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Fe Element Analysis in the Spleen Tissue by Using EDS and ICP-MS after Aminated Silica Shelled Magnetite (Fe_3O_4) Administered to the Tail Vein of Mice

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The suppression of cytokine storm in severe coronavirus disease 2019 (COVID-19) patients can be treated with monoclonal antibody therapy against CD3 for T cell receptor inhibition. An optimized liquid phase as a CD3 antibody-magnetic nanoparticle (Ab-MNP) conjugate can inhibit the overactivation of T cells. We aim to analyze the distribution of Fe in the spleen after acute administration of silica-conjugated amine magnetite (Fe_3O_4) nanoparticles (35 nm) delivered by intravenous injection. The Fe element distribution and concentration levels in spleen tissue were analyzed using energy dispersive spectroscopy (EDS) and inductively coupled plasma-mass spectrometry (ICP-MS). The experimental result is a difference in the concentration of Fe elements, which was 1.89×10^3 mg/kg in the spleen of a control mouse not administered with MNPs, whereas increases significantly to 1.93×10^3 mg/kg in that of a mouse administered with MNPs. Further, time kinetic analysis of biochemical and immunological parameters is required to confirm its suitability in bio-administration.

Keywords : magnetic nanoparticle (MNP), Fe element, spleen, inductively coupled plasma-mass spectrometry (ICP-MS), energy dispersive spectroscopy (EDS)

1. Introduction

Magnetite nanoparticles (MNPs) have gained interest in imaging, drug delivery, and therapeutics. Superparamagnetic nanoparticles avoid their self-agglomeration and only show magnetic properties in the presence of an external magnetic field. The hyperthermic properties of MNPs in the presence of a pulse magnetic field are utilized to destroy tumor cells [1]. Besides, the outer surfaces of MNPs are functionalized by different molecules, including inorganic molecules, small organic molecules, and polymeric materials [2]. Silica-coated amine MNPs are specially prepared to conjugate antibodies, enzymes, and other protein molecules by covalent binding with the amine functional group.

The pandemic coronavirus SARS-CoV-2 infected more than 500 million and caused around 7 million death worldwide [3]. Cytokine storm (CS) results in hyper-inflammatory features, including acute respiratory distress syndrome regarded as one of the leading causes of death [4]. Targeting T cells via binding the cluster of differentiation 3 (CD3) to prevent allograft rejection in heart, kidney, and liver transplantation brought the novel prize in 1979. Moreover, the excessive side effects of anti-CD3 were overcome by introducing humanized anti-CD3. Furthermore, it was confirmed that the binding of anti-CD3 monoclonal antibodies on the Fc receptors of antigen-presenting cells and T cells activates T cells and induces inflammation [5]. Conjugation of anti-CD3 with MNPs (Ab-MNP) substantially blocks the Fc region in binding with T cells and subsequent activation of inflammation [6, 7].

The distribution half-life of MNPs largely depends on their size. The particles smaller than 5 nm are excreted through the kidney, whereas particles larger than 20 nm and smaller than 100 nm are drained to the reticuloendothelial system (e.g., spleen and lymph nodes). Therefore, the therapeutic administration of MNPs primarily relies on disposition and biocompatibility in the living system. In the current study, the amine-functionalized silica-coated MNPs (NH_2-SiO_2-Fe_3O_4) were investigated for their biodistribution in the spleen by measuring the

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concentration of Fe element in spleen tissue.

2. Materials and Methods

2.1. Animal experiments

Mouse dissection experiments follow the Animal Experiment Regulations (No. 2021-02) approved by the Animal Ethics Committee (IACUC) of Sangji University. Female BALB/c mice 6-8 weeks old and weighing about 20 g were used for in vivo experiments. Animals were housed at a controlled temperature (25 ± 2 °C). The mice were given a routine rodent diet from DBL, Chungbuk, Korea, and water ad libitum. The mice were acclimatized in the laboratory for one week before starting the experiments. For anesthesia, zoletil-rompun (25 μL) was injected into the intraperitoneal cavity of mice. Then, mice were anesthetized, and a single dose of MNPs (15 mg/kg) in 100 μL phosphate buffer saline (PBS), pH 7.4, was injected through the tail vein. Control mice received only PBS. Mice were sacrificed on day 10 post-MNPs injection.

