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Enzymatic preparation of hollow magnetite microspheres for hyperthermic treatment of cancer

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Abstract Ferrimagnetic materials can be expected to be useful as thermal seeds for hyperthermic treatment of cancer, especially where the cancer is located in deep parts of body, as they can generate heat by magnetic hysteretic loss when they are placed in an alternating magnetic field. In this study, hollow magnetite (Fe_3O_4) particles were prepared using an enzymatic reaction of urease. A hollow particle was obtained by using a Pasteur pipette. The particle was 500 μm in size and was composed of Fe_3O_4. Its saturation magnetization and coercive force were 57 emu·g^{-1} and 183 Oe, respectively. Its heat generation under an alternating magnetic field of 300 Oe at 100 kHz was estimated to be 45 W·g^{-1}. Microspheres 30 μm in diameter were also successfully obtained by using a spray gun.

1. Introduction

Cancer cells are generally destroyed at above 43°C because of their intrinsic microenvironments including low pH, whereas normal cells are not damaged, at even higher temperatures [1, 2]. In addition, tumors are more easily heated than the surrounding normal tissues, as their vascular and nervous systems are poorly developed [3–5]. Therefore, hyperthermia is expected to be a very useful treatment of cancer with minimal side effects [1, 2]. Various techniques for heat delivery, such as treatments with hot water, infrared rays, ultrasound and microwaves have been attempted. However, deep-seated tumors cannot be heated effectively and locally using these techniques. Ferrimagnetic microspheres 20–30 μm in diameter are useful as thermoseeds for inducing hyperthermia in cancers, especially in tumors located deep inside the body. These spheres are entrapped in the capillary bed of the tumors when they are implanted though blood vessels, and can heat cancers locally by their hysteretic loss when placed in an alternating magnetic field.

So far, glass-ceramics containing lithium ferrite ($LiFe_5O_8$) in a biocompatible matrix of hematite (α-Fe_2O_3) and a SiO_2–P_2O_5 glassy phase [6–8]; magnetite (Fe_3O_4) in a matrix of β-wollastonite (β-$CaSiO_3$) and a CaO–SiO_2–B_2O_3–P_2O_5 glassy phase [9–16]; α-Fe [17]; Fe_3O_4 in a B_2O_3-free CaO–SiO_2–P_2O_5 glassy phase [18]; or zinc-iron ferrite in a CaO–SiO_2 glassy phase [19] have been developed for this purpose. However, none has been produced in the form of microspheres 20–30 μm in diameter, or has shown a high heat generating ability.

Recently, we developed ferrimagnetic microspheres 20–30 μm in diameter, in which silica glass microspheres were coated with ferrimagnetic maghemite (γ-Fe_2O_3) [20]. Their saturated magnetization and coercive force were 53 emu·g^{-1} and 156 Oe, respectively. Their heat generation was estimated to be 41 W·g^{-1} under an alternating field of 300 Oe at 100 kHz. It is of concern, however, that these microspheres implanted into a tumor may accumulate in the dorsal blood vessels of the patients because of their high density.

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On the other hand, hollow hydroxyapatite particles have been developed using an enzymatic reaction [21]. These particles were prepared via enzymatic decomposition of urea within alginate gel template particles. The hydroxyapatite particles are about 1 mm in diameter and have a shell 150 μm thick. Porous alumina (Al_2O_3) particles can also be obtained by a similar technique [22]. The Al_2O_3 particles are about 1–2 mm in diameter and have sharp pore size distribution profiles around 0.1–0.01 μm. The Al_2O_3 particles can be made either hollow or filled, depending on the aging time after precipitation of the precursor.

In this study, we prepared hollow particles containing ferrimagnetic magnetite (Fe_3O_4) by using an enzymatic reaction of urease, and investigated the structure and the magnetic properties of the particles obtained.

2. Experimental

2.1. Preparation of particles

Ammonium alginate sol solution and iron (III) nitrate solution were prepared as follows. Ammonium alginate (309.3 mg; Wako Pure Chemical Industries, Osaka, Japan), urease (1 mg, made from jack bean; Wako Pure Chemical Industries) and ultra-pure water (10 ml) were mixed in a glass beaker for 1 h at room temperature using a magnetic stirrer (solution A). Iron (III) nitrate enneahydrate (4.04 g; Nacalai Tesque, Kyoto, Japan), urea (246.3 mg; Nacalai Tesque) and ultra-pure water (100 ml) were mixed in a glass beaker for 10 min at room temperature using a magnetic stirrer (solution B).

