The effect of surface charge on the uptake and biological function of mesoporous silica nanoparticles in 3T3-L1 cells and human mesenchymal stem cells

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Abstract

Cellular uptake of nanoparticles for stem cell labeling/tracking is considered as the most promising method. Recently mesoporous silica nanoparticles (MSNs) are emerging as an ideal agent for efficient stem cell labeling. The objective of this study was to evaluate the effect of surface charge on the highly efficient cellular uptake and in vitro cytotoxicity of MSNs in human mesenchymal stem cells (hMSCs). The surface charge was varied by the degree of surface modification with *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride and the uptake of MSNs was detected by flow cytometry. 3T3-L1 cells were also used to compare the uptake behavior of MSNs between cell types. A clear correlation of positive surface charge and the number of fluorescence-labeled cells was mainly observed in 3T3-L1 cells. In both cells, uptake of unmodified MSNs was inhibited by phenylarsine oxide (PAO) and cytochalasin D (Cyt D) suggesting a clathrin- and an actin-dependent endocytosis were involved. With strongly positive-charged MSNs, the inhibitory effects were observed in 3T3-L1 cells but not in hMSCs. Without regard to the surface charge, uptake of MSNs into both cells did not affect their viability, proliferation, and differentiation. Our results show that MSNs uptake by hMSCs can be regulated by a threshold of positive surface charge but also imply that the modulation of surface charge on MSNs uptake is specific to cell type.

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1. Introduction

Mesoporous silica nanoparticles (MSNs), in the form of stable aqueous dispersion, are emerging as an ideal agent for biomedical applications [1–6]. The properties of high surface area, large pore volume, and uniform porosity

make MSNs easily multi-functional. We are developing MSNs as a platform for stem cell tracking. Tracking the distribution of stem cells is crucial to their therapeutic use. To date, cellular labeling with magnetic and fluorescent nanoparticles is considered as the most promising methods [5,7–10]. The applications certainly require the stem cells to internalize the nanoparticles efficiently. Hence, methods for improving the capturing efficiency of MSN-carriers and associated cargos into the targeted cells are important.

Considerable efforts have been devoted to improving the labeling efficiency by the attachment on the nanoparticle surface with protein transduction domains (PTDs) [7,11], by utilizing transfection agents [12], or by modifying the surface

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with new coating materials or chemical functionalization [13,14]. However, surface characteristics of nanoparticles not only determine the uptake efficiency of these materials [13–16] but also may play an essential role in evoking the deleterious effects on cellular functions. Gupta et al. [13] have shown that pullulan coated superparamagnetic iron oxide nanoparticles (Pn-SPION) were non-toxic and induced changes in cytoskeleton organization different from that observed with SPION in human dermal fibroblasts. Huang et al. [17] showed that the degree of deacetylation had a greater influence than molecular weight on the uptake and cytotoxicity of chitosan nanoparticles in A549 cells. However, study about the effects of surface characteristics of nanoparticles in human stem cells is lacking. For stem cell tracking, the biological effects of the internalized nanoparticles surely have higher priority over other considerations. Recently, we have shown the MSNs without specific transfection agent can be efficiently internalized into human mesenchymal stem cells (hMSCs) while cell viability, growth, and differentiation are not affected. On the other hand, silica particles are rather inert and hence are suitable for studying the influence of surface functionalization.

In this study, we would like to evaluate the effect of surface charge on the highly efficient cell-uptake of MSNs and to elucidate the uptake mechanism. MSNs were modified with positively charged *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride (TMAC). 3T3-L1 and hMSCs were chosen for comparing the uptake behavior of MSNs between cell types. Furthermore, we investigated the effects of different charge profiles of MSNs on the cytotoxicity and differentiation capacity to evaluate the potential of the charge-modification for improving the labeling efficiency and subsequently in vivo stem cell tracking.

