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# Cellular Recognition of Functionalized with Folic acid Nanoparticles*

Shu Taira and Takahiro Hatanaka
*Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida City, Tokyo 194-8511, Japan*

Shinji Moritake, Yukino Kai, and Yuko Ichiyanagi
*Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida City, Tokyo 194-8511, Japan, and Department of Physics, Graduate School of Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan*

Mitsutoshi Setou†
*Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida City, Tokyo 194-8511, Japan, and Nano Structural Physiology, National Institute for Physiological Sciences, National Institute of Natural Sciences, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan*

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We have prepared extremely small functionalized nanoparticles (NPs) and showed that they could be introduced into living cells without modification (e.g. cationic coating) to enhance endocytic internalization. However, the internalization depended on physical adsorption of the particles on the cell surfaces, and therefore the internalization process was nonselective. Here, we prepared NPs conjugated with folic acid and a coumarin fluorophore so that the NPs ($d = 2.6$ nm) would be recognized by folate receptors on the cell surface. The presence of the folic acid and coumarin on the surface of the NPs was confirmed by Fourier transform infrared spectroscopy. The modified NPs were internalized by human pharyngeal cancer cells (KB cells) after an incubation time that was short compared with the time required for internalization of NPs without folic acid. This result indicates that the folic acid receptor on the KB cell membrane recognized the folic acid-conjugated NPs. Cellular recognition of NPs may lead to the development of cell-specific delivery systems.
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Keywords: nanoparticle; surface modification; folic acid; living cell; endocytosis; delivery system

## I. INTRODUCTION

Nanoparticles (NPs) with diameters in the 100-1000-nm range are widely used as carriers for macromolecules (such as plasmid DNA [1] and siRNA [2]), peptides [3], genes [4], and small molecules (such as a corticosteroid [5] and alkaloids [6]), with the goal of improving the therapeutic efficacy of cellular drug-delivery systems as well as bioengineering applications [7–9] and the field of fundamental physics [10]. The surface on NPs can be loaded with impermeable biomolecules and other chemical compounds. NPs can assist in the transport of such molecules into living cells. For facilitating endocytic introduction of the carriers into cells; and for large NPs, cationic residues are sometimes necessary for efficient introduction of the particles [11, 12]. So far, however, this method has been used only for nonspecific endocytosis processes; that is, the destination of the particles is not strictly controlled. In addition, cationic residue showed cytotoxicity. Controlling the destination of a drug via NPs that shows no cytotoxic property might become feasible if our NPs could be modified not only with the drug but also with a moiety that is recognized by specific cell types [13, 14].

The folate receptor (FR) is overexpressed on the surfaces of human tumor cells [13, 15, 16], and folic acid (FA) has been used as a ligand to target tumor cells. When the $\gamma$-carboxyl group of FA is linked to a drug or an imaging reagent, the linked compound binds strongly to the FR ($K_D \sim 10^{-10}$), and receptor-mediated endocytosis occurs smoothly [17]. FA-conjugated NPs have also been investigated for use in imaging and drug-delivery systems. However, the method for the preparation of the modified NPs is complicated in that several steps are required. In addition, the diameter of the NPs is more than several tens of nanometers.

In previous studies, we have prepared NPs surrounded by amorphous $SiO_2$ by mixing aqueous solutions of $3d$ transition-metal chlorides ($MCl_2 \cdot nH_2O$) and sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$) to obtain monodispersed single-digit nanosize particles in a single step [18–23]. We have also prepared NPs functionalized with amino groups on the particle surface via a silanization procedure to covalently load target molecules without increasing the particle diameter. We showed that these extremely small particles can gain access to the surfaces of cells without steric hindrance between the plasma membrane and the particles; therefore, the particles can be introduced into the cells without the necessity for a cationic coating [24].