Solution A was added dropwise into solution B by using a Pasteur pipette or a spray gun (nozzle caliber: 1 mm; pressure: 1 kg·cm^{-2}; Kyoto Compressor, Kyoto, Japan). The solution obtained was then kept standing for 3 days at 36.5°C. Resulting particles were washed several times with ultra-pure water and ethanol, and freeze-dried (FD-1000, Tokyo Rikakiki Co., Tokyo, Japan).

2.2. Heat treatment of particles

The particles obtained by using a Pasteur pipette were placed in an alumina boat, heated to various temperatures (600–900°C) at a rate of 5°C·min^{-1} in an SiC electric furnace, and kept at the given temperature for 1 h in reductive atmospheres of mixtures of CO_2 and H_2 gases in various ratios (70%CO_2 + 30%H_2 to 40%CO_2 + 60%H_2). The particles were allowed to cool to room temperature in the furnace under the given atmosphere.

Alternatively, particles obtained by using the spray gun were placed in an alumina boat, heated up to 800°C at a rate of 5°C·min^{-1} in an SiC electric furnace, and kept at 800°C for 1 h in an atmosphere of 50%CO_2 + 50%H_2. The particles were allowed to cool to room temperature in the furnace under the same atmosphere.

2.3. SEM observation and powder X-ray diffraction of particles

The structures of particles obtained by using a Pasteur pipette and the heat-treated particles were observed using a field-emission scanning electron microscope (FE-SEM; S-4700, Hitachi, Tokyo, Japan).

The crystalline phases precipitated in the heat-treated particles were characterized by powder X-ray diffraction (RINT-1400, Rigaku Co, Tokyo, Japan), using the following settings: X-ray source, Ni-filtered CuKα radiation; X-ray power, 40 kV, 200 mA; scanning rate, $2\theta = 2^\circ\cdot\text{min}^{-1}$; and sampling angle, 0.02°. The crystalline phases precipitated in the specimens were identified by referring to data from the Joint Committee on Powder Diffraction Standards.

2.4. Magnetic properties of particles

The saturation magnetization and coercive force of the heat-treated particles were measured using a magnetic property measurement system (MPMS-XL, Quantum Design, Tokyo, Japan) under magnetic fields up to 10 kOe at room temperature. The heat generation (P) of the products was calculated from the area of the hysteresis loop under a magnetic field of up to 300 Oe, which is a practically available alternating magnetic field, using the following equation [23]:

$$P = f \bullet \oint H \mathrm{d}B \times 10^{-7} \tag{3}$$

where f, H and B are the frequency (hertz), the magnetic field strength (oersted) and magnetization (electromagnetic units per gram), respectively.

2.5. Transmission electron microscope (TEM) observation of particles

The particles heated to 800°C in a 50%CO_2 + 50%H_2 atmosphere were pulverized into fine particles using an alumina pestle and mortar, and suspended in 5 ml of acetone. A few drops of the suspension were placed on a collodion film supported on a copper microgrid mesh, and then observed using a transmission electron microscope (TEM: JEM-2000 FX-III, JEOL, Tokyo, Japan) at 200 kV equipped with energy dispersive X-ray analyzer (EDX: Voyager III, NORAN Instruments, Middleton, WI).