2. Materials and methods

2.1. Nanoparticle preparation

Red emitting rhodamine B dye (RITC) attached MSNs (RITC-MSNs) with well-ordered hexagonal pore structure were synthesized by a method described in a previous study [3] except replacing FITC with RITC. Nanoparticles with various surface charge were synthesized by modification of the extracted RITC-MSNs with different amount of *N*-trimethoxysilylpropyl-*N,N,N*-TMAC (Gelest). Twenty milliliters ethanolic RITC-MSNs suspension, corresponding to 125 mg particle in dry weight, and various amount of TMAC (0.14, 0.28, 1.4 mmol) were stirred under reflux for 6 h. Products were collected by centrifuging, washed with deionized water and ethanol. Solid products were obtained by centrifuge and dried in the centrifuge tube at 60 °C overnight for further characterization.

2.2. Characterization of RITC-MSNs

Powder X-ray diffraction (XRD) experiments were performed on a Philips X' Pert PRO system using a Cu K_{α} radiation source. Nitrogen adsorption-desorption measurements were carried in a Micromeritics ASAP 2010 device at 77 K. The surface area, pore volume, and pore size were obtained using Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) methods, respectively. Zeta potential was generated

using Malvern Zetasizer 3000 HS. Particle size was estimated by dynamic light scattering (DLS) with laser particle analyzer LPA-3100. The morphology of mesoporous nanoparticles was characterized with transmission electron microscope (JEOL-JSM-1200EX II operating at an acceleration voltage of 80 kV). FTIR spectra were collected with a Nicolet FT-IR 380.

2.3. Cell culture

hMSCs were isolated from bone marrow of normal donors as described in a previous study [1]. 3T3-L1 cells, a mouse embryonic cell line with differentiation activity to adipocyte, were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in regular growth medium consisting of high-glucose DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 100 U/ml penicillin, and 100 µg/ml streptomycin. All cultures were kept in an atmosphere of 5% CO₂ and 95% air at 37 °C.

2.4. Flow cytometry analysis

The determination of RITC-MSNs uptake by flow cytometry was performed as follows. hMSCs and 3T3-L1 cells were seeded at 1.2×10^5 cells per well in 6-well plates and allowed to attach for 24 h. To determine the RITC-MSNs uptake and loading extent, the cells were incubated with 100 µg/ml of RITC-MSNs suspension in serum-free medium for 1 h. Treated cells were then washed three times with phosphate-buffered saline (PBS), and then harvested by trypsinization. The red emitting rhodamine B dye incorporated in RITC-MSNs serves as a marker to quantitatively determine their cellular uptake analyzed by FACSCalibur flow cytometry and CellQuest Pro software (Becton Dickinson, Mississauga, CA). To study the mechanism of RITC-MSNs uptake, cells were incubated with various pharmacological inhibitors (Sigma-Aldrich), including PAO, filipin, Cyt D and nocodazole, and with a suspension of RITC-MSNs (100 µg/ml) for 1 h before flow cytometry determination.

2.5. Cell viability and proliferation assay

In vitro cell viability experiments were performed as follows. After incubation with 100 µg/ml of RITC-MSNs suspension in serum-free medium for 2 h, cells were incubated with fresh serum-free medium containing 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 1 h at 37 °C for cytotoxicity assay. For proliferation assay, the cells after treatment with 100 µg/ml of RITC-MSNs for 1 h were allowed to grow in regular growth medium for 24 h followed by incubation with MTT reagent. The dark blue formazan dye generated by the live cells was proportional to the number of live cells and the absorbance at 570 nm was measured using a microplate reader. Cell numbers were determined from a standard plot of known cell numbers vs. the corresponding absorbance density.

2.6. In vitro differentiation and cytochemical assay

The effects of RITC-MSNs uptake on cellular differentiation were performed as follows. To induce adipogenic differentiation, hMSCs and 3T3-L1 cells were first incubated with 100 µg/ml of RITC-MSNs for 1 h followed by PBS wash, and then cultured in adipogenic medium or regular growth medium for 14 days. Medium changes were carried out twice weekly. Adipogenic medium consists of high-glucose DMEM supplemented with 0.5 mM isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), 10 ng/ml insulin (Sigma-Aldrich), 50 µM indomethacin (Sigma-Aldrich), and 10% FBS. To induce osteogenic differentiation, hMSCs were incubated first with 100 µg/ml of RITC-MSNs for 1 h followed by PBS wash, and then treated with osteogenic medium for 7 days, changing the medium twice weekly. Osteogenic medium consists of α -MEM (GIBCO) supplemented with 1 µM dexamethasone, 50 µM β -glycerolphosphate (Sigma-Aldrich), and 50 µg/ml

ascorbic acid (Sigma-Aldrich). Adipogenic differentiation was assessed by the cellular accumulation of neutral lipid vacuoles after cells were fixed with 4% formaldehyde, stained with oil-red O (Sigma-Aldrich). For evaluation of osteogenic differentiation, cells were fixed with citrate buffered acetone and stained with Fast Blue RR Salt (Sigma-Aldrich) for detecting the alkaline phosphatase activity.

