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Preparation of Amino Acid Conjugated Nano-Magnetic Particles for Delivery Systems*

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Nanotechnologies to allow the nondisruptive introduction of carriers in vivo have wide potential for therapeutic delivery system. Iron oxide (γ-Fe_2O_3) nano-magnetic particles (NMPs) were functionalized by silanization with (3-aminopropyl)triethoxysilane. For the purpose of functionalizing the surface of the particles with amino groups for subsequent cross-linking with pharmaceuticals and biomolecules. We have evaluated physical characterization of obtained NMPs by Fourier transform infrared spectroscopy, a superconducting quantum interference device, X-ray diffraction and transmission electron microscopy to confirm particle preparation. The particles were successfully introduced into living cells without any further modification to enhance endocytic internalization, such as the use of a cationic help. Furthermore, the functionalized NMPs were conjugated with an amino acid via a cross-linker, *N*-(ε-maleimidocaproyloxy) sulfosuccinimide ester, with the goal of selective uptake of NMPs by cells. To confirm the amino acid modification of the NMPs, the amino acid-conjugated NMPs were labeled with a fluorescent reagent (dansyl chloride), and the fluorescence was observed by confocal laser scanning microscopy. The amino acid-conjugated NMPs have great potential for use in cell-selective delivery systems involving amino acid transporter proteins. [DOI: 10.1380/ejssnt.2007.60]

Keywords: nanoparticles; iron oxide; magnetic properties; delivery system; amino acid

I. INTRODUCTION

Nanobioengineering [1–3] is one of the most interesting areas of study to reveal basic questions and realize current challenges in biology and medicine by devising new experimental approaches that are enabled by nanotechnological platform. Especially, the efficient introduction of target molecules into the cell using nanomaterials [4–7] is significant in medical field to realize the nondisruptive analysis and safety treatment for the disease [8].

Nanoscale magnetic particles [9, 10] have attracted much attention because they were able to be transported to and concentrated at targeted locations by means of an external magnetic field. Recent reports indicate that magnetic nanoparticles with diameters < 100 nm may be useful for medical applications, such as magnetic resonance imaging [11–13] and delivery systems [14–17]. Meanwhile, the existing particles are coated by synthetic polymer to stabilize particle shape and property [18]. But it induced an increase of particles diameter and difficulty of internalization of the particles. To enhance the uptake of the particles by the cells, the method of introduction of cationic residues to the particle which load target molecules is well used to facilitate endocytic introduction into living cells on most studies.

Previously, we obtained monodispersive nano-magnetic particles (NMPs) with unique magnetic properties in a single step by mixing aqueous solutions of $3d$ transition-metal chlorides ($MCl_2 \cdot nH_2O$) and a sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$) [19–24]. The particle size can be controlled by adjusting the annealing temperature and time. We have also reported that extremely small particles can readily contact the surfaces of cells without steric hindrance between plasma membrane and themselves; therefore, NMPs can be introduced into the cells without the necessity for a cationic coating to help non-specific endocytic internalization [25]. Here, we designed amino acid conjugated-NMPs to recognize specific cells and to be incorporated into the cells by the aid of amino acid transporter. We have already taken advantage of the recognition ability of an amino acid transporter on the surfaces of cell membranes for a cell-specific delivery system [26]. We first prepared the functionalized NMPs

