells greater than the native ferritn when PEG derivatives are attached to the outer surface of the ferritin cages, it is one of the useful methods in which the diameters of the ferritin–PEG hybrids are measured

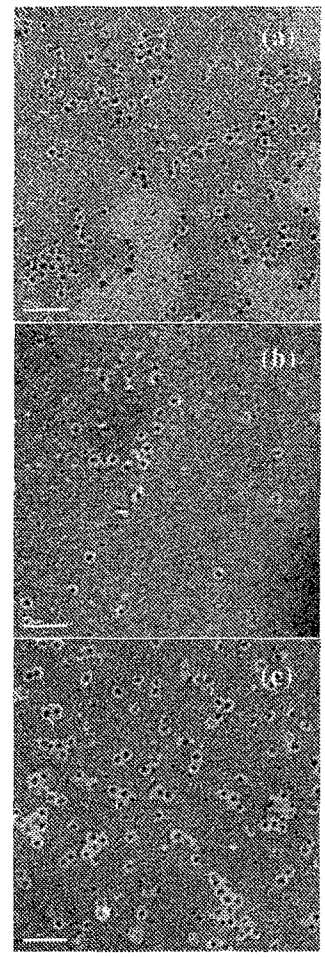

Fig.4 Transmission electron micrographs of (a) the native ferritin, (b) the products reacted with MeO–PEG–NHS (MW 2000), and (c) the products reacted with MeO–PEG–NHS (MW 5000). Scale bars are 50 nm.

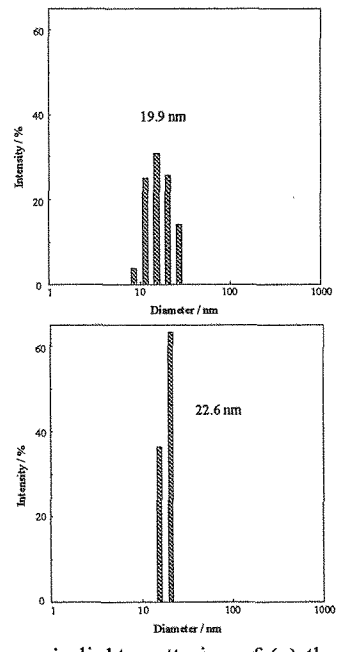

Fig.5 Dynamic light scattering of (a) the products reacted with MeO–PEG–NHS (MW 2000) indicating a diameter of 19.9 nm and (b) the products reacted with MeO–PEG–NHS (MW 5000) indicating a diameter of 22.6 nm.

by DLS in order to prove the chemical modification of the ferritin cages. As shown in Fig.5, the DLS of the ferritin–PEG hybrids indicated an icrease in the diameters. Furthermore, the hybrid samples that reacted with the PEG derivatives with MW 5000 showed a cage having a diameter (22.6 nm) greater than that with MW 2000 (19.6 nm), probably due to the length of the polymer chains on the exterior of the ferritin cage. These data suggest that the chemical modification of the PEG derivatives can be achieved on the exterior of the ferritin cage.

4. CONCLUSIONS

The ferritin–PEG hybrids were synthesized using the succinimidyl ester of poly(ethylene glycol) and quantitative modification was achieved by analyzing the fluorescein derivative of PEG attached to the exterior of the ferritin cage, indicating that a maximum of 24 polymer chains per ferritin cage was modified on the exterior surface. The hybrids were characterized by utilizing TEM and DLS. These results suggest that the ferritin proteins can be used as a nanoblock for constructing nano–scaled architectures by combining various polymers. The hybrid materials of the protein with a polymer will be applicable for nano–biomedicine such as a drug delivery system (DDS) and biosensor.

5. REFERENCES

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