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Electron Microscopy of the Al and UO_2 Nanophase Particles Synthesized in Horse Spleen Ferritin

Hyang-Ran Mun, Kyung-Suk Kim[1,*] and Jung-Hoo Lee
School of Science and Technology, [1]Faculty of Biological Sciences, Chonbuk National University, Chonju, 561-756, Korea

말 비장 Ferritin에서 합성된 Al과 UO_2 나노 입자의 전자현미경 연구

문 향 란, 김 경 숙[1,*], 이 정 후
전북대학교 자연과학대학 과학기술학부, [1]생물과학부

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ABSTRACT

Synthesis of inorganic nanophase particles was performed to verify and understand the binding of non-ferrous metal ions including Al and UO_2 to the apoferritin molecules. Reconstituted inorganic particles of Al or UO_2 were identified by TEM as discrete electron dense cores encapsulated within the protein shell. The corresponding EDXA spectra confirm the presence of metal ions in the reconstituted ferritin. The Al cores of ferritin has been studied by TEM for the first time. Bimetallic cores with Al/Fe and UO_2/Al were also produced and examined under TEM. Mixed metal cores encapsulated in the protein shell are well formed and its corresponding EDXA spectra also confirm the presence of metal ions in the mineral cores. Therefore, the present study proves that ferritin can be used to synthesize inorganic nanophase particles of Al and UO_2.

Key words : TEM, EDXA, Nanophase particle, Ferritin, Al, UO_2

INTRODUCTION

The synthesis of inorganic nanophase particles is currently of great interest in the developing field of nanotechnology. The synthesis was developed with the use of reaction systems such as surface-bound organic groups, polymers, porous glasses, zeolite, phospholipid vesicles, etc. (Meldrum et al., 1991; Mann, 1996). In the biological system, an iron storage protein, ferritin, can be used in the synthesis of nanophase particles (Dickson, 1994). Ferritin functions not only as a reaction cage but also as an enzymatic catalyst (Meldrum et al., 1991). The ferritin consists of a sph-

* Correspondence should be addressed to Dr. Kyung-Suk Kim, Faculty of Biological Sciences, Chonbuk National University, Chonju, 561-756, Korea. Ph: (0652) 270-3341, FAX: (0652) 270-3399, E-mail: sukkim@moak.chonbuk.ac.kr

erical protein shell (apoferritin) composed of 24 polypeptide subunits, in which a large amount of Fe atoms (up to 4500 atoms) can be stored (Harrison et al., 1989). Inorganic nanophase particles that are previously synthesized within the ferritin core are potentially biocompatible and bioactive, and among examples are uranyloxide (Meldrum et al., 1991; Mann & Meldrum, 1991), manganese oxide (Mackle et al., 1993) and magnetite (Meldrum et al., 1992).

Sczekan and Joshi (1989) reported the binding of non-ferrous metal ions including Al to phytoferritin (plant ferritin) and suggested that ferritin was capable of acting as a detoxificant for Al. The toxicity of Al has been implicated in the Alzheimer's disease (Fleming & Joshi, 1987; Joshi et al., 1995), although another study reported no significant increase in the Fe and Al content of ferritin in the cerebral cortex of Alzheimer's disease (Dedman et al., 1992a). They also reported that horse spleen apoferritin binds Al poorly upon the addition of aluminium citrate (Dedman et al., 1992b). However, the Al cores of ferritin produced in the presence of Al has not yet been studied by transmission electron microscopy (TEM).

The uranyl cation, UO_2, is known to bind on the internal surface of apoferritin (Meldrum et al., 1991). Addition of $(CH_3COO)_2UO_2$ to an apoferritin solution resulted in discrete electron-dense cores of mean diameter 6 nm, but the core mineralization was not limited within the core. It also appears as external UO_2 binding to the surrounding protein shell (Meldrum et al., 1991; Mann & Meldrum, 1991). Reconstitution of ferritin with UO_2 has also been intended for tumor therapy (Hainfeld, 1992).

