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Cellular uptake of magnetic nanoparticle is mediated through energy-dependent endocytosis in A549 cells

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Biocompatible silica-overcoated magnetic nanoparticles containing an organic fluorescence dye, rhodamine B isothiocyanate (RITC), within a silica shell [50 nm size, MNP@SiO_2(RITC)s] were synthesized. For future application of the MNP@SiO_2(RITC)s into diverse areas of research such as drug or gene delivery, bioimaging, and biosensors, detailed information of the cellular uptake process of the nanoparticles is essential. Thus, this study was performed to elucidate the precise mechanism by which the lung cancer cells uptake the magnetic nanoparticles. Lung cells were chosen for this study because inhalation is the most likely route of exposure and lung cancer cells were also found to uptake magnetic nanoparticles rapidly in preliminary experiments. The lung cells were pretreated with different metabolic inhibitors. Our results revealed that low temperature disturbed the uptake of magnetic nanoparticles into the cells. Metabolic inhibitors also prevented the delivery of the materials into cells. Use of TEM clearly demonstrated that uptake of the nanoparticles was mediated through endosomes. Taken together, our results demonstrate that magnetic nanoparticles can be internalized into the cells through an energy-dependent endosomal-lysosomal mechanism.

Key words: A549 cells, cellular uptake, endocytosis, magnetic nanoparticle

Introduction

Nanoparticles are increasingly used in different applications, including bioimaging, diagnostic technology, and drug/gene delivery. Among them, magnetic iron oxide nanoparticles have been used for many years as magnetic resonance imaging contrast agents or as drug delivery applications [4]. Tissue- and cell-specific gene/drug delivery by these magnetic nanoparticles (MNPs) can be achieved by employing nanoparticle coatings or carrier-drug or -gene conjugates that contain a ligand recognized by a receptor on the target cell. However, significant concern exists regarding the potential toxicity of nanoparticles. More specifically, inhalation and dermal uptake appear to be the most likely routes of exposure to humans. Therefore, proper surface coating with biocompatible materials such as silica (SiO_2) is necessary for the prevention of potential toxicities [1].

The synthesis of biocompatible silica-coated magnetic nanoparticles (MNP@SiO_2) has been studied extensively by various research groups, and size-controllable and multifunctional core-shell nanoparticles have gained much attention. Among the shell-coating materials, silica is a very promising candidate because it contains inorganic materials with good biocompatibility and chemical stability [14]. Silica-coated core-shell nanoparticles have recently been synthesized using various methods, and organic fluorescence dyes also have been incorporated into the silica shell for more extensive application [6,7]. We recently synthesized a biocompatible silica-overcoated magnetic nanoparticle containing organic fluorescence dye (rhodamine B isothiocyanate), MNP@SiO_2(RITC)s, within a silica shell of controllable thickness, and reported that the MNPs were incorporated into cells and the nanoparticle-uptaken cells could be driven by external magnetic force [17]. For future application of the MNPs into biomedicine for use in drug or gene delivery, detailed information of the cellular uptake process of the nanoparticles is essential. Since our preliminary experiments have shown that human lung cancer cells (A549) could uptake nanoparticles rapidly, this study was performed to elucidate the precise mechanism by which the lung cancer cells uptake the nanoparticles. Here, we report that biocompatible magnetic nanoparticles can penetrate the cells through energy-dependent endocytosis.

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Materials and Methods

Preparation of biocompatible MNP@SiO_2(RITC)s

Biocompatible magnetic nanoparticles containing organic fluorescence dye, rhodamine B isothiocyanate (RITC; Sigma-Aldrich, USA), within a silica shell [50 nm size, MNP@SiO_2(RITC)s] were synthesized according to a previously described method [17]. In brief, pre-synthesized cobalt ferrite magnetic nanoparticles (average size is about 9 nm) were added to the aqueous polyvinylpyrolidone (PVP, Sigma-Aldrich, USA) solution. The PVP-stabilized cobalt ferrite nanoparticles were separated by the addition of acetone and subsequent centrifugation. The precipitated particles were redispersed in ethanol. Trimethoxysilane (Gelest, USA) modified by RITC was prepared from 3-aminopropyltriethoxysilane (Gelest, USA) and RITC under nitrogen. The synthesized silane modified dye solution was then mixed with tetraethoxysilane (TEOS; Gelest, USA) and injected into the PVP-stabilized cobalt ferrite ethanol solution. The solution was subsequently polymerized on the surface of PVP-stabilized cobalt ferrites by the addition of ammonia as a catalyst to form RITC-incorporated silica-coated magnetic nanoparticle, MNP@SiO_2(RITC)s. The synthesized magnetic naoparticles were confirmed by transmission electron microscopy (TEM).