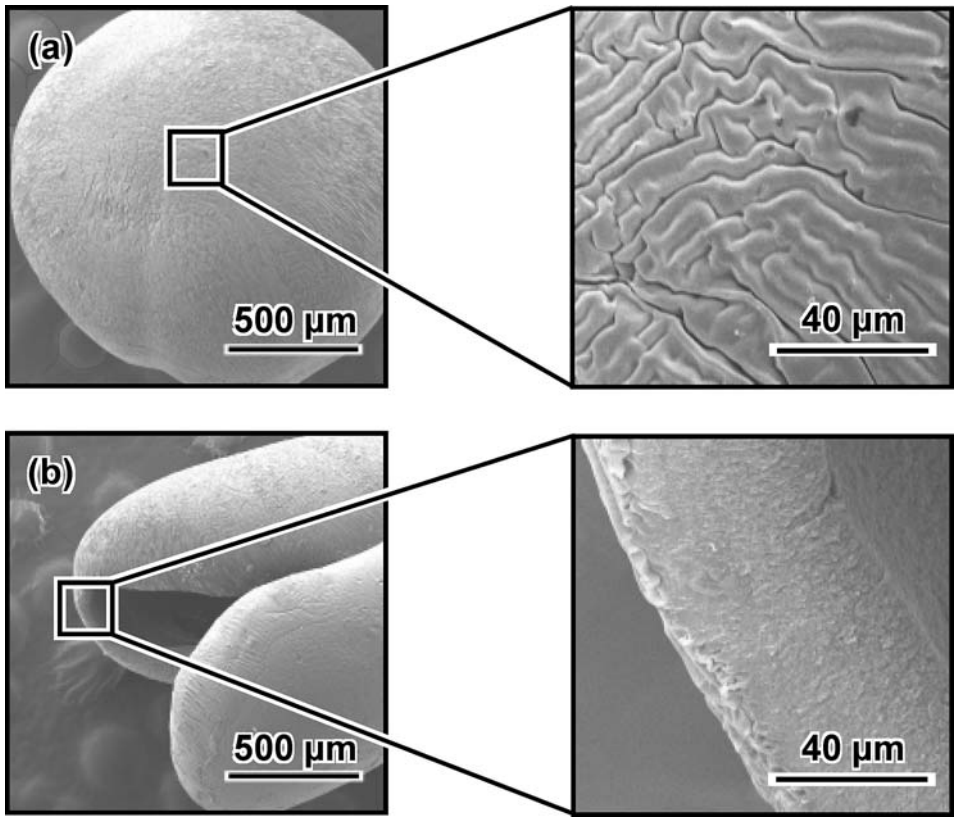

Fig. 1 FE-SEM photographs of hollow particles prepared using a Pasteur pipette.

3. Results and discussion

3.1. Structure of particles prepared by using a pasteur pipette

Bright yellow gels 2 mm in size were obtained using a Pasteur pipette. Fig. 1 shows FE-SEM photographs of the particles obtained using a Pasteur pipette. The particles obtained had wrinkles on their surface as shown Fig. 1 (a), and had a hollow structure as shown in Fig. 1 (b). It is considered that the iron precursor is formed by hydrolysis of iron nitrate by enzymatic decomposition of urea, as shown in reactions (1) and (2).

$$NH_2CONH_2 + 3H_2O \rightarrow 2NH_4^+ + 2OH^- + CO_2 \quad (1)$$

$$Fe^{3+} + xOH^- + nH_2O \rightarrow FeO_{y/2}(OH)_{3-y} \cdot mH_2O \quad (2)$$

Hydrolysis of urea by urease causes an increase in the pH of the solution on the surface of ammonium alginate gel particles (reaction (1)). As a result, iron hydroxide ($FeO_{y/2}(OH)_{3-y}\cdot mH_2O$) with low solubility is formed by hydrolysis of Fe^{3+} (reaction (2)).

Fig. 2 shows FE-SEM photographs of the particles after heat treatment at 800°C in an atmosphere of 50%CO_2 + 50%H_2. The particle size decreased from 2 mm to 0.5 mm, and a large number of small cracks were formed by the heat treatment. This is attributed to the dehydration and shrinkage of the hollow particles by the heat treatment. The formation of the cracks should be suppressed, because the cracks cause the decrease in the mechanical properties of the particles, and hence increase the risk of the fracture of the particle during the treatment. If the particles are fractured at the interior of the patients' bodies, the resultant fragments of the particle might give severe damages to the blood vessels of the patients.

Fig. 3 shows the powder X-ray diffraction patterns of hollow particles prepared using a Pasteur pipette and then heat-treated at 800°C for 1 h in various atmospheres. The particles prepared in this manner gave a halo at about 23° at 2θ. This indicates that these particles took on an amorphous structure. The particles heat-treated in an atmosphere of 40%CO_2 + 60%H_2 gave peaks assigned to ferrimagnetic magnetite (Fe_3O_4) and ferromagnetic α-Fe. The particles heat-treated in an atmosphere of 50%CO_2 + 50%H_2 yielded peaks assigned to Fe_3O_4 only. The particles heat-treated in 60%CO_2 + 40%H_2 and 70%CO_2 + 30%H_2 atmospheres gave peaks assigned to Fe_3O_4 and small peaks assigned to weak ferromagnetic hematite (α-Fe_2O_3). It is expected from these results that particles fully composed of Fe_3O_4 would be obtained by heat treatment in an atmosphere of 50%CO_2 + 50%H_2.