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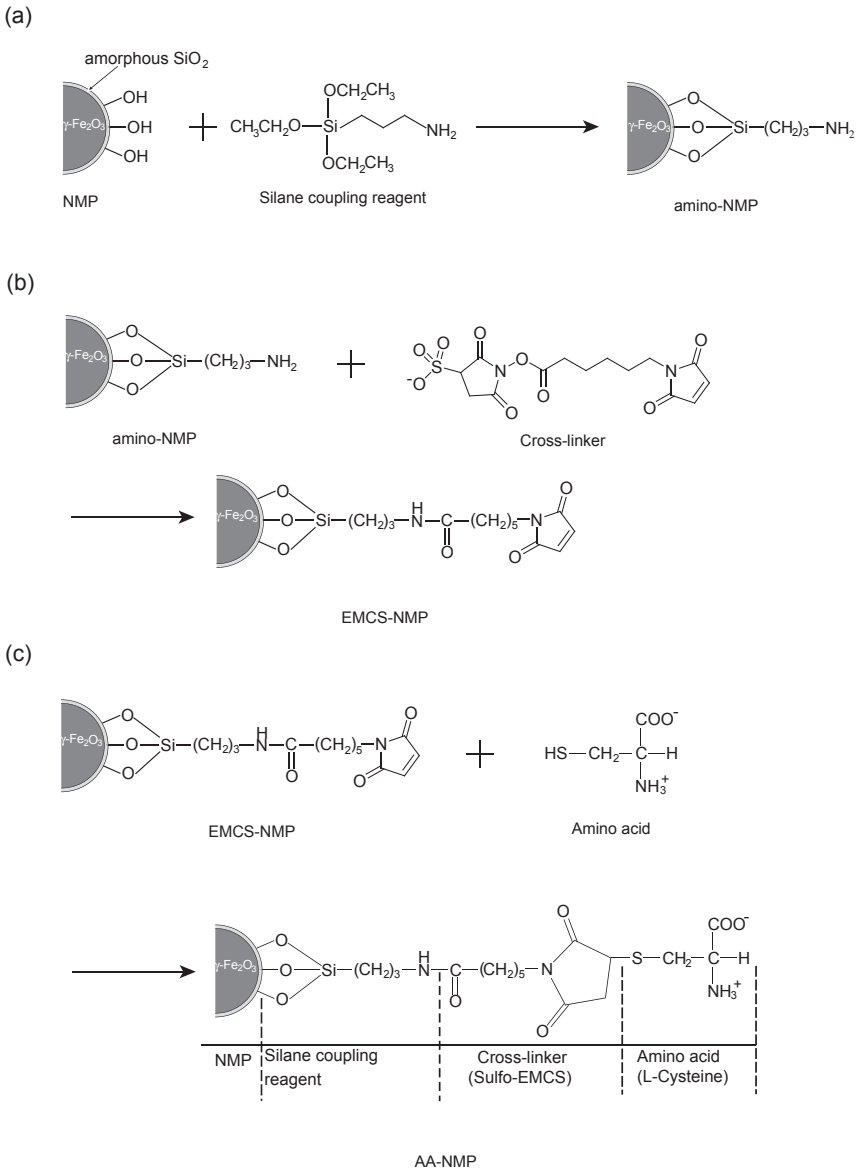

FIG. 1: Functionalization of NMPs (a), conjugation of cross-linker to the surface of the amino-NMPs (b), and modification of the NMPs surface with an amino acid (c).

that are added to functional group such as amino group on particle surface via a silanization procedure to covalently load targeted molecules and investigate the effect of single-digit nano-scale particles on their cellular uptake. Consequently, we succeeded in the preparation of amino acid (AA)-conjugated particle by the use of our functionalized NMPs.

II. EXPERIMENTAL

A. Preparation and functionalization of NMPs

γ-Fe_2O_3 NMPs surrounded by amorphous SiO_2 (a-SiO_2) were prepared from aqueous solutions of $FeCl_2 \cdot 4H_2O$ (Wako Pure Chemical Industries, Osaka, Japan) and $Na_2SiO_3 \cdot 9H_2O$ (Junsei Chemical Co., Tokyo, Japan) according to a previously described method [24].

For modification of the surface of the NMPs with amino groups (Fig. 1a), 500 μL of (3-aminopropyl)triethoxysilane (γ-APTES; Shin-Etsu Chemical Co., Tokyo, Japan) was added to the solid NMPs, and the reaction mixture was stirred at room temperature for 10 minutes and then heated to 403 K. After 20 hours at that temperature, the mixture was cooled and then washed three times with ultrapure water ($\geq$ 18.2 Ω, Milli-Q SP, Millipore Co., Massachusetts, USA) and ethanol. The CuK_α X-ray diffraction (XRD) patterns of the NMPs and the amino-NMPs were measured to confirm particle structure. The morphology of the NMPs and amino-NMP were investigated by transmission electron microscopy (TEM; JEM-1230, JEOL, Tokyo, Japan). Magnetization measurements were performed with a superconducting quantum interference device (SQUID) magnetometer (MPMS, Quantum Design, San Diego, California, USA) under an external field between