In this study, we synthesized some new functionalized NPs designed to recognize certain cell types for specific delivery. The NPs were covalently modified with FA on the particle surfaces so that the particles would bind to the FR. We expected that the FA-conjugated NPs would bind to FR on human pharyngeal cancer cells (KB cells) and be selectively and preferentially introduced to the target cells (Fig. 1)

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†Corresponding author: setou@nips.ac.jp

  

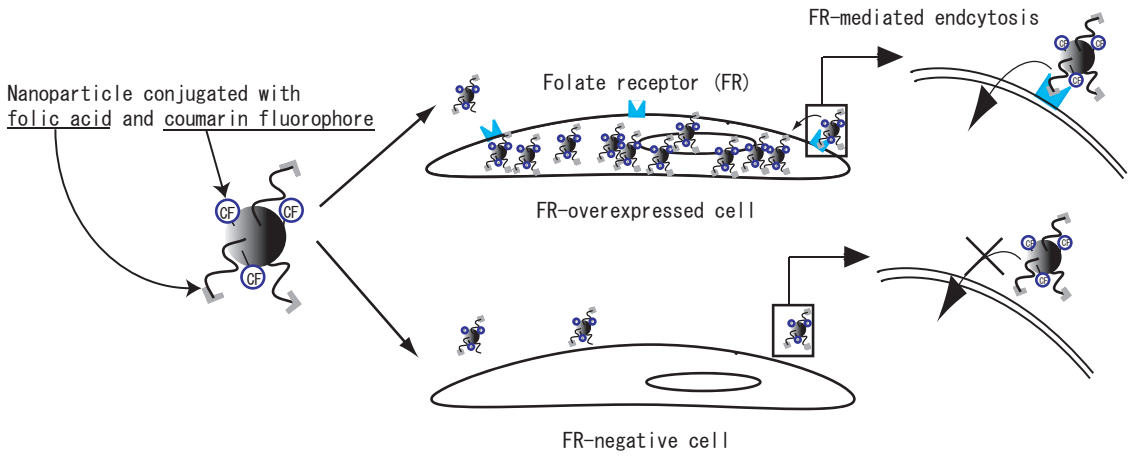


FIG. 1: Schematic of cell-specific uptake of FA-modified NPs.

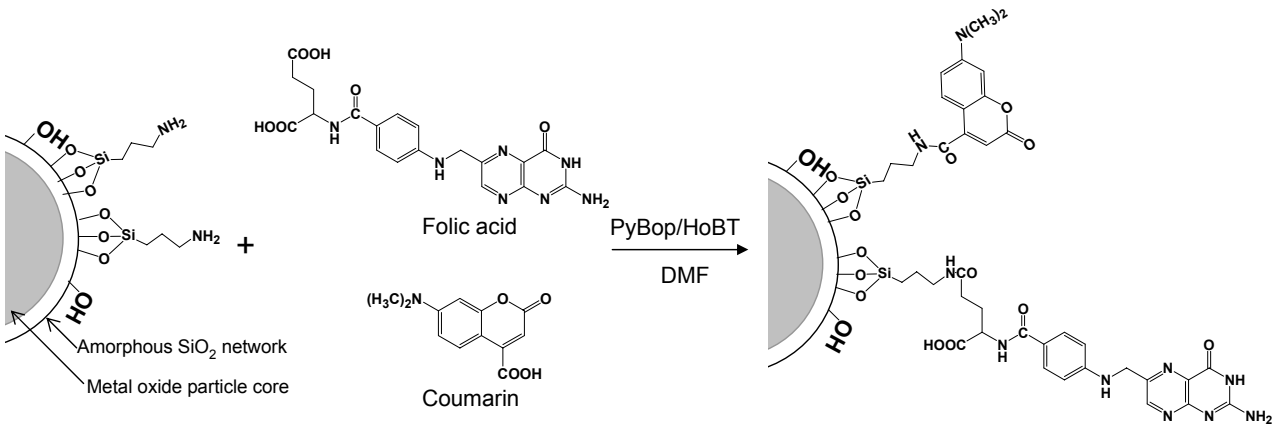


FIG. 2: Preparation of FA-CF-conjugated NPs.

## II. EXPERIMENTAL

### A. Modification of nanoparticles with folic acid and coumarin

$\gamma$-$Fe_2O_3$ NPs surrounded by amorphous $SiO_2$ ($a$-$SiO_2$) were prepared by mixing 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 previously described methods [18–23].

NPs modified with surface amino groups (amino-NPs; Fig. 2) were prepared by adding (3-aminopropyl)triethoxysilane ($\gamma$-APTES; Wako Pure Chemical Industries) to the solid $\gamma$-$Fe_2O_3$ NPs and allowing the silanization reaction to proceed at 403 K for 6 h.