2.7. Statistical analysis

Data are presented as the mean $\pm$ standard error of mean (SEM) for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by a *t*-test and *P*-values less than 0.05 were considered significant.

3. Results and discussion

3.1. Characterization of MSNs

RITC-MSNs, were synthesized for this purpose following the method described in our previous study [3]. After removal of surfactants with acidic ethanol (complete removal of surfactants as indicated by FTIR which is not shown), RITC-MSNs were surface modified with various amount of TMAC in ethanol. The products were characterized with TEM, XRD, N_2 adsorption–desorption, zeta potential and elemental analysis. The zeta potential became more positive as the extent of modification increased and four samples of RITC-MSNs were obtained as: uRITC-MSN (unmodified), wRITC-MSN (weakly positive-charged), mRITC-MSN (moderately positive-charged) and sRITC-MSN (strongly positive-charged). Table 1 summarizes the properties of as-synthesized RITC-MSNs (suspended in PBS, pH = 7.35). TEM (Fig. 1) shows the well-organized mesoporous structure remained after modification and the average particle size around 100 nm. Typical MCM-41 XRD patterns (Fig. 2) were observed for all samples, which exhibited three hexagonal characteristic reflection peaks (100), (110), and (200). The d_{100} spacing of extracted RITC-MSN was 4.1 nm. The physical properties of these MSNs were analyzed by N_2 adsorption–desorption isotherms (Fig. 3 and Table 1) and the surface area (1050–780 m²/g), pore volume (0.87–0.55 cm³/g), and BJH pore size distribution (2.6–2.0 nm) were obtained. All four RITC-MSNs exhibited characteristic type IV BET isotherms and no hysteresis

loop was observed. The surface area, pore volume, and pore size decreased as the extent of modification increased.

3.2. Effects of surface charge on the cellular uptake of RITC-MSNs

As RITC-MSNs contained the fluorescent dye, the degree of MSNs uptake was quantified with flow cytometry by determining the red fluorescence emitted from RITC-MSNs-treated cells. The logarithmic fluorescence intensity of untreated cells was set between 10⁰ and 10¹, hence cells that exhibited red fluorescence intensity higher than 10¹ were taken as positively-labeled cells with RITC-MSNs. After incubation with various RITC-MSNs at the same concentration of 100 μ g/ml for 1 h, most of hMSCs were labeled. For example, in the case of uRITC-MSN, more than 80% of hMSCs and about 70% of 3T3-L1 cells were labeled with uRITC-MSN. Typical flow cytometry plots of hMSCs (top panels) and 3T3-L1 cells (bottom panels) were shown in Fig. 4A. The data suggested highly efficient cellular labeling of MSNs in hMSCs in agreement with our previous report [1]. When comparing the uptake efficiency with the zeta potential of various RITC-MSNs,

Fig. 1. Transmission electron micrograph (TEM) of sRITC-MSN.

Table 1
Characterization of different-charged RITC-MSNs

Samples	S_{BET} (m ² /g) ^a	W_{BJH} (nm) ^a	V_{P} (cm ³ /g) ^a	Quaternary ammonium (mmol/g) ^b	Zeta potential ζ (mV) ^c	Particle size (nm) ^d
uRITC-MSN	1050	2.6	0.87	—	−4.90	115
wRITC-MSN	980	2.4	0.84	0.29	+7.90	110
mRITC-MSN	930	2.2	0.73	0.98	+14.6	111
sRITC-MSN	780	2.0	0.55	1.7	+19.0	108

^aNitrogen adsorption–desorption measurements were carried in a Micromeritics ASAP 2010 device at 77 K. The surface area, pore volume, and pore size were obtained using Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) methods, respectively.