2.2. Magnetic nanoparticles (MNP)

The 35 nm MNPs are silica-coated amine magnetite (III, Fe_3O_4) nanoparticles purchased from nanoCmposix, San Diego, CA, USA [6, 7].

2.3. TEM and EDS analysis of MNPs and Fe element

The amine-functionalized Si-MNPs after in-vivo administration were visualized by TEM (JEM-F200, JEOL Ltd, Japan) [6]. Further, energy dispersive X-ray spectroscopy (EDS) was performed for elemental analysis. Briefly, after sacrifice, spleen tissue was separated and immediately dissociated into single cell suspension. The suspension cells were then taken into pure carbon film with a 400 mesh Cu TEM grid (Ted Pella, Inc., Redding, CA, USA).

2.4. Analysis of Fe elements by ICP/MS

Samples were prepared based on acid digestion methods described elsewhere [8]. Briefly, the frozen spleen tissues were taken in 2 ml plastic tubes, and 1 mL HPLC-grade distilled water (dH_2O) was added. The spleen tissue was then homogenized using a tissue homogenizer. Five hundred μL homogenates taken in a separate tube and kept at -80 °C for one day. Finally, the samples were freeze-dried at -40 °C and vacuum gauge 20 Pa for 24 hours. The dry weight of the lyophilized samples was recorded. The acid digestion step was performed under a chemical fume hood. Briefly, the freeze-dried tissue samples were taken in a beaker, and 5 ml of 60 % HNO_3 (metal grade) (Merck KGaA, Darmstadt, Germany) was added. The samples were then heated at 160 °C for 3 hours by putting a watch glass on top of the beaker. Samples were then cooled at room temperature, and 15 ml dH_2O was added. Blank samples were prepared with the same process. The instrumental condition of ICP/MS includes: Model: NexION 350D (PerkinElmer Inc., MA, USA); source: argon plasma (6000 K); mass resolution: 0.3-3.0 amu; detection limit: 9Be <15 ppt, 59Co < 2 ppt, 115In < 0.5 ppt; RF Power: 1100 W. The flow rate was maintained at 1.00 ml/min.

3. Results and Discussion

3.1. Characterization of amine-functionalized Si-MNPs

The surface image structure of MNPs with pure amines is analyzed from the TEM image. Figure 1(a) shows that several amine-MNPs observed by the TEM images are clustered together in a round shape within 35 nm. The TEM mapping image for the atomic strength of SEI (solid electrolyte interface) is shown using EDS analysis, an energy dispersive spectroscopy method, as shown in Fig. 1(b). The mapped image of Fe, the main element of MNPs, is shown in Fig. 1(c). As shown in Fig. 1(c), the superparamagnetic properties and physically and chemically stable magnetic nanoparticles used in this study were coated with silica (SiO_2) on the core magnetite (Fe_3O_4). The particles with a diameter of less than 35 nm with an amine-group (NH_2) attached to the surface [9, 10]. Amine-functionalized silica coated on the Fe_3O_4 core is helpful for binding studies, conjugation with carboxyl-containing molecules through EDC/NHS coupling, or binding to dyes and molecules with isothiocyanate (ITC) or amine-reactive esters. In addition, the amines at the colloid surface can be protonated at acidic pH to yield particles with a significant positive zeta potential [11].

The MNPs solution contained in distilled water (ddH_2O) is changed to PBS×1 (phosphate buffered saline, pH 7.4, Introgen, USA) buffer so that the concentration is 1 to 2 mg/mL. The diameter size of the silica shell MNPs of amination of iron oxide (Fe_3O_4) used in this study is 35 nm, as shown in Fig. 1(d). The model (#SCM0067) manufactured by the nanoComposix (San Diego, CA 92111, USA) is a sphere-type shape [11]. MNPs (15 mg/kg) in 100 μL PBS are injected along the tail vein of the anesthetized mouse, and 300 μg, the total amount of MNPs, is slowly and carefully injected as shown in Fig. 1(e). The MNPs are injected into the tail vein of the mouse and go through the corresponding route to the spleen, which produces T cells and red blood cells. On the first day of the dissection experiment, PBS and MNPs