Cells and selection of nanoparticle concentration

A549 human lung cancer cells (A549 cells; ATCC, USA) were maintained in RPMI 1640 medium supplemented with 10% FBS (GibcoBRL, USA) and gentamycin (500 μg/ml; GibcoBRL, USA). The concentration of MNP@SiO_2(RITC)s was determined using the IC_{50} value derived from a cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] assay. The IC_{50} value was 4 mg/ml. Thus, 0.4 mg/ml, 1/10th of IC_{50}, was chosen for future cell studies.

Cell treatment protocol

To determine whether the uptake of MNPs into lung cells was energy-dependent or, more generally, cell function-dependent, the cells were incubated with MNP@SiO_2(RITC)s under varying metabolic conditions. The uptake studies were performed at 37°C or 4°C, and in the presence of sodium azide (0.1%), sucrose (0.45 M), and bafilomycin A (0.05 μM), respectively. After initial passage in tissue culture flasks, cells were grown on 8-chamber mounted Permanox slides with covers (2×10^4 cells seeded per chamber, Lab-Tek Chamber Slide; Nalge, USA). After the cells had reached 80-90% confluence, the cells were preincubated at 4°C with 0.1% sodium azide [15], hyperosmotic 0.45 M sucrose [8], and 0.05 μM bafilomycin A [2] for 10 min, respectively, and were then treated with MNP@SiO_2(RITC)s for an additional 30 min (cells preincubated at 4°C were incubated for an additional 30 min at 4°C. Remaining treated and control cells were incubated for 30 min at 37°C). Uptake was terminated by washing the cells three times with PBS. After washing, cells were fixed with 4% paraformaldehyde in PBS for 1 h and washed with PBS. Individual coverslips were mounted cell-side down onto fresh glass slides with fluorescence-free glycerol-based mounting medium (Fluoromount-G; Southern Biotechnology Associates, USA). Cells were viewed in order to determine the differential interference contrast and to obtain fluorescence images with confocal microscopy (Zeiss, Germany) that would be used to evaluate the intercellular localization of MNP@SiO_2(RITC)s.

Protocol of TEM study

To study the intracellular translocation of magnetic nanoparticles, treated cells were fixed with 1% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, at 4°C. The samples were then washed with PBS followed by washing in 0.1 M cacodylate buffer, pH 7.2, and post-fixed in 1% osmium tetraoxide in 0.1 M cacodylate buffer for 1.5 h at room temperature. The samples were then washed briefly in dH_2O, dehydrated by a graded ethanol series, infiltrated using propylene oxide and EPON epoxy resin (Structure Probe, USA), and finally embedded with only epoxy resin. The samples that had been mixed with epoxy resin were loaded into capsules and polymerized at 60°C for 24 h. Thin sections were cut using an RMC MT-X ultramicrotome and collected on copper grids; these sections were not stained with any reagent for detecting the uptake of nanoparticles into the cells. Images were collected using a JEOL transmission electron microscope (JEM-1011; Japan) at 80 kV with a GATAN digital camera (Gatan, USA).

Results

Synthesis of ~50 nm-sized MNP@SiO_2(RITC)s

The silica-coated magnetic core-shell nanoparticles [MNP@SiO_2(RITC)s] were successfully prepared, and the size (about 50 nm) and shape were characterized by TEM (Fig. 1). As shown in Fig. 1, the narrow size distribution of the nanoparticles was determined using a low magnititude image. In the high magnitude image (insert image), core-shell structure was clearly shown by different image contrast, with the cores of the MNPs appearing darker than the silica shell due to the differing electron densities. The thickness of the silica shell could also be controlled effectively by ratios of TEOS and MNPs at the synthesis step (data not shown).