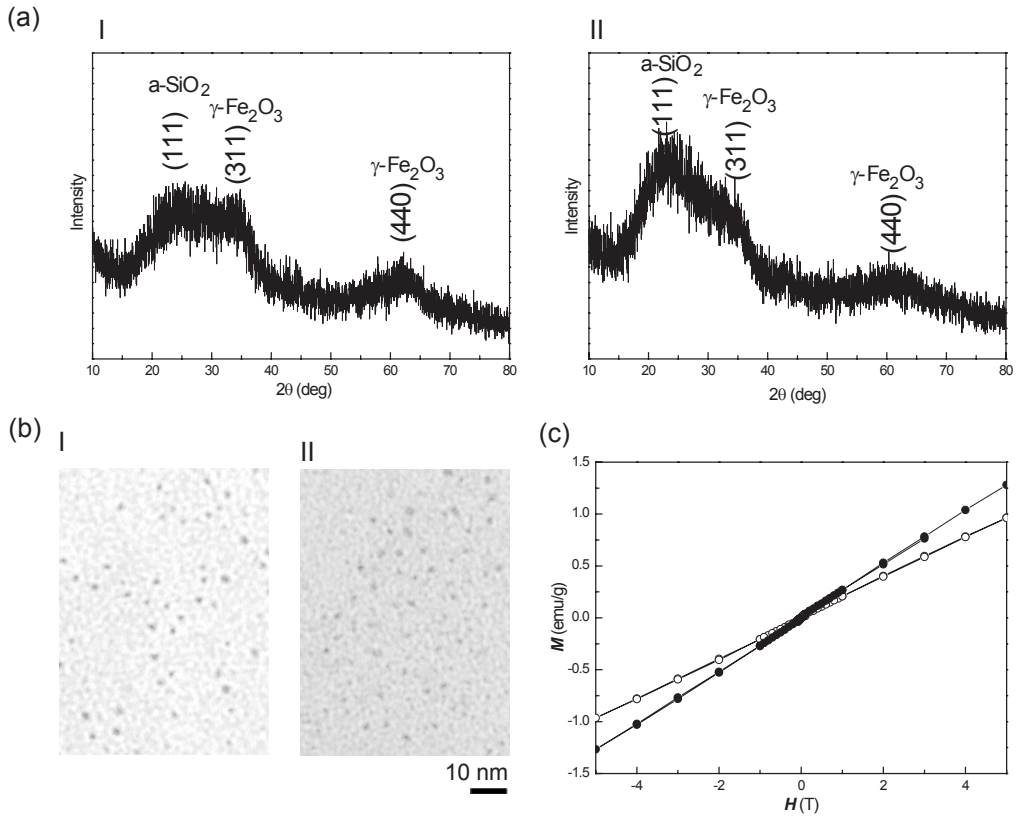

FIG. 2: The CuK$_\alpha$ XRD patterns of the unmodified NMPs (a-I) and amino-NMPs (a-II). TEM images of unmodified NMPs (b-I) and amino-NMPs (b-II). *M-H* curves for unmodified NMPs (○) and amino-NMPs (●).

−5 and 5 tesla (T) at 300 K. The presence of the amino groups was confirmed by Fourier transform infrared spectroscopy (FT-IR; FT-720, Horiba, Kyoto, Japan).

To confirm the ability of the amino-NMPs to bind molecules to their surfaces and to visualize the introduction of NMPs into cells, rhodamine fluorophore (RF)-labeled NMPs were prepared as follows. A 0.1 M dimethyl sulfoxide solution of 5- and 6-carboxytetramethyl rhodamine succinimidyl ester (SIGMA Co.; final concentration, 10 mM) was added to an aqueous solution of the amino-NMPs, and the total volume of the solution was brought to $\sim 500\ \mu$L. The labeling reaction was allowed to proceed at 310 K for 10 minutes and was then quenched for 30 minutes at room temperature by the addition of formaldehyde (final concentration, 0.1 M). After reduction of the Schiff base by $NaBH_4$ (final concentration, 0.1 M), the resulting precipitates were washed several times with ultrapure water. The colloidal stability and surface electric charge of the RF-NMPs were investigated by running the RF-NMPs in 1% agarose gel (100 V, 10 minutes). These chemicals in the all experiments were used without further purification.