Fig. 4 shows the powder X-ray diffraction patterns of hollow particles heat-treated at various temperatures in an atmosphere 50%CO_2 + 50%H_2. The particles heat-treated

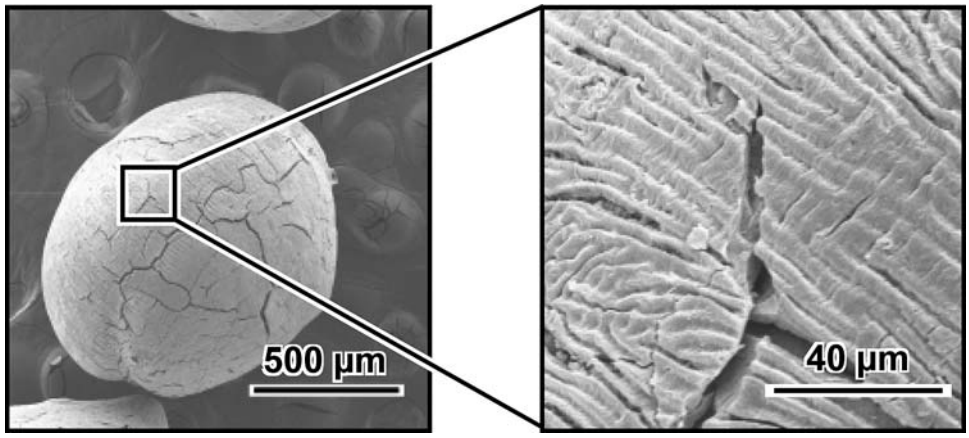

Fig. 2 FE-SEM photographs of hollow particles prepared using a Pasteur pipette and then heat-treated at 800°C for 1 h in an atmosphere of 50%CO_2 + 50%H_2.

at 600°C and 700°C gave broad peaks assigned to Fe_3O_4, whereas those heat-treated at 800°C gave the sharp peaks of Fe_3O_4. This indicates that crystallization of Fe_3O_4 is fully achieved by the heat treatment at 800°C. The particles heat-treated at 900°C gave peaks assigned to Fe_3O_4, α-Fe_2O_3 and antiferromagnetic wustite (FeO). However, according to the phase diagram for Fe-O binary system [24], the coexistent of these three phases can not be achieved in an equilibrium state. Therefore, it is speculated that the particles were not in equilibrium state under the present heat treatment condition.

3.2. Magnetic properties of particles prepared by using a pasteur pipette

Fig. 5 shows magnetization curves in fields of 10 kOe and 300 Oe for particles heat-treated at various temperatures for 1 h in an atmosphere of 50%CO_2 + 50%H_2. Table I summarizes the magnetic properties of these particles. The heat generation was calculated from the area of the magnetization curves under a magnetic field of 300 Oe as shown in Fig. 4, using equation (3), where the frequency (f) was set at 100 kHz. The saturation magnetization of the products increased from 15 to 57 emu·g^{-1} when the heat treatment temperature increased from 600 to 800°C. The increase in the saturation magnetization is attributed to the increase in the amount of ferrimagnetic Fe_3O_4 crystals above 10 nm in size constituting the heat-treated particles. Magnetite shows superparamagnetism when its crystallite size is below 10 nm [25]. The saturation magnetization of the products decreased from 57 to 28 emu·g^{-1} when the heat treatment temperature increased from 800 to 900°C. This is attributed to the precipitation of α-Fe_2O_3 and FeO instead of Fe_3O_4 by heat treatment at the higher temperature.

The coercive force of the products increased from 56 to 203 Oe with increasing heat treatment temperature up to 900°C. This might be attributed to an increase in the number of Fe_3O_4 crystals of approximately 40 nm in size. Generally, a Fe_3O_4 particle has a single domain and shows a maximum coercive force when its crystallite size is 10–40 nm [26].

For the present samples, particles heat-treated at 800°C in an atmosphere of 50%CO_2 + 50%H_2 with a saturation magnetization of 57 emu·g^{-1} and a coercive force of 183 Oe were estimated to show the maximum heat generation, 45 W·g^{-1} in 300 Oe at 100 kHz.