^bThe content of quaternary ammonium was determined by elemental analysis.

^cThe zeta potential of RITC-MSNs (suspended in PBS, pH = 7.35) was generated using Malvern Zetasizer 3000 HS.

^dThe particle size of RITC-MSNs suspended in D.I. water was estimated by dynamic light scattering (DLD) with laser particle analyzer LPA-3100.

Fig. 2. XRD patterns of differently charged RITC-MSNs.

Fig. 3. BET nitrogen adsorption-desorption isotherms (a) and BJH pore size distributions (b) of differently charged RITC-MSNs.

a positive correlation between positive surface charge and the number of labeled cells was observed for 3T3-L1 cells. As shown in Fig. 4B-a, there is about 15 percent increase

from uRITC-MSN to sRITC-MSN on the labeled 3T3-L1 cells. In contrast, though one can see some increase of cell-uptake in sRITC-MSNs, the differences in uptakes among uRITC-MSN-, wRITC-MSN-, mRITC-MSN- and sRITC-MSN-treated hMSCs were not significant (Fig. 4C-a). It appears that for hMSCs the cellular uptake of MSNs were so highly efficient that the improvement of labeling efficiency induced by surface charge only shows for the highly positively charged MSN. The different profiles of the uptake improvement by surface charge in various cell types agree with a recent report which showed a clear correlation of surface charge of polystyrene nanoparticles and fluorescence intensity in different cell lines with the exception of MSCs [16]. However, in both cells, as shown in Fig. 4B-b and C-b, no significant correlation between positive surface charge and the mean fluorescence intensity (MIF) was observed. The data suggested that MSNs have a high cellular labeling efficiency and subsequently the positive surface charge had negligible effect on the uptake efficiency of MSNs.

3.3. Effects of surface charge on the mechanism of RITC-MSN uptake

Our previous study showed that internalization of FITC-MSNs is mediated by clathrin- and actin-dependent endocytosis in both hMSCs and 3T3-L1 cells. To further investigate the effect of surface charge on the mechanism of endocytosis, we tested the cellular uptake of these different MSNs in the presence of various pharmacological inhibitors as described below. PAO, a trivalent arsenical, inhibits clathrin-mediated endocytosis by reacting with vicinal sulfhydryls to form stable ring structures [18,19]. Filipin interacts with 3- β -hydroxysterols in the plasma membrane to form filipin-sterol complexes and subsequently cause the filamentous caveolin-1-coat to rapidly disassemble which leads to inhibition of caveolae-mediated endocytosis [20]. Cyt D, a potent inhibitor of actin polymerization, was used to inhibit actin-related phagocytosis and non-clathrin-non-caveolae-dependent endocytosis [21,22]. Nocodazole disrupts microtubules by binding to β -tubulin and preventing the formation of one of the two interchain disulfide linkages, thus inhibiting microtubule dynamics and blocking endocytotic vesicle trafficking [21–23]. For hMSCs using uRITC-MSNs-treated cells as control, the cellular uptake of uRITC-MSN, wRITC-MSN and mRITC-MSN were inhibited by PAO and Cyt D (Fig. 5A and B) but neither by filipin nor by nocodazole (Fig. 5C); this suggested a clathrin- and an actin-dependent endocytosis were involved in the RITC-MSNs uptake. The inhibition of particle uptake was dependent on the concentration of inhibitors used. Interestingly, the uptake of sRITC-MSN was not inhibited by any of the above inhibitors (Fig. 5A–C). It is reasonable that the insusceptibility of the sRITC-MSN uptake to the endocytotic inhibitors in hMSCs was induced by a yet unrevealed mechanism; its onset depends on a threshold of positive

Fig. 4. (A) Flow cytometry determination of RITC-MSNs uptake in hMSCs (top panels) and 3T3-L1 cells (bottom panels). The number of positively labeled cells was represented as the percentage of total counting cells in each panel. The effect of zeta potential on the cell numbers of RITC-MSN-uptake in 3T3-L1 cells (B-a) and hMSCs (C-a). Data are expressed as mean $\pm$ SEM of three to four independent experiments. *** $P < 0.001$ as compared with uRITC-MSN (control). The effect of zeta potential on the mean fluorescence intensity (MIF) of RITC-MSN-labeled cells in 3T3-L1 cells (B-b) and hMSCs (C-b). Data are expressed as mean $\pm$ SEM of three to four independent experiments as compared with uRITC-MSN (control).