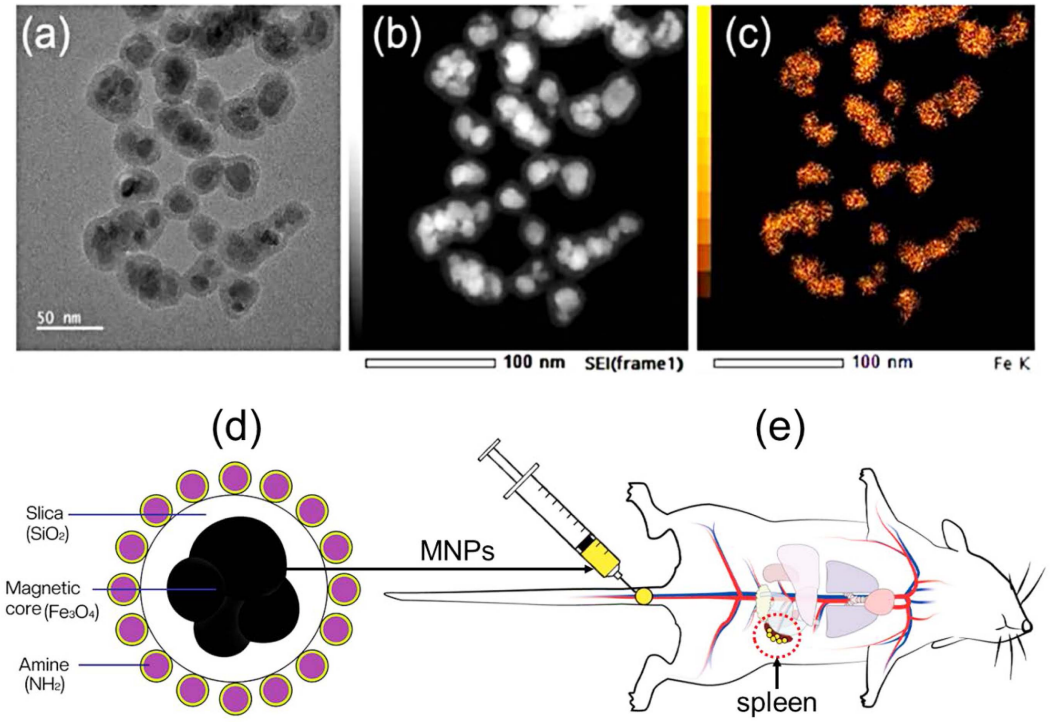

Fig. 1. (Color online) (a) TEM image of amine-SiO_2-Fe_3O_4 nanoparticles (nanoComposix) with the surface area of diameter ~ 33 ± 5 nm and aminated particle surface [10, 11]. Mapping TEM images for atomic intensities of (b) SEI (solid electrolyte interphase), (c) Fe analyzed by EDS, Scale bars: (a) 50 nm, (b) and (c) 50 nm. (d) Schematic structure of one aminated silica-shelled magnetite (Fe_3O_4). (e) Feature for injecting MNPs into the tail veins of the mouse and a schematic diagram showing particles accumulating in the spleen through the blood.

are injected into the tail vein of 2 mice. The control mice were sacrificed on day 1 after injection of saline (PBS), and the MNPs injected mice were sacrificed on day 10.

3.2. TEM/EDS analysis of MNPs in the spleen

Immediately after analyzing the TEM images, the bonding elements in the spleen tissue are investigated using EDS mapping. The main constituent elements of aminated silica shelled MNPs are N, O, Si, and Fe. Figure 2(a) is a TEM image of MNPs on a grid after removing liquid from a spleen tissue in PBS. The diameter distribution of MNPs is within the range of 25-35 nm. When an electron beam is directed at the amine group on the surface of the MNPs, characteristic x-rays are emitted as the outer electrons of the atom transition to the positions of the vacant central orbital electrons. The observed image in Fig. 2(b), is a TEM image of the thin SEI layer caused by the electrode-electrolyte interface reaction [12]. Figures 2(c), 2(d), 2(e), and 2(f) show the characteristic x-rays emitted from nitrogen (N), oxygen (O), Si, and Fe. We assume that the N, O, Si, and Fe are from amine receptors of MNPs, silica shells, and magnetites. It can be seen that the elemental mapping of O in Fig. 2(d) and Fe in Fig. 2(f) appear as it is, depending on the shape of the MNPs forming clusters noticed by one dotted circle in Figs. 2(a) and 2(b) in the spleen tissue.