B. Cellular uptake of labeled NMPs

To determine whether NMPs had cytotoxic effects on the cells, RF-NMPs suspension and large-size particle as control (FluoreSphere beads (d = 200 nm; Invitrogen Co., California, USA)) [final number concentration, 4.8×10^7 mL^{-1}] were independently injected into rat kangaroo kidney epithelium (PtK2) cells in cultured media. The dishes were washed three times with culture medium to remove loosely bound particles from the cell surfaces after 24 hours, and the living cells were observed with a fluorescence microscope (ECLIPSE TE2000-U, Nikon Co., Tokyo, Japan). The living cells were observed again after 5 days.

To confirm the existence of NMPs in the cells, the cells containing the RF-NMPs were cultured for 24 hours. After the culture dishes were washed with phosphate-buffered saline buffer, PtK2 cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 15 minutes at room temperature and then washed with PB. Osmium (0.1%) in 0.1 M PB was added to the cells at 277 K. The cells were rinsed with distilled water, dehydrated with ethanol, and embedded in epoxy resin (Epon 812). Ultrathin sections were cut with an ultramicrotome (Reichert-Nissei Ultracut N, Nissei Sangyo Co., Tokyo, Japan), collected on copper slot grids, stained with uranyl acetate/lead citrate, and then observed by TEM (Tecnai G2 Sphera, FEI, Hillsboro, OR, USA).

C. Amino acid modification of amino-nano magnetic particles (NMPs)

Amino-NMPs (5 mg) were dispersed in 100 μL of *N,N*-dimethylformamide (DMF), and 7.2 mg of *N*-(-maleimidocaproyloxy) sulfosuccinimide ester (sulfo-

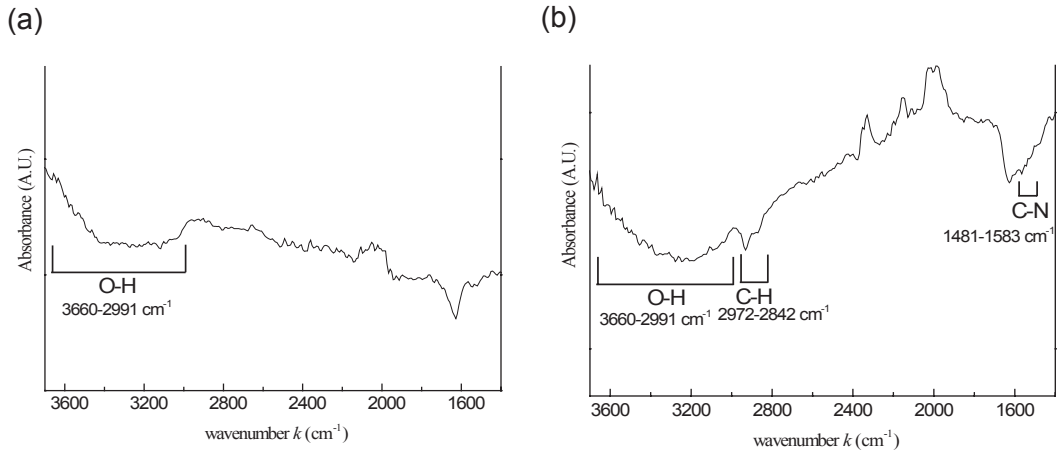

FIG. 3: FT-IR spectra of unmodified NMPs (a) and amino-NMPs (b).