Low temperature disturbed the uptake of the magnetic nanoparticles into the cells

To confirm whether the uptake of the magnetic nanoparticles was mediated by energy-dependent endocytosis, the cells were incubated with MNP@SiO_2(RITC)s at 37°C and 4°C, respectively. As shown in Fig. 2, incubation of A549 cells

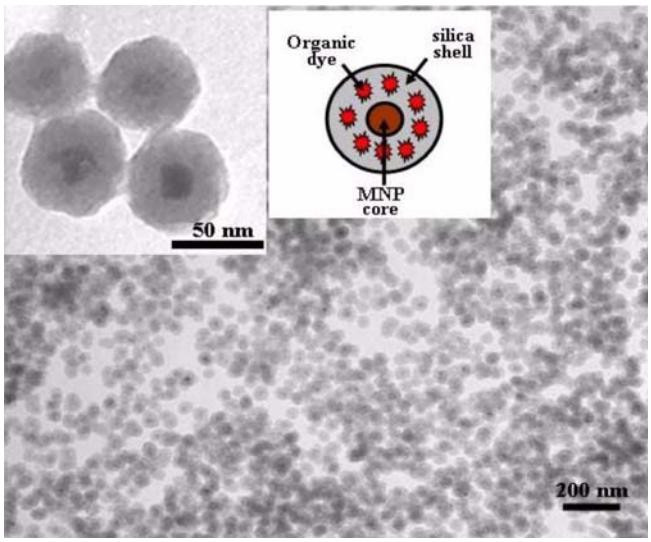

Fig. 1. Representative transmission electron micrograph (TEM) of cobalt ferrite magnetic-silica (core-shell) nanoparticles, MNP@SiO_2(RITC)s. The average size of the particle is approximately 50 nm. In the low magnitude image, the size distribution is revealed to be narrow (scale bar = 200 nm). In the high magnitude image (left insert image), core-shell structure is clearly shown by different image contrast, which shows the core of MNPs being darker than the silica shell due to electron density. The right insert image represents the detailed structure of MNP.

with MNP@SiO_2(RITC)s at 4°C significantly impeded uptake (Fig. 2B), while the uptake occurred successfully at 37°C (Fig. 2A). Together, our results demonstrate that uptake of MNPs into the cells requires an appropriate temperature.

Metabolic inhibitors prevented the delivery of the magnetic nanoparticles into the cell

Localization of MNP@SiO_2(RITC)s in A549 cells showed that uptake of the nanoparticles was energy-dependent. Compared to the cellular localization of MNP@SiO_2(RITC)s upon incubation at 37°C (Fig. 3A), incubations with several metabolic inhibitors, including sodium azide (Fig. 3B), sucrose (Fig. 3C), and bafilomycin A (Fig. 3D), did not deliver the magnetic nanoparticles into A549 cells.

Uptake of magnetic nanoparticles was mediated through endosomes

To confirm that the uptake of nanoparticles is mediated through endosomes, TEM analysis was performed. Nanoparticles were initially uptaken through the endosomes (Fig. 4A & B). The beginning of uptake occurred by the initiation of plasma membrane invagination. After being brought into the cells, the nanoparticles were found to be clumped in the lysosomes (Fig. 4C). The TEM study clearly revealed that nanoparticles were uptaken through the endosomes.