In the present work, inorganic nanophase particles were synthesized to verify and understand the binding of non-ferrous metal ions including Al and UO_2 to apoferritin. Bimetallic cores with Al/Fe and UO_2/Al were also produced. TEM and energy dispersive X-ray analysis (EDXA) were used to identify the reconstituted inorganic nanophase particles.

MATERIALS AND METHODS

Horse spleen ferritin was purchased from Sigma Chemical Co. (USA) and apoferritin was prepared by chemical reduction using 1% thioglycolic acid (pH 5.0), followed by dialysis against 0.5% sodium bicarbonate (pH 8.0). All steps were carried out at 4°C. After dialysis, the sample was filtered and the concentration of protein was measured by the modified Lowry method (Hess et al., 1978) with bovine serum albumin as protein standard.

For reconstitution, apoferritin (2 μM) was incubated with the addition of $AlCl_3$, $(CH_3COO)_2UO_2 \cdot 2H_2O$

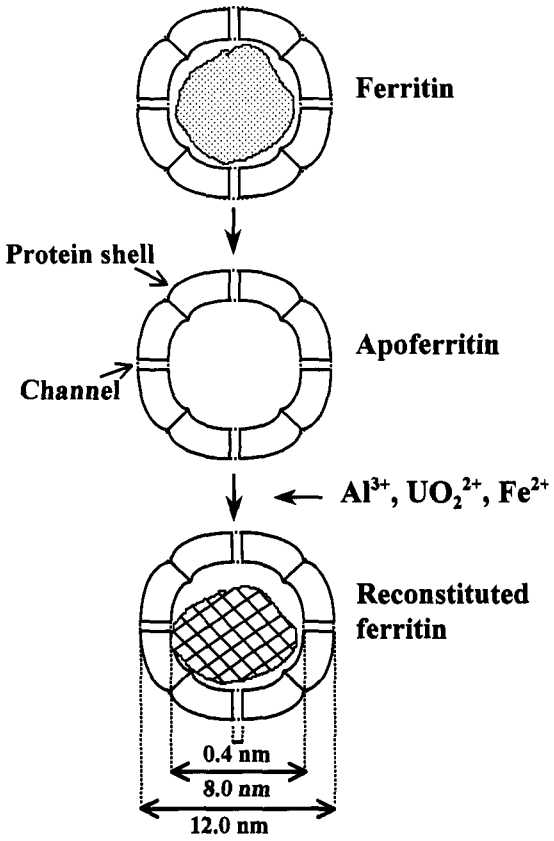

Fig. 1. A schematic representation of the synthesis of inorganic nanometer particles in ferritin.

and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ solutions in 25 mM TES buffer (pH 8.0). A general scheme for the reaction is shown in Fig. 1. Each metal solution was added to give a concentration of 2 mM (theoretically corresponding to a loading of 1000 metal ions per molecule of apoferritin). Mixed metal cores were produced by successive addition of 1000 Al/1000 Fe and 1000 UO_2/1000 Al atoms/molecule of protein, respectively. Fe solution was prepared under anaerobic condition by providing N_2 gas. UO_2 was added in a dark condition. Following reactions with the metal ions at room temperature for 24 hr, dialyes were performed against d-H_2O. The reconstituted ferritins were filtered using 0.45 μm filter (Millipore) to remove aggregates that might have occurred during reconstitution.

The reconstituted ferritin samples were prepared for TEM, electron diffraction (ED) and EDXA by air-drying small drops of ferritin onto formvar-coated copper grids, and examined under a JEM1010 (Jeol) and a EM912 Omega (Carl Zeiss) operated at 80 KeV. Stained samples were prepared using 2% phosphotungstic acid. ED and EDXA were carried out on the selected area of the unstained sample.

RESULTS

Horse spleen ferritins reconstituted with metal ions including Al, UO_2 and Fe were studied by TEM and EDXA. The addition of Al to the apoferritin resulted in a clear colorless solution. A negatively stained image of the reconstituted ferritin loaded with 1000 Al atoms/ molecule of protein is shown in Fig 2a. There are discrete ferritin cores encapsulated within the protein shell and some cores are partially filled (arrows in Fig. 2a). Its electron diffraction shows a diffuse ring pattern, indicating that the inorganic particles are of poor crystallinity (data not shown). The EDXA spectra of selected area clearly show the presence of Al in the reconstituted ferritin (Fig. 2b).