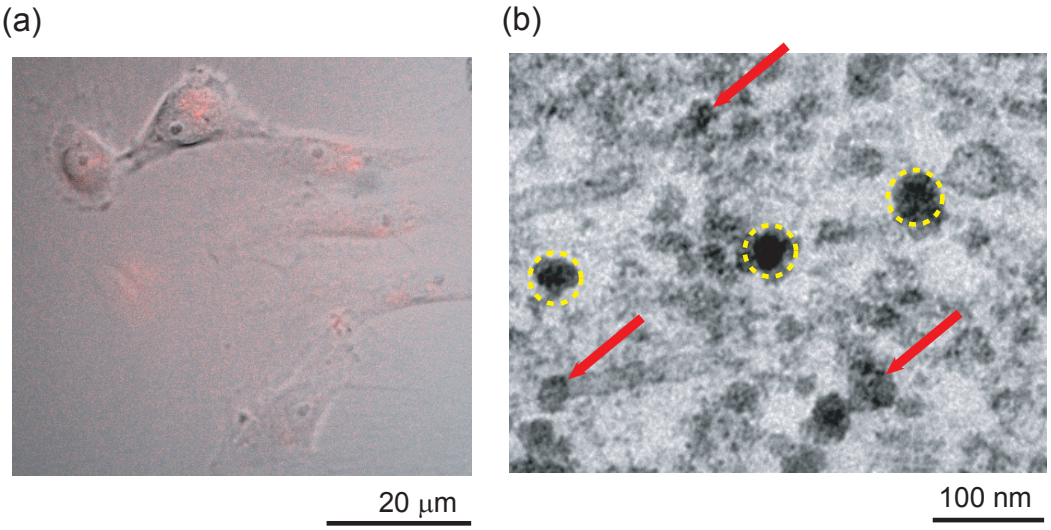

FIG. 4: PtK2 cells incubated with RF-NMPs for 24 hours (a) and TEM image of PtK2 cells incubated with RF-NMPs (the yellow dashed circles indicate aggregated NMPs and the red arrows indicate ribosomes).

EMCS; Pierce Biotechnology, Rockford, USA) was dissolved in 100 μL of DMF. The NMPs dispersion and the sulfo-EMCS solution were mixed and then stirred at 277 K for 30 minutes and at room temperature for 90 minutes. The reaction mixture was cooled in an ice bath and quenched with formaldehyde (3 minutes on ice and 15 minutes at room temperature; final formaldehyde concentration, 0.175 M). After reduction of the Schiff base with $NaBH_4$ (final concentration, 0.175 M), excess reagents were removed by centrifugation (3 times at 5000 rpm), and the solvent was changed to 100 μL of K-PIPES buffer (0.1 M, pH 6.8). Conjugation of the cross-linker to the surface of the functionalized NMPs was confirmed by FT-IR. These particles are referred to as EMCS-NMPs (Fig. 1b).

For modification of the surface of the EMCS-NMPs with an amino acid (Fig. 1c), 100 μL of 0.175 M L-cysteine dissolved in K-PIPES was added to EMCS-NMPs, and the reaction mixture was stirred at 277 K for 30 minutes and at room temperature for 90 minutes. The suspension had been washed three times with 1 mL of K-PIPES. The solvent was replaced to 100 μL of 20 mM citric acid buffer (pH 8) and 80 μL of acetone after centrifugation of particle suspension (3 minutes at 5,000 rpm). To confirm amino acid modification of the EMCS-NMPs, the particles were conjugated with 20 μL dansyl chloride (DNS) in acetone (final concentration, 20 mM). Excess reagents were removed by centrifugation (3 times at 5000 rpm). The morphology of amino acid-conjugated NMPs (AA-NMPs) was investigated by TEM and the fluorescence of DNS-NMPs was observed by confocal laser scanning microscopy (LSM5, Carl Zeiss Co., Oberkochen, Deutschland).

III. RESULTS AND DISCUSSION

In the XRD pattern of the γ-Fe_2O_3 NMPs (Fig. 2a-I) and the amino-NMPs (Fig. 2a-II), the peaks at $2\theta = 35°$ and 60° originated from the (311) and (440) reflections of the spinel γ-Fe_2O_3 structure, respectively, and the peaks below $2\theta = 30°$ originated from a-SiO_2 [24]. These results