The EDS spectrum obtained from the TEM image intensity analysis of tissues in the spleen on the 10th day after the injection of MNPs is shown in Fig. 3. The green peaks of the spectrum show the Ka peaks for N, O, Si, and Fe, which are the main elements constituting the aminated silica-shelled magnetite (NH_2-SiO_2-Fe_3O_4). Here, N, O, Si, and Fe are the main elements constituting the framework of the MNPs. The characteristic energies of N, O, Si, and Fe are 0.392 keV, 0.525 keV, 1.739 keV and 6.498 keV, respectively [13]. Since element O and element N exist in the background, the counter value of the main peak is very high, over 14,000. However, the larger Fe peak than the Si peak indicates that the distribution of MNPs in the spleen exists as a value with a significant size. Therefore, according to the results of the EDS analysis, it can be seen that the MNPs injected into the living body move along the vascular system circulation path and accumulate in the spleen.

Table 1 shows the analysis of the results based on the TEM image in Fig. 2 and the EDS spectrum in Fig. 3 for

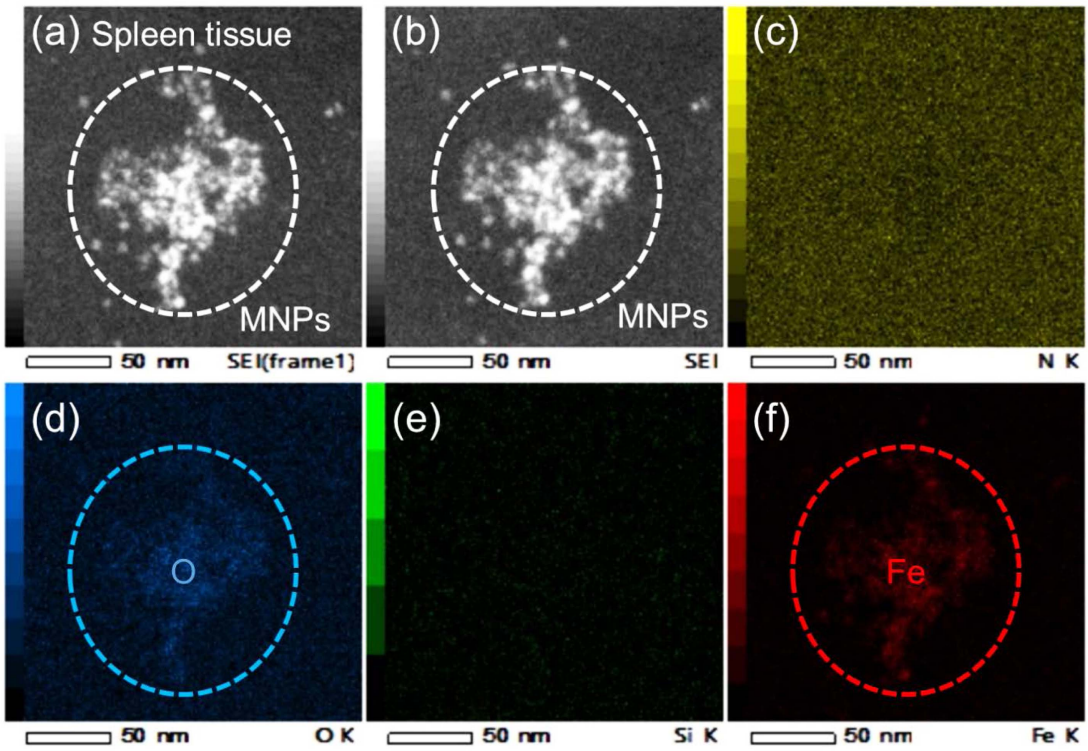

Fig. 2. (Color online) Images of the transmission electron microscope (a) and atomic intensities of (b) SEI, (c) N, (d) O, (e) Si, and (f) Fe for elements of aminated silica-shelled magnetite (NH_2-SiO_2-Fe_3O_4) MNPs with a diameter of 25-35 nm. Depending on the shape of the MNPs forming clusters noticed by one dotted circle (a) and (b) in the spleen tissue, it can be seen that the elemental mapping of (d) O and (f) Fe appears as it is.