The amino-NPs were modified with FA and coumarin by means of the following procedure (Fig. 2). The vessel containing the DMF suspension of the amino-NPs (25 mg) was cooled in an ice bath. 1-Hydroxy-1$H$-benzotriazole (HoBT) (23 mM), Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBop) (23 mM), and $N$-Ethyl-$N$-(1-methylethyl)-2-propanamine (DIEA) (2.3 mM) were added to the particle suspension. A mixture of 12.5 mg each of FA and 7-dimethylaminocoumarin-4-acetic acid fluorophore (CF) (230 $\mu$M) in dry DMF was added dropwise over a period of 10 min, and then the reaction was allowed to proceed for 20 h in the dark. After the reaction was complete, the crude suspension was washed three times with dry DMF and then quenched for 30 minutes at room temperature with formaldehyde (final concentration, 0.1 M) in methanol. After reduction of the Schiff base with $NaBH_4$ (final concentration, 0.1 M), the resulting precipitates were washed several times with ultrapure water. For control experiments, NPs conjugated with the CF were prepared by the above method. The FA-CF-conjugated NPs was characterized by Fourier transform infrared spectroscopy (FT-IR; Horiba FT-720). The colloidal stability and surface electric charge of the FA-CF- (or only CF) conjugated NPs were investigated by running these NPs in 0.75% agarose gel (100 V, 10 minutes)

### B. Cellular uptake of FA-CF-conjugated NPs

Human pharyngeal cancer cells (KB cells) as a model tumor cell line and rat kangaroo kidney epithelium (PtK2) cells as control were chosen. Approximately $2.0 \times 10^4$ KB cells or PtK2 were initially cultured in dishes

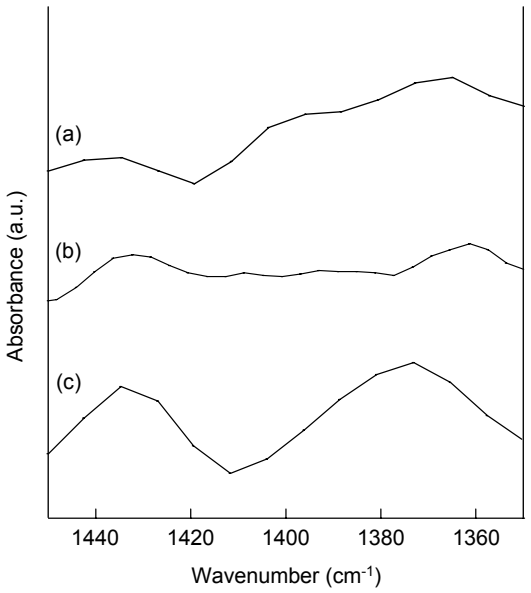


FIG. 3: FT-IR spectra of (a) FA-CF-conjugated NPs, (b) amino-NPs, and (c) folic acid.

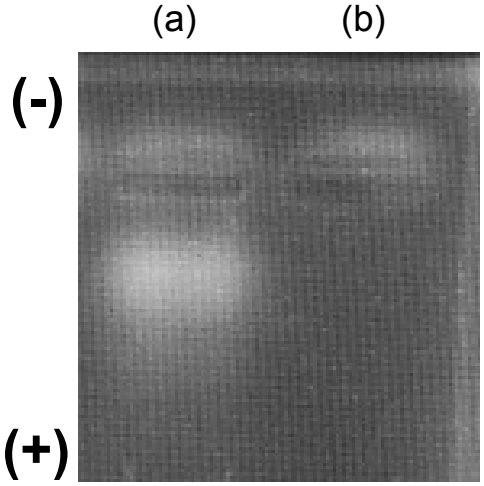


FIG. 4: Gel electrophoretic analyses of (a) FA-CF-conjugated NPs and (b) CF-conjugated NPs. After electric migration, the gel was exposure UV light to detect CF light.

with 2 ml of medium and were then incubated for 48 h, respectively.

Either FA-CF- or CF-conjugated NPs were added to the cell-culture dishes for 2 h as a cellular uptake of NPs. To confirm the shape of the KB or PtK2 cells, 0.8 $\mu$g/ml DiI (Invitrogen) was added to the dishes after 1 h as a staining reagent for the cell surfaces. Cellular uptake of NPs was investigated by confocal laser scanning microscopy (LSM5, Carl Zeiss Co.). To confirm specific introduction of FA-CF-conjugated NPs, a competitive uptake inhibition assay was examined. FA-CF-conjugated NPs were coincubated with free folic acid (1.1 mM) to KB cells.