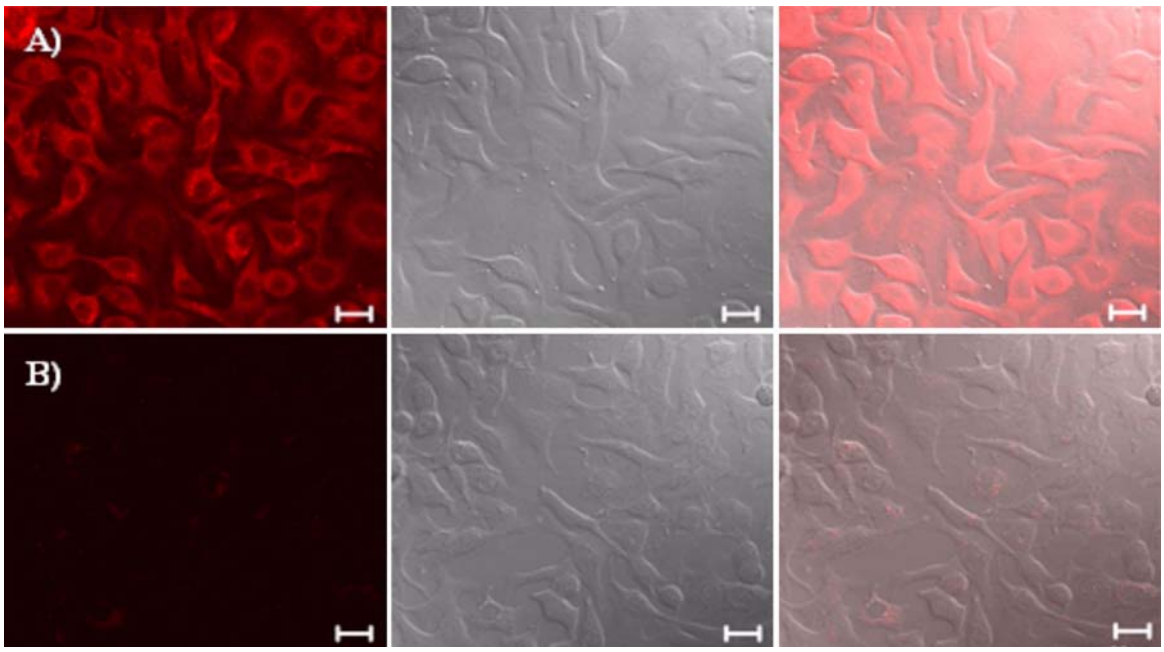

Fig. 2. Confocal laser scanning microscope (CLSM) images of MNP@SiO_2(RITC) uptake under low temperature conditions. In order to confirm whether nanoparticle uptake was possible at low temperature, A549 cells were incubated at 37°C and 4°C (after pre-incubation at 4°C for 10 min) for 30 min. Concentration of magnetic nanoparticles is 0.4 mg/ml, and the uptake pattern was observed by CLSM. A549 cells were incubated at 37°C (A) and 4°C (B) for 30 min. The left panel shows the fluorescence image (emission spectrum is 488 nm.), the middle panel shows the optical microscopic image, and the right panel shows the images merged together. bars = 20 μm.