Ferritin reconstituted with 1000 Al/1000 Fe atoms/

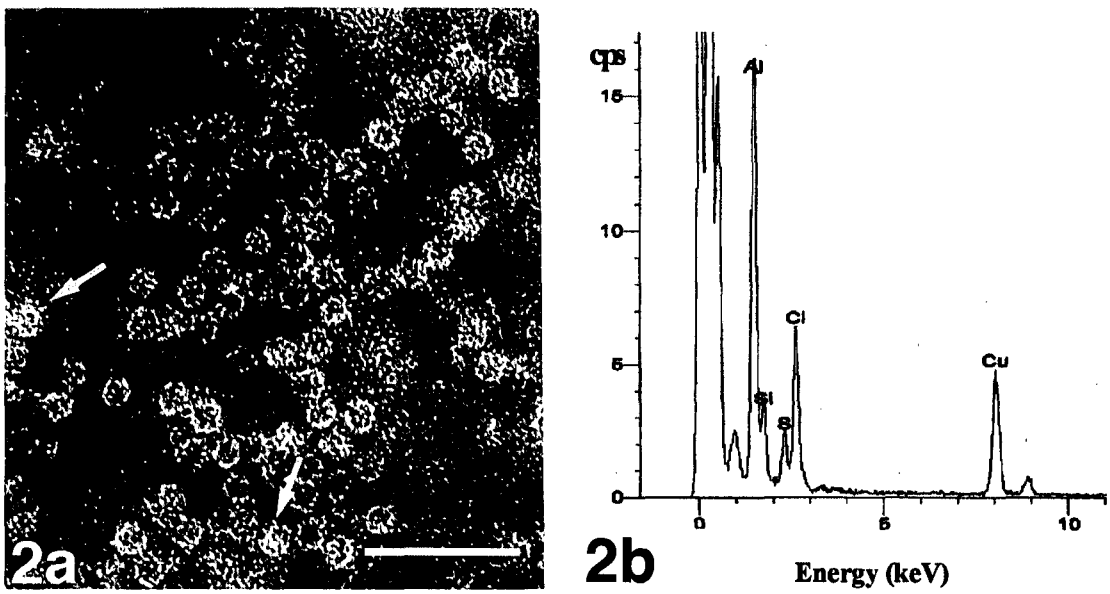

Fig. 2. Stained TEM image of inorganic nanophase cores (1000 Al atoms/molecule of protein) encapsulated within the protein shell of ferritin. (a) Stained photomicrograph. Scale bar represents 50 nm. The arrows show partially-filled cores. (b) Corresponding EDXA spectrum. Cu peaks arise from the electron microscope grids. Other peaks are contaminants possibly from the buffer and reagents.