## III. RESULTS

### A. Physical characterization of NPs

The presence of the amino groups on the amino-NPs was confirmed by FT-IR. The typical O-H peak (3100-3700 $cm^{-1}$) of the NPs and the C-H peak (2822-2982 $cm^{-1}$) of the $\gamma$-APTES group were detected in the spectrum of the amino-NPs [24]. FA-CF-conjugated NPs showed a peak at 1419 $cm^{-1}$ corresponding to the *p*-amino benzoic acid moiety of FA [25] (Fig. 3a); no that of peak was found from amino-NPs (Fig. 3b). The IR peaks of the coumarin fluorophore could not be identified, because the coumarin peaks overlapped the FA peaks. The presence of the coumarin moiety on the NPs was confirmed by fluorescence microscopy (data not shown). In addition, regarding the colloidal stability surface electric charge of the FA-CF- (or only CF) NPs, FA-CF-conjugated NPs had migrated toward the anode. For CF-conjugated NPs, no migration toward either the anode or the cathode occurred after 10 minutes at 100 mV (Fig. 4). This indicated that FA-CF-NPs have anionic property due to modification of FA that has $\alpha$-carboxyl group.

Transmission electron microscopy showed that there was no significant difference between the shape of the FA-CF-conjugated NPs, as in the caption for the (Fig. 5a) and that of the amino-NPs (Fig. 5b). The number-average diameter of the FA-CF-conjugated NPs was determined to be about $2.6 \pm 0.05$ nm (mean±SEM).

### B. Selective Uptake of NPs by living cells

We have evaluated cellular uptake of nanoparticle in three dimensions. After the KB cells had been cultured for total 3 hours, fluorescence was observed from the cells treated with FA-CF-conjugated NPs. The lateral image clearly shows that the aggregated particles were present inside the cells (Fig. 6a). In contrast, no fluorescence was observed from inside the cells treated with CF-conjugated NPs (Fig. 6b), although weak and aggregated strong fluorescence were observed from the cell surface and hollow region, respectively, owing to nonspecific adsorption of particle. Untreated KB cells showed no fluorescence (Fig. 6c), which indicates that the fluorescence observed from the KB cells treated with the FA-CF-conjugated NPs was not autofluorescence originating from the cells themselves. Normalized fluorescence intensities (F.I.)/$\mu m^2$ were estimated. All fluorescence intensities were normalized with respect to the fluorescence intensity of the untreated control cells (Fig. 6d). The F.I. ratio of the FA-CF-conjugated NPs internalized in the cells to the CF-conjugated NPs to the untreated cells was 158:2:1. In addition, no fluorescence was observed in cells treated with FA-CF-conjugated NPs and excess free FA at the same time. As another control experiment, FA-CF-conjugated NPs were added to rat kangaroo kidney epithelium (PtK2) cells. No fluorescence was observed from both inside and surficial the cells treated with FA-CF-NPs. For CF-conjugated NPs, the existence of its particle was confirmed on cell surface although no fluorescence was observed from inside the cells (data not shown).