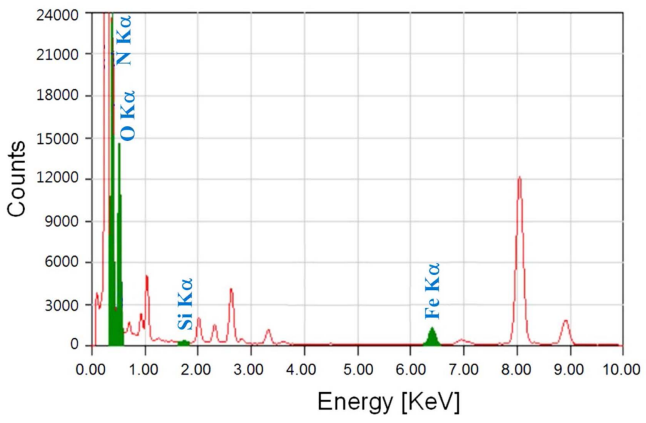

Fig. 3. (Color online) The EDS spectrum of aminated silica shelled magnetite (NH_2-SiO_2-Fe_3O_4) in the spleen tissue after being administered to the tail vein of mice to analyze their atomic stoichiometric characteristics. The characteristic energies for the N, O, Si, and Fe elements constituting the framework of the MNPs are 0.392 keV, 0.525 keV, 1.739 keV, and 6.498 keV, respectively.

the spleen tissue samples, including N, O, Si, and Fe, which are four major elements constituting MNPs, 10 days after MNPs are injected into the tail vein. The EDS spectrum analysis, an acceleration voltage, and a probe current of the beam scanned by the TEM (JEM-F200) device were 200.0 kV and 7.475 nA, respectively. Also, real-time and live times are 98.19 s and 70.82 s, respectively, and dead time is 27 %. The counting rate is 21813 cps, and the fitting coefficient is 0.8242 by standardless quantitative analysis. The mass ratio and atomic ratio of element O/Fe element accumulated in the spleen for 10 days are 0.40 %/5.55 % and 0.22 %/1.52 %, respectively.

Table 1. The standardless quantitative analysis of the EDS spectrum with the acquisition parameters: an acceleration voltage of 200.0 kV, a probe current of 7.475 nA, a real-time of 98.19 s, a live time of 70.82 s, a dead time of 27 %, a counting rate of 21813 cps, and a fitting coefficient of 0.8242. The mass ratio(%) for four elements (N, O, Si and Fe) is proportional to the counts.

Element	(KeV)	Mass%	Counts	Atom%
N Kα	0.392	66.52	135169.53	72.15
O Kα	0.525	27.52	75533.96	26.12
Si Kα	1.739	0.40	1159.12	0.22
Fe Kα	6.398	5.57	15567.08	1.52
Total		100.00		100.00

These results analyze the quantitative Fe element in the spleen of the control mice not injected with MNPs and mice 15 days after injection of MNPs using the ICP-MS device.

3.3. Biodistribution of MNPs

For the analysis of Fe in the spleen, only the spleen collected through the previous dissection experiment is separated and pre-treated using an ICP-MS device. This mass spectrometer type separates ions from a sample solution contained in distilled water to analyze elemental components and quantity in the sample [8]. The operation principle of such ICP-MS is as follows. First, no change occurs when a sample in a spray state, that is, argon (Ar) in a gaseous state, is injected into the sample introduction part. However, when a high frequency is continuously applied to the induction coil (RF), collisions between charged particles occur, and a magnetic field is induced. As a result, argon (Ar) in the gaseous state is ionized, and plasma, a flame, is generated. After confirming the plasma, if the previously prepared pre-treated sample is introduced in an aerosol form, the sample becomes a cation and an ion in an electronic state. The mass-to-charge ratio is calculated according to the strength of the electric field through the mass spectrometer. Since ions have a positive charge, repulsive force is applied at the positive (+) pole, and an attractive force acts at the negative (−) pole to pass through. The heavier particles arrive at the ion detector in the order of detection. It goes through a series of processes analyzing the number of seconds detected through the system.