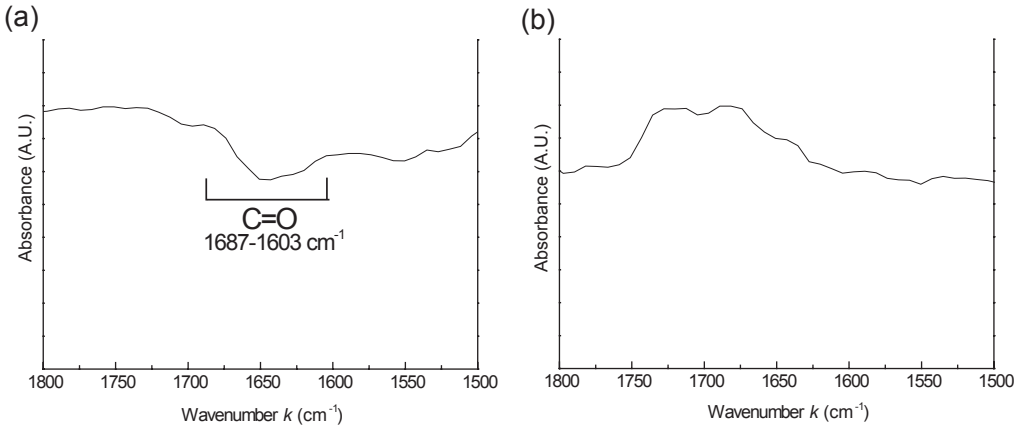

FIG. 5: FT-IR spectra of EMCS-NMPs (a) and amino-NMPs (b).

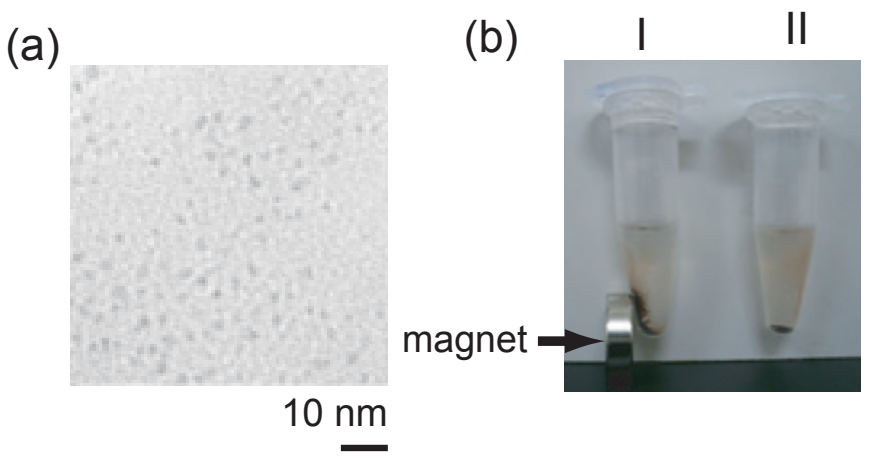

FIG. 6: TEM image of AA-NMPs (a). Photographs showing attraction of AA-NMPs by an external magnetic field (340 mT) (b-I) and without an external magnetic field (b-II).

suggest that functionalization of the NMPs with amino groups did not affect their structure.

The TEM images showed no significant difference between the shape of the NMPs (Fig. 2b-I) and that of the amino-NMPs (Fig. 2b-II). The number-average diameter of the amino-NMPs was determined to be about 2.0 nm±0.04 nm (mean±SEM). The SQUID results indicated that the magnetizations of both the NMPs and the amino-NMPs increased linearly with increasing magnetic field; that is, both types of particles showed paramagnetism (Fig. 2c).

The presence of the amino groups on the NMPs was confirmed by FT-IR. The typical O-H peak ($3660-2991$ cm^{-1}) was detected in both spectra of unmodified NMPs (Fig. 3a) and amino-NMPs (Fig. 3b). The C-H peak ($2972-2842$ cm^{-1}) and the C-N peak ($1583-1481$ cm^{-1}) were observed in the spectrum of the amino-NMPs (Fig. 3b). These results indicated that O-H peak was derived from a-SiO_2 of NMPs and C-H peak and C-N peak were derived from γ-APTES. Our functionalized NMPs with amino groups via silanization permit covalent attachment of carbonyl compounds to the particle surface. As a fact, we have succeeded in the attachment of RF to NMPs surface to visualize particle shape. Therefore, we used RF-NMPs to evaluate cellular uptake of NMPs.