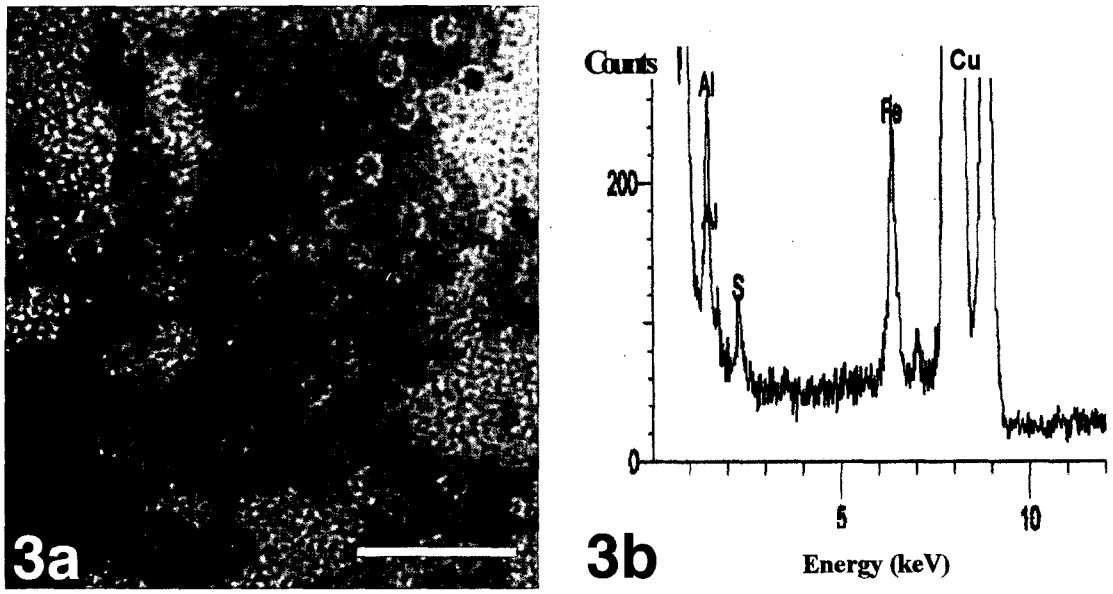

Fig. 3. Stained TEM image of inorganic nanophase cores (1000 Al and 1000 Fe atoms/molecule of protein) encapsulated within the protein shell of ferritin. (a) Stained photomicrograph. Scale bar represents 50 nm. (b) Corresponding EDXA spectrum.

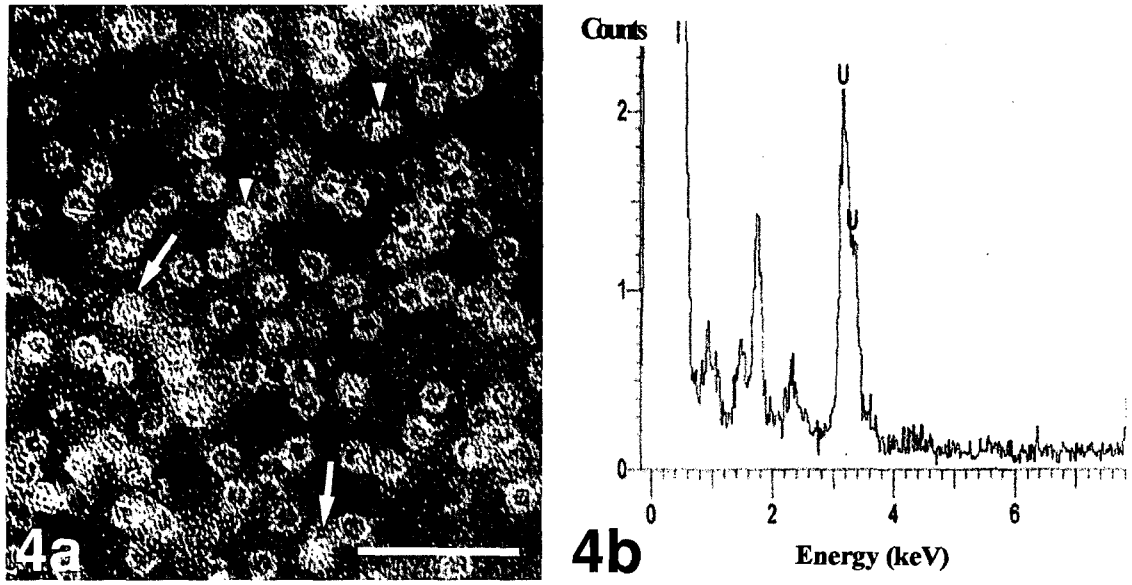

Fig. 4. Stained TEM image of inorganic nanophase cores (1000 UO_2 atoms/molecule of protein) encapsulated within the protein shell of ferritin. (a) Stained photomicrograph. Scale bar represents 50 nm. Arrows, empty cores; Arrowheads, partially-filled cores. (b) Corresponding EDXA spectrum.

molecule of protein resulted in a clear yellowish-brown solution. Its negatively stained image is shown in Fig. 3a. The mineral cores encapsulated in the protein shell are generally irregular in morphology. Inter-