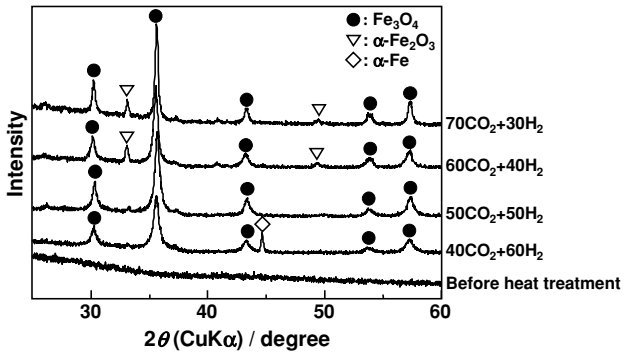

Fig. 3 Powder X-ray diffraction patterns of hollow particles prepared using a Pasteur pipette and then heat-treated at 800°C for 1 h in various atmospheres.

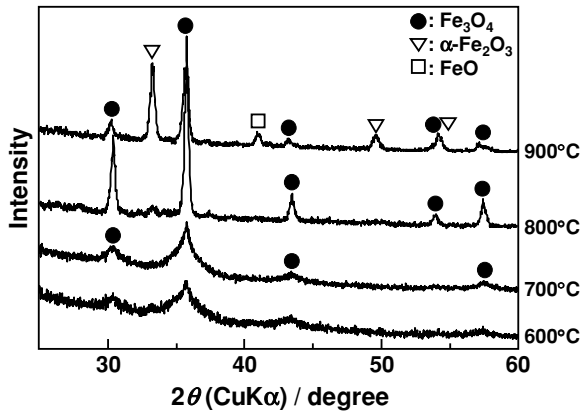

Fig. 4 Powder X-ray diffraction patterns of hollow particles prepared using a Pasteur pipette and then heat-treated at various temperatures for 1 h in an atmosphere of 50%CO_2 + 50%H_2.

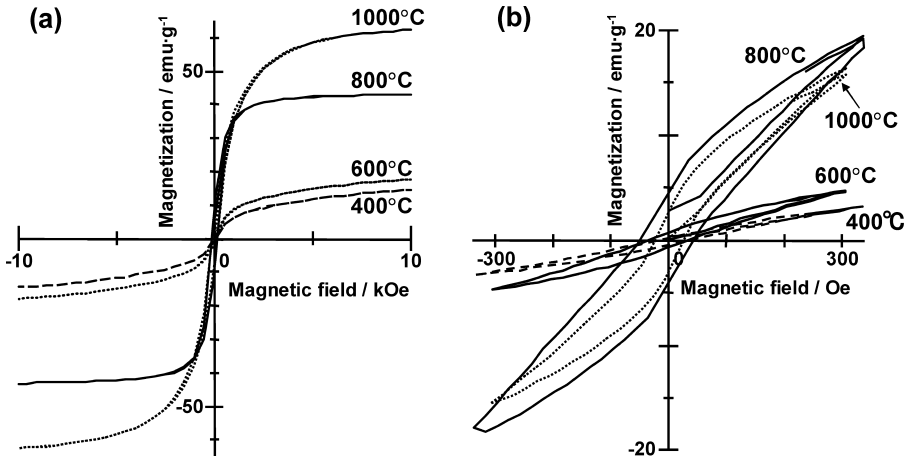

Fig. 5 Magnetization curves of hollow particles, heat-treated at various temperatures for 1 h in an atmosphere of 50%CO_2 + 50%H_2, in magnetic fields of (a) up to 10 kOe and (b) up to 300 Oe.

Table 1 Magnetic properties of hollow particles heat-treated at various temperatures for 1 h in 50CO_2 + 50H_2 atmosphere

Heat treatment temperature/°C	Saturation magnetization (*Ms*)/emu·g^{-1}	Coercive force (*Hc*)/Oe	Heat generation under 300 Oe, 100 kHz (*P*)/W·g^{-1}
600	15	56	1.4
700	22	62	2.8
800	57	183	45
900	28	203	7

3.3. TEM observation of particles prepared by using a pasteur pipette

Fig. 6 shows TEM photographs of Fe_3O_4 precipitated in the products heat-treated at 800°C in an atmosphere of 50%CO_2 + 50%H_2. The magnetite had a wide distribution in its crystallite size, ranging from 5 nm (Fig. 6 (a)) to 500 nm (Fig. 6 (b)).

As mentioned in the previous section, Fe_3O_4 shows maximum coercive force when its crystallite size is about 40 nm. If the crystallite size of magnetite could be precisely controlled at about 40 nm, the products would show higher coercive force and therefore higher heat generating ability.