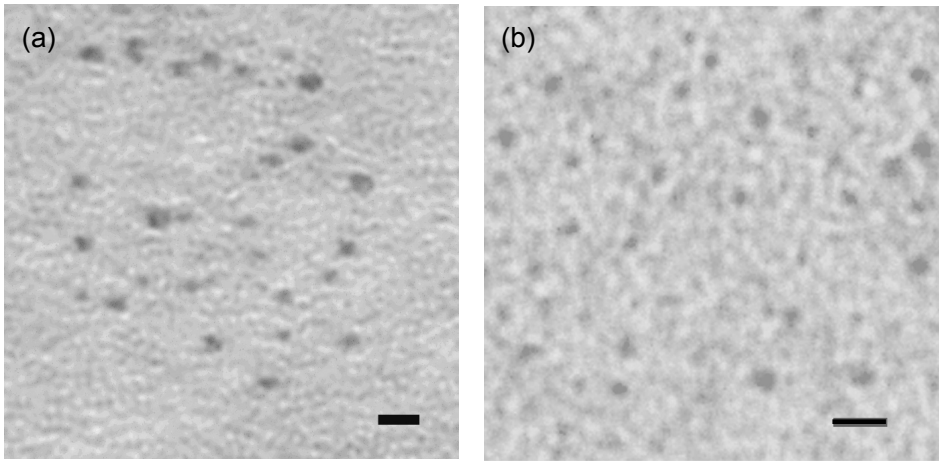


FIG. 5: TEM image of (a) FA-CF-conjugated NPs and (b) amino-NPs. Scale bar=5 nm.

These results indicate that FA-CF-conjugated NPs were internalized in the KB cells via the FR-mediated endocytosis rather than by a nonspecific endocytosis process that occurred without ligandreceptor interaction.

## IV. DISCUSSION

We demonstrated that folic acid-conjugated NPs can recognize cell type and are internalized to tumor cell. We have conducted simulations to determine the number of amino groups per particle [24]. Assuming that the amino groups are symmetrically distributed on the particle surface, they should be existing on an icosahedral pattern. Hence, we expected the number of amino groups per particle to be approximately 20. This value implies that the number of FA and CF groups per particle is less than 20. Conjugation of the NPs with multiple ligands was achieved: FA was used to bind to the receptor protein on the cell surface, and the coumarin fluorophore was used to visualize the particle shape. We hope to be able to exploit this technique to develop a drug-delivery system in a future study.

The small size of the NPs allowed them to approach the surfaces of the KB cells more readily than particles with diameters of 100 nm or greater [26], owing to less steric hindrance. The FA groups on the particles served as ligands for binding to the FR of the cells, and FR-mediated endocytosis occurred. FA-CF-conjugated NPs could not internalize to PtK2 cells that has no FR on plasma membrane. In contrast, CF-conjugated NPs could adsorb on both KB and PtK2 cells surface. We have confirmed FA-CF-conjugated NPs showed anionic property due to modification of FA and CF-conjugated NPs showed nonionic property, respectively. It implied that electrostatic repulsion between anionic cell surface and anionic FA-CF-conjugated NPs occurred. Nonionic CF-conjugated NPs could non-specifically adsorb on any cell type surface. However, receptor-mediated endocytosis did not occur; viz. we have observed no internalization of CF-conjugated NPs. In addition, FA-CF-conjugated NPs could not internalize to KB cell with the excess amount of free FA. Therefore, introduction of FA-CF-conjugated NPs to KB cell was selectively induced by the interaction between FA on NPs and FR on cell surface. One possible explanation for the aggregated NPs observed in the cells (Fig. 6a) is that multiple unaggregated NPs on the cell surface may have been be included in a FR-mediated endocytosed vesicle and then become aggregated after endocytosis. For the effect of our NPs to the cells, after 24 hours, the cells containing the internalized particles were still thriving, which indicates that the NPs did not show cytotoxicity that inhibited cell division; however, fluorescence was not detected after 24 hours, because the internalized particles were dispersed by cell division. The cells containing the internalized particles continued to thrive, which implies that they were not damaged by incorporation of the NPs.

Previously, we succeeded in introducing NPs by a nonspecific endocytosis process that took 24 hours. In the current study, we shortened the time required for internalization of the NPs to 3 hours and achieved selective transportation of the NPs via binding to the FR on the plasma membrane.

We believe that cellular recognition of NPs has great potential for the development of selective drug-delivery applications, because the surfaces of NPs can be modified with various types of molecules. For example, we have already taken advantage of the recognition ability of an amino acid (AA) transporter on the surfaces of cell membranes and designed AA-based prodrugs for a cell-specific delivery system [14]. In addition, we prepared AA-modified NPs to realize a specific recognition of the NPs via the transporter [27]. Our NPs also have magnetic properties. We may be able to take advantage of these properties to deliver therapeutic agents to diseased tissue by application of a magnetic field and thus achieve selective delivery at the cellular level using our NPs.