To evaluate the internalization of the NMPs into the cells, we cultured the PtK2 cells after addition of the RF-NMPs. After the cells were cultured for 24 hours, fluorescence was observed from inside the cells even after washing (Fig. 4a); in contrast, no fluorescence was observed from the untreated cells as control. This suggested the obtained fluorescence from rhodamine fluorophore on NMPs, not autofluorescence originating from the cells themselves. In addition, the PtK2 cells treated with the NMPs divided and continued to grow even after 5 days, although we observed weak and diffuse fluorescence. These results indicate that the PtK2 cell division was not inhibited by the NMPs.

Regarding the colloidal stability surface electric charge of the RF-NMPs, no migration toward either the cathode or the anode occurred after 10 minutes at 100 mV, which indicates that the quench procedure of residual amino group on a particle succeeded and particle surface was nonionic. Therefore, extremely small particle can internalize into the cell without conventional cationic reagent, although we suppose that this internalization of NMPs into the cells was induced by non-specific endocytosis.

We investigated the intracellular space of the living

PtK2 cells by TEM to confirm the introduction of the RF-NMPs into the cells. In the TEM image of the cells into which NMPs had been introduced showed high-density spots (Fig. 4b; yellow dashed circles). These can be identified as the aggregated NMPs. In the control TEM image, no large high-density spots that show control particles (d = 200 nm) were observed, which indicates that no particularly large particles were introduced by endocytosis. For small dots indicated as red arrows in Fig. 4b, estimating from the shape and the massive number, they were not determined as particles however possibly ribosomes. One possible explanation for resulting of aggregated NMPs in the cell is that non-aggregated plural NMPs on cell surface may be included in an endocytosed vesicle, and aggregate together after non-specific endocytosis occurred. We confirm these findings in the present study [25]. We newly determine degree of dispersion for amino-NMPs diameter from TEM result.@

Conjugation of the cross-linker to the amino-NMPs was also confirmed by FT-IR. The C=O peak ($1687 - 1603$ cm^{-1}) deriving from the amide bond was detected only in the spectrum of the EMCS-NMPs (Fig. 5).

The TEM image of the AA-NMPs showed no significant difference between the shape of the AA-NMPs (Fig. 6a) and that of the unmodified NMPs (Fig. 2b-I). The number-average diameter of the AA-NMPs was determined to be about 2.4 ± 0.05 nm (mean±SEM). The diameter increased compared with amino-NMPs (2.0 nm). This may be due to the modification of EMCS as a linker.

After modification of EMCS-NMPs with amino acid, the magnetization of the particles was retained; that is, the magnetization was sufficient that the AA-NMPs were attracted by the permanent magnet (340 mT; NIROKU Seisakusyo, Shiga, Japan) (Fig. 6b).

DNS fluorescence was observed from the AA-NMPs labeled with dansyl chloride (DNS-NMPs), and no fluorescence for DNS was observed from the amino-NMPs. This indicated that DNS was modified with α-amino group of amino acid (L-cysteine) on NMPs. Besides, we have succeeded in preparation of amino acid conjugated NMPs.

IV. CONCLUSIONS

We studied the feasibility of *in vivo* introduction of functionalized NMPs. NMPs conjugated with functional group such as amino group was readily prepared by silanization procedure. We have achieved detail physical characterization of functionalized NMPs. Obtained functionalized NMPs can modify with desired molecule and exhibits paramagnetism and original particle structure. We have succeeded in cellular uptake of NMPs via non-specific endocytosis without conventional cationic reagent.

Finally, we prepared amino acid-conjugated NMPs with the eventual goal of achieving cell-selective uptake of the conjugated particles. In a previous work, we have already taken knowledge of the recognition ability of an amino acid transporter on the surfaces of cell membranes and achieved drug delivery system using the recognition ability of amino acid [25]. Our AA-NMPs have great potential for use in drug-delivery applications because the surfaces of the NMPs can be modified with multiple molecules [27]. The AA-NMPs can be exploited for cellular recognition delivery tool.

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