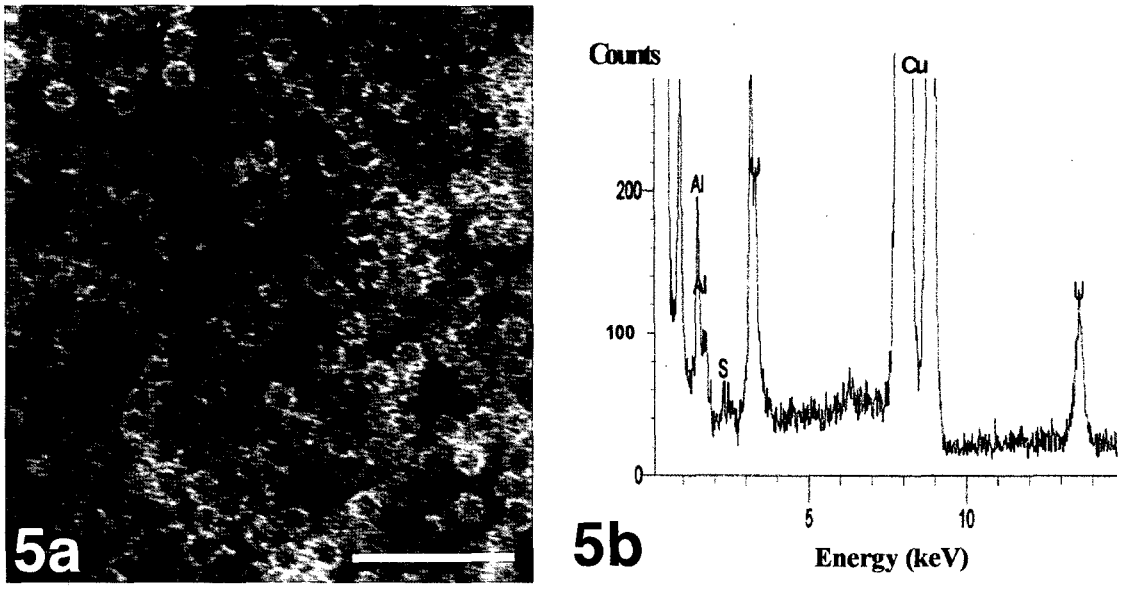

Fig. 5. Stained TEM image of inorganic nanophase cores (1000 UO_2 and 1000 Al atoms/molecule of protein) encapsulated within the protein shell of ferritin. (a) Stained photomicrograph. Scale bar represents 50 nm. (b) Corresponding EDXA spectrum.

estingly, the cores are variable in their electron density and the reason so far is not known. In our experiment, a direct competition between the metal ions was established as apoferritin was reconstituted simultaneously with 1000 Al ions and 1000 Fe ions. The mixed metal oxides formed in the core of ferritin were analyzed by EDXA spectra, and they show the presence of Al and Fe in the mineral cores (Fig. 3b).

Ferritin reconstituted with UO_2 resulted in a clear yellowish solution. In a negatively stained TEM photomicrograph, there are discrete electron-dense cores enclosed within the protein shell (Fig. 4a). Some empty (arrows in Fig. 4a) and partially-filled (arrowheads in Fig. 4a) cores are also seen in the photomicrograph. Its electron diffraction shows a diffused ring pattern, indicating that the inorganic particles are amorphous. The EDXA spectra confirm the presence of U in the reconstituted ferritin (Fig. 4b).

The simultaneous addition of 1000 UO_2/1000 Al atoms/molecule of protein to the apoferritin resulted in a clear yellowish solution. Its negatively stained image is shown in Fig. 5a, indicating that metal ions enter the apoferritin cavity. Its corresponding EDXA spectrum (Fig. 5b) confirms the presence of Al and U in the mineral cores.

DISCUSSION

The reconstituted Al cores within the apoferritin shell has not yet been examined under TEM, although analytical experiments were achieved for ferritin reconstituted with Al (Sczekan & Joshi, 1989; Dedman et al., 1992a). The results described here clearly indicate that discrete Al cores formed when apoferritin was reconstituted in the presence of aluminium chloride. Previously, apoferritin is known to bind only 7.6 Al atoms/molecule on incubation with aluminiumn citrate (Dedman et al., 1992b). The approximate core size (Fig. 2a) of ferritin reconstituted with Al apparently suggests that the concentration of Al is much greater in the presence of aluminium chloride (this study) than of aluminium citrate (Dedman et al., 1992b). Dif-

fuse electron diffraction patterns are unable to identify Al phases of ferritin cores.