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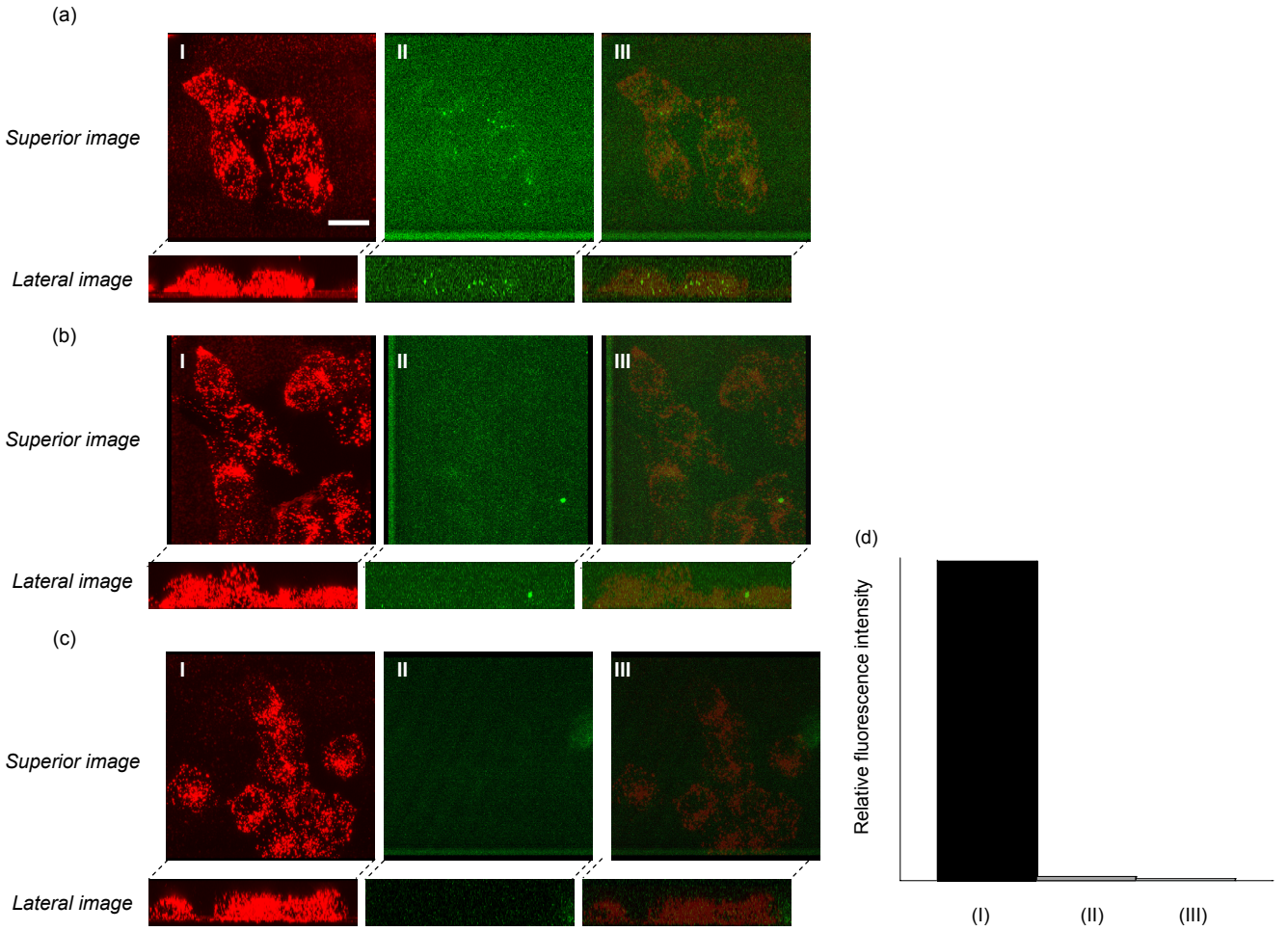


FIG. 6: Confocal laser scanning microscopy images of (a) KB cells treated with FA-CF-conjugated NPs, (b) KB cells treated with CF-conjugated NPs, and (c) untreated KB cells as a control. (I) DiI fluorescence images (cell), (II) coumarin fluorescence images (NPs), and (III) merged images. (d) Relative fluorescence intensities determined by dividing the sum of all pixel intensities of coumarin. (I) FA-CF-conjugated NPs internalized in the cells, (II) CF-conjugated NPs, and (III) untreated cells. Scale bar = 20 $\mu$m.

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