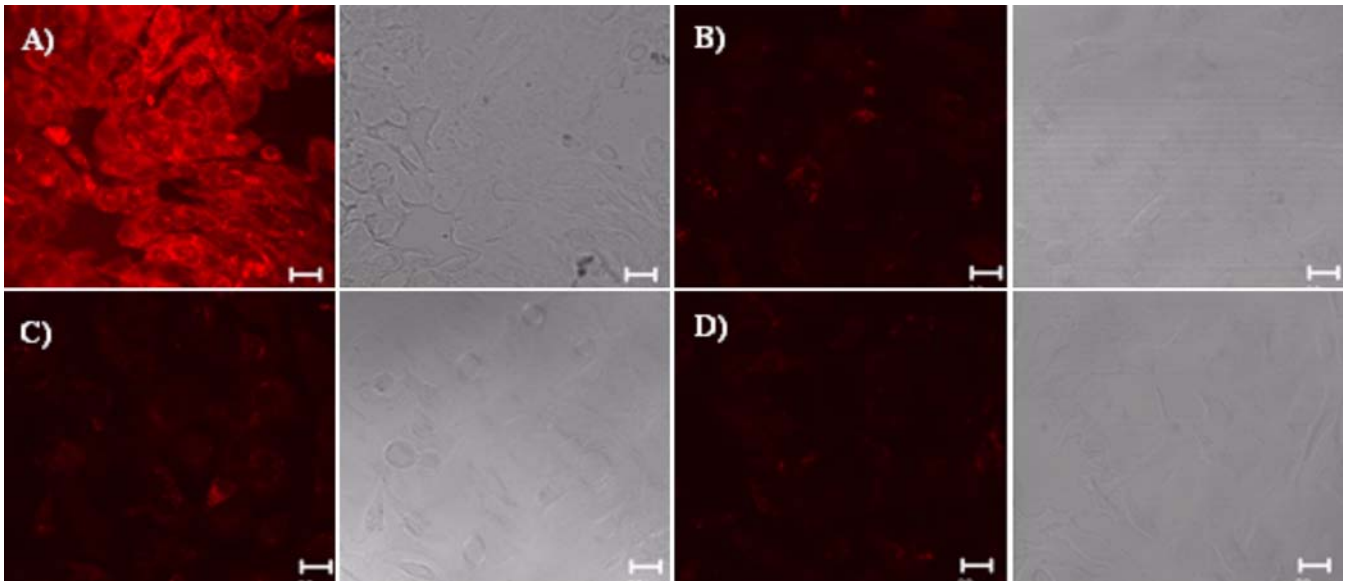

Fig. 3. Confocal laser scanning microscope (CLSM) images of MNP@SiO_2(RITC) uptake in the presence of several metabolic inhibitors. To confirm whether MNP uptake was possible under metabolic inhibition, A549 cells were co-incubated with MNP@SiO_2(RITC)s and metabolic inhibitors. (A) A549 cells were incubated with the magnetic nanoparticles only, (B) A549 cells pretreated with 0.1% sodium azide, (C) 0.45 M sucrose, or (D) 0.05 mM bafilomycin A were incubated with the nanoparticles at 37°C for 30 min. The left panel shows the fluorescence image (emission spectrum is 488 nm), and the right panel shows the optical microscopic image. bars = 20 μm.

Discussion

Nanoparticles have been considered as effective delivery vehicles, and have been studied extensively for the purpose of delivering drugs/genes into cells of interest [12]. In fact, targeted entry into cells is an important area of research in drug and gene delivery. Thus, site-specific delivery of drugs and therapeutics can significantly reduce the potential toxicity of a drug and increase its therapeutic effects. To maximize the efficiency of nanoparticle-mediated gene delivery, one should have detailed information regarding how the nanoparticles can translocate into the cells. With such information available, efficient and specific nanoparticle-mediated drug or gene delivery systems may be possible. In this study, we hypothesized that internalization and transport processes were responsible for cellular uptake of MNPs. To test this hypothesis, MNPs were synthesized and labeled by fluorescence. The fluorescence label, RITC, was stable enough not to be photobleached due to the aid of a SiO_2 overlayer. Recent line of evidence indicating that core-shell silica methodology provides the strongest photostability [10] also supports our finding. Photostability is a particularly important criterion when using nanoparticles as fluorescent markers in complex biological environments, where it is desirable to observe markers for extended periods of time against the background of intrinsic cellular emissions. With the discovery of such biocompatible and photostable nanoparticles, diverse studies for assessing cellular and biological fates may be possible.

Since nanoparticle uptake into cells could go through different processes, including phagocytosis and endocytosis, we performed several studies using metabolic inhibitors. Internalization of MNPs was halted completely at 4°C. The results clearly demonstrated that MNPs entered the cells in an energy-dependent manner, and this uptake was influenced by temperature. Sodium azide is widely used both *in vivo* and *in vitro* as an inhibitor of cellular respiration. It acts by inhibiting cytochrome C oxidase, the last enzyme in the mitochondrial electron transport chain, and thereby produces a drop in intracellular ATP concentration [15]. The uptake of MNPs into lung cells pretreated with sodium azide was completely blocked, thus suggesting that the uptake mechanism occurs through an energy-dependent process.

Clathrin-coated pits are the primary plasma membrane specialization involved in the uptake of a wide variety of molecules by endocytosis [11]. Two broad functions have been attributed to these regions of membrane: (a) molecular determinants associated with the clathrin lattice may cause receptors to become clustered; and (b) the clathrin lattice may somehow control the invagination of the membrane to form endocytic vesicles [5]. To understand the molecular mechanisms underlying these two aspects of coated pit function, one approach is to search for treatments that inhibit endocytosis, and to then characterize the effects of these treatments on coated pit function. One such treatment for the inhibition of endocytosis is to expose cells to hypertonic media [8]. In this study, hyperosmotic 0.45 M sucrose was utilized to suppress the coated pit function. The results