surface charge. However, with 3T3-L1 cells, the uptakes of those RITC-MSNs were generally inhibited by clathrin (Fig. 5D) but neither by filipin nor nocodazole (data not shown), suggesting a clathrin-dependent mechanism was still responsible for MSNs uptake regardless of their

surface charge. These data not only indicated that the uptake of MCM-41-type MSNs by hMSCs can be regulated by a threshold of positive surface charge but also implied that the modulation of surface charge on MSNs uptake is specific to cell type.

Fig. 5. The effects of various inhibitors on RITC-MSNs uptake. Cellular uptake at a single dose of different-charged RITC-MSNs: (A) co-treatment with PAO and (B) co-treatment with Cyt D inhibited the uRITC-MSN, wRITC-MSN and mRITC-MSN dose-dependently but not the sRITC-MSN uptake in hMSCs. (C) Co-treatment with either filipin or nocodazole did not inhibit any of RITC-MSNs uptake in hMSCs. (D) In 3T3-L1 cells, PAO inhibited all of RITC-MSNs uptake. Data are expressed as mean $\pm$ SEM of three to four independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001 as compared with respective RITC-MSN control.

3.4. The biocompatibility of surface charge modification of MSNs

Besides the uptake efficiency, the biocompatibility of nanoparticles is another key issue for ideal cellular labeling and tracking, especially for stem cells. To examine the biocompatibility of RITC-MSNs with different degree of positive surface charge, cell viability was examined by MTT reduction assay. Cytotoxicity was not observed at 2 h after treatment with different surface-charged

RITC-MSNs (Fig. 6A); nor was the cell proliferation affected by any of these RITC-MSNs after hMSCs were incubated in growth medium for 24 h (Fig. 6B). In addition, we investigated the differentiation potential of labeled hMSCs into adipocytes and osteocytes to examine possible adverse effects of surface charge on the functions of stem cells. Adipogenic differentiation with the formation and accumulation of lipid vacuoles and osteogenic differentiation with alkaline phosphatase activity were observed by oil-red O stain and Fast Blue stain,

Fig. 6. Cellular effects of positively surface charged RITC-MSNs on acute cytotoxicity (A), proliferation (B) and differentiation (C) in hMSCs. The cell viabilities of hMSCs incubated with a dose (100 µg/ml) of differently charged RITC-MSNs either for 2 h (A) or for 1 h followed by additional 24 h growth (B) were not affected as compared with untreated control cells. After 1-h incubation, neither adipogenic differentiations (C) nor osteogenic differentiations (D) were affected by any of RITC-MSNs.

respectively. As shown in Fig. 6C, adipogenic differentiation of hMSCs was only observed in cells grown in adipogenic medium but not in regular growth medium. hMSCs, treated with or without any of these different surface-charged RITC-MSNs, did differentiate into adipocytes, and there were no obvious differences in oil-red O stain assay between particle-treated cells and untreated control cells. Fig. 6D also showed that these RITC-MSNs

did not affect the osteogenic differentiation induced by incubating hMSCs with osteogenic medium. These data indicated that surface charge functionalization did not affect the biocompatibility of MSNs. Similar results of the biocompatibility experiments were also observed in 3T3-L1 cells as demonstrated by cytotoxicity assay, cell proliferation examination and adipogenic differentiation (data not shown).

4. Conclusions

In summary, we have demonstrated surface functionalization with different amounts of positively charged quaternary ammonium group is quite biocompatible and can enhance the uptake of MCM-41 type MSNs for more efficient cellular labeling. However, the charge effect of cell-uptake is cell type and surface charge dependent. At low surface charge, the normal clathrin- and actin-dependent mechanisms operate, which by themselves are already quite efficient for hMSC and 3T3-L1 cells. Above a certain threshold of surface charge, a new unrevealed charge-dependent mechanism starts to be effective for hMSC. The highly positive-charged MSNs with specific endocytosis pathways could serve as a new approach to study the uptake mechanism and to improve the application for stem cell tracking.

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