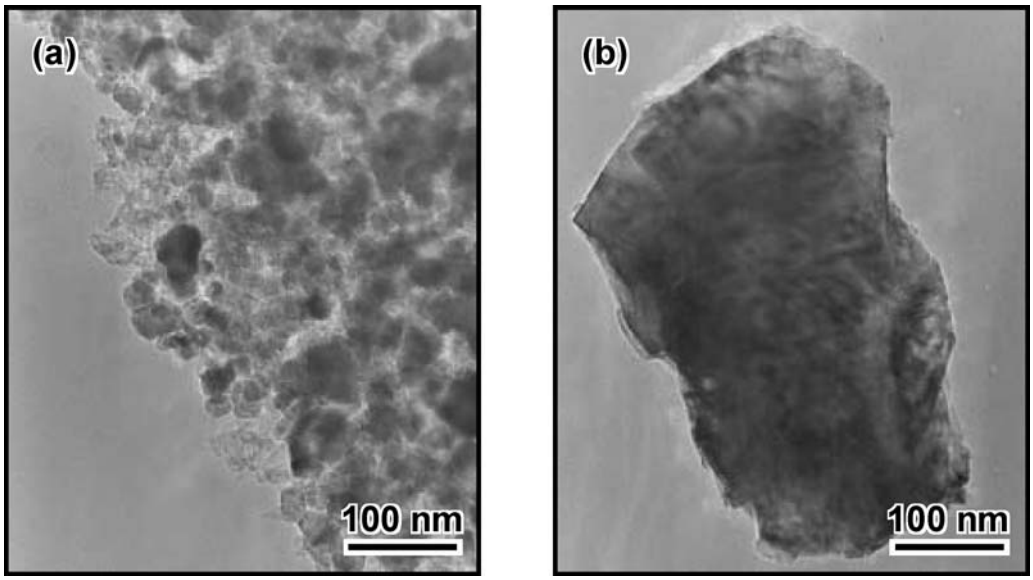

Fig. 6 TEM photographs of magnetite precipitated in the hollow particles heat-treated at 800°C for 1 h in an atmosphere of 50%CO_2 + 50%H_2.

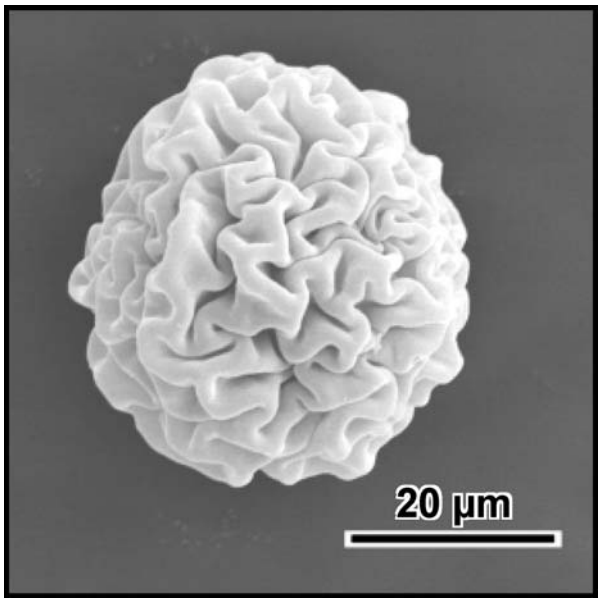

Fig. 7 FE-SEM photograph of a hollow particle prepared using a spray gun.

3.4. Structure of particles prepared by using a spray gun

Fig. 7 shows FE-SEM photographs of particles obtained using the spray gun method. Microspheres 30 μm in diameter were observed. This indicates that microspheres 20–30 μm in diameter can be obtained when a spray gun is used instead of a Pasteur pipette. The optimum conditions for obtaining the microspheres 20–30 μm in diameter, and the structure and magnetic properties of the resultant microspheres should be determined in a future study.

4. Summary

Bright yellow gels 2 mm in size were obtained when ammonium alginate sol solution with urease was dropped into iron (III) nitrate solution with urea using a Pasteur pipette. The particles heat-treated at 800°C in an atmosphere of 50%CO_2 + 50%H_2 were composed of ferrimagnetic magnetite, and took on a hollow structure; their saturated magnetization and coercive force were 57 emu·g^{-1} and 183 Oe, respectively. Their heat generating ability in an alternating magnetic field of 300 Oe at 100 kHz was estimated to be 45 W·g^{-1}. Microspheres 30 μm in size were obtained when ammonium alginate sol solution with urease was added into iron (III) nitrate solution with urea using a spray gun instead of a Pasteur pipette.

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