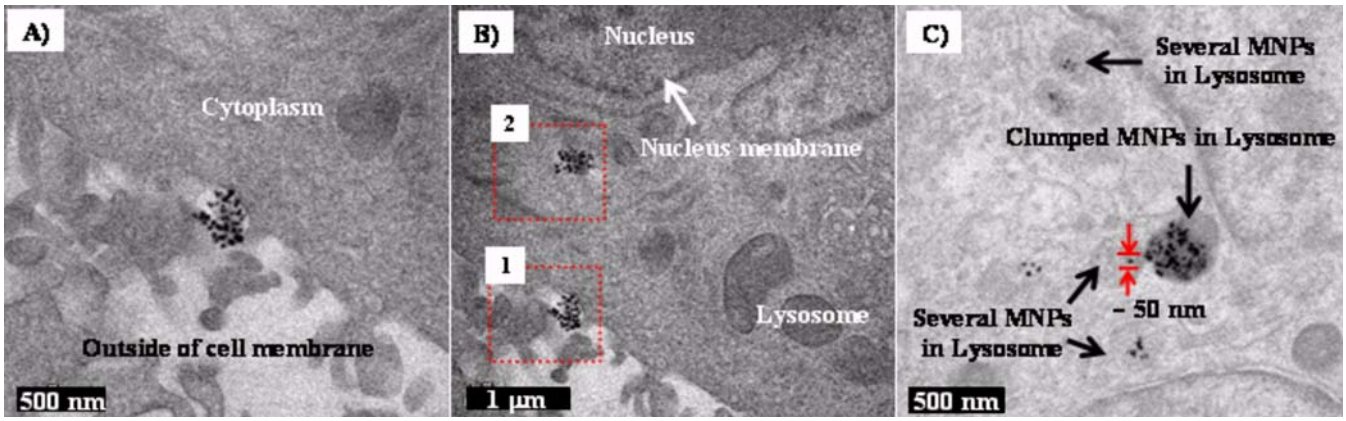

Fig. 4. Representative transmission electron micrographs (TEM) of A549 cells treated with MNP@SiO_2(RITC). To elucidate the detailed information of MNP uptake through the endosome-lysosomal mechanism, TEM was performed. The treated cells were fixed with glutaraldehyde, paraformaldehyde, and osmium tetroxide, and were subsequently embedded with epoxy resin. Thin sections were cut using an ultramicrotome; sections were not stained with any reagent for detecting of uptake of nanoparticles into the cells. Images were collected using a transmission electron microscope and a digital camera. (A) Uptake of MNPs was initiated upon the invagination of the plasma membrane. (B) Some nanoparticles had already been internalized into the cells (solid line box 2), while some cells still in the process of uptake at the plasma membrane (solid line box 1). (C) Uptaken MNP@SiO_2(RITC)s were trapped inside the lysosome.

suggest that uptake of MNPs occurred through clathrin-mediated endocytosis in A549 cells.

Vacuolar-type A ATPase (V-ATPase) is a complex, heteromultimeric protein consisting of a peripheral, catalytic V1 complex and a membrane-bound, ion-translocating Vo complex. V-ATPases in eukaryotes appear to be exclusive proton pumps that energize intracellular membranes of all cells as well as plasma membranes in a variety of mammalian cells [16]. V-ATPase plays crucial roles in many cellular processes, and may also be involved in diseases such as cancer [9]. Bafilomycin A, a plecomacrolide antibiotic containing a 16-membered lactone ring, was reported to be a specific inhibitor of V-ATPase [13]. Pretreatment with bafilomycin A completely suppressed the uptake of MNPs, thus indicating that uptake occurred through V-ATPase-dependent transport. Energy-dependent endocytic uptake of the MNPs was shown by TEM study. At the beginning of uptake, coated pits of the plasma membrane wrapped the nanoparticles and brought them into the cell. Together, our results clearly demonstrated that MNPs translocated the cells through energy-dependent endocytosis.

Chemical transfection is typically based upon a two-compartment system where the carrier, or transfection reagent, is complexed with DNA. Regardless of the transfection reagent, DNA is condensed by a cationic moiety, which protects the DNA in the extracellular environment and masks the charge of DNA to allow cellular uptake. Efficient DNA transfection, therefore, is critical for biological research. Our studies strongly suggest that the MNPs can be used as transfection agents because they allow for effective, energy-dependent endocytic uptake to occur. Our findings can be further supported by other researchers who have investigated silica nanoparticles as a stand-alone transfection reagent [3]. Since the chemistry of magnetic nanoparticles may affect cellular internalization as well as complex formation with drugs- or genes-of-interest, additional research regarding the optimal modification of surface of the MNPs is needed.

In conclusion, our results clearly demonstrate that MNP@SiO_2(RITC)s can be translocated into the cells through an energy-dependent endosomal-lysosomal mechanism. Moreover, our data strongly suggest that the MNPs can be used as transfection reagent. New methods designed to utilize our MNPs in broad applications such as transfection, bioimaging, and biosensor technologies without added toxicity is currently in development by our group.

Acknowledgments

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