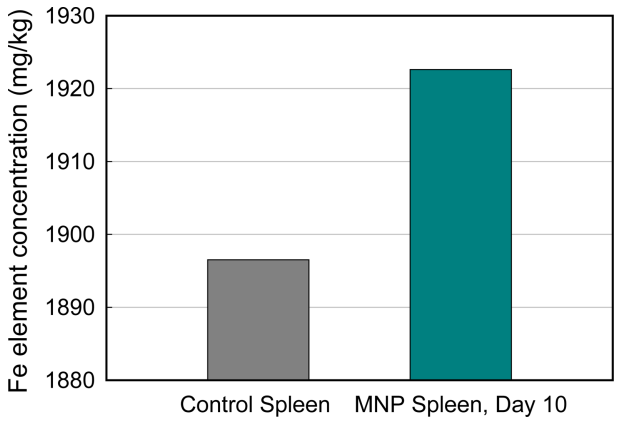

Fig. 4. (Color online) Fe concentration by drainage of MNPs in spleen tissues after a mouse administered with MNPs. The reason for choosing the spleen from the mouse organ is that it stores MNPs with a particle size of 35 nm administered in vivo. Therefore, the result is a difference in the concentration of Fe elements, which was 1.89×10^3 mg/kg (=1.89×10^6 ppb) in the spleen of a control mouse not administered with MNPs, whereas increases significantly to 1.93×10^3 mg/kg (=1.93×10^6 ppb) in that of a mouse administered with MNPs.

Figure 4 shows the ICP-MS investigation of Fe levels in the spleen of control mice and mice on the 10th day to obtain quantitative information on Fe inorganic elements among the sample components. The bar graphs show that the control concentration level significantly increased the Fe level on the 10th day of the MNPs injection [14]. The spleen is a significant organ for cellular immunity expression. T cells are distributed in the lymphocytes surrounding the central artery of the spleen, and B cells are distributed in the lymph nodes located in the periphery of the spleen [15, 16]. The blood opens through the arterioles and finally enters the sinuses of the red nasal sinus, the arterial endothelium (arterial endothelial) macrophages clean old cells and foreign particles. Then, it enters the red aqueous vein and the newly-born blood cells as the nasal gland (red axon) that fills the gap in the sinus, collects in the nasal vein and returns to the circulation system. Therefore, the result that can be inferred based on the above is that the injected MNPs migrate through veins and accumulate in the spleen by the 10th day. After that, it can be seen that the level decreases as it exits the spleen through the circulatory system and circulates through the body [17].

4. Conclusion

The treatment of suppression of cytokine storm in severe coronavirus disease 2019 (COVID-19) patients includes cytokine receptor suppression and depletion of specific immune cells in addition to steroid treatment. It is a monoclonal antibody therapy against CD3, one of the critical signaling molecules for T cell receptor activation. MNPs used as biocompatible materials contain Fe, an essential trace element constituting living organisms, and are used in various bio-application fields such as drug delivery and thermotherapy. These MNPs are a group of substances expected to have high utility in biomedical aspects, and it is time to study the basic characteristics such as distribution in the body. In this study, it is possible to conjugate an antibody (targeting T cells) to MNPs coated with silicone, a biocompatible compound, to eliminate toxicity. After the magnetic nanoparticles were injected into the tail vein of the mouse, the Fe element concentration in the spleen was analyzed using ICP-MS. After 10 days, the period of maximum accumulation in the spleen by moving the veins through the vascular circulation, the magnetic nanoparticles increased by 4×10^3 mg/kg from 1.89×10^3 mg/kg to 1.93×10^3 mg/kg. Therefore, MNPs used for therapeutic purposes

can effectively suppress the overactivation of T cells generated in the spleen and improve treatment and diagnostic efficiency by delivering drugs to a target site.

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