The synthesis of mixed metal cores of nanometer size particle was confirmed as proposed by Dedman et al. (1992b). Metals of Al, Be, Cd and Zn were reported to bind to phytoferritin with similar affinities (Kd ≈ 10^{-6} M; Sczekan & Joshi, 1989). Our results indicate that ferritin is capable of binding and perhaps detoxifying metal ions.

The formation of UO_2 cores in ferritin was examined by TEM. Our investigation is to pursue possibility of using hydrolytic polymerization as inorganic precipitation within the protein shell. Upon the incubation of apoferritin with $(CH_3COO)_2UO_2 \cdot 2H_2O$ in 25 mM TES buffer (pH 8.0), a clear yellowish brown solution was obtained in contrast with Meldrum et al. (1991), in which extensive nonspecific precipitation was reported outside the protein (Meldrum et al., 1991; Douglas, 1996). In our experiment nonspecific precipitation might have been reduced since we added 1000 UO_2 per molecule of protein rather than 4000 UO_2 of Meldrum et al. (1991). Our result of discrete electron dense UO_2 cores agrees with Meldrum et al. (1991). The hydrolytic polymerization appeared to proceed competitively in the initial stage, and metals once bound cause the preferred catalysis of subsequent hydrolysis and crystal growth over the nucleation. It is shown as the difference in contrast in Fig. 4a (dark cores are filled, white cores are empty).

When horse spleen apoferritins were incubated with $AlCl_3$, $(CH_3COO)_2UO_2 \cdot 2H_2O$ and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, mixed metal oxide cores are well formed. Until now, the only mixed metal oxides of Fe and Mn were formed in ferritin, and the core mineral was a two-phase layered system of crystalline Fe oxide and amorphous Mn oxide (Medrum et al., 1995). Pead et al. (1995) also suggested that Fe and Mn are likely to bind to ferritin only by carboxylate coordination and their behavior is similar. Thus, it was infered that reconstitution of ferritin with a simultaneous addition of Fe and Mn may proceed competitively for the bind-ing site inside the protein shell and the core mineral grows independently to two-phase layered system. Electron diffraction of all reconstituted monometallic and bimetallic cores exhibited diffuse ring patterns implying that the inorganic particles are poorly crystalline or amorphous.

In this study we postulated that the mechanism of Al and UO_2 mineralization seems analogous to the iron mineralization of ferritin, showing preferred hydrolytic polymerization over nonspecific precipitation. Individual metal ions of Al and UO_2 may affect the formation and phase of minerals. Further studies are required to understand how much effect and what relation each component in the inorganic nanophase particles has on the structural and compositional properties.

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<국문초록>

본 연구에서는 Al과 UO_2의 나노미터 크기의 무기물 입자를 합성하기 위하여 생체 시스템인 철단백질 ferritin을 이용하였다. Ferritin에서 합성된 Al과 UO_2의 무기물 입자를 TEM을 이용하여 관찰한 결과, 단백질 내부에 나노미터 크기의 구형인 미네랄(mineral) core를 확인하였다. 그 입자들에 대한 EDXA 분석 결과 각각 Al과 UO_2로 구성된 미네랄임을 확인하였다. Ferritin을 이용하여 합성된 Al core는 이번 연구에서 처음으로 전자현미경으로 관찰되었다. 그리고 두 종류의 다른 금속 즉, Al/Fe 및 UO_2/Al의 존재하에 ferritin core를 합성하여 TEM을 관찰한 결과, 역시 나노미터 크기의 구형인 전자밀도 core를 관찰하였고 EDXA 분석 결과 구형인 core가 합성시킨 두 금속 원소의 미네랄로 구성되어 있음을 증명하였다. 그리하여 본 연구는 철단백질을 이용하여 철이 아닌 Al과 UO_2로 구성된 나노미터 크기의 무기물 입자를 합성할 수 있음을 증명하였다.