Efficient Labeling of Porcine Hematopoietic Cells by Fluorescence-Conjugated Nanoparticles

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ABSTRACT

Nanotechnology is currently receiving considerable attention in various fields of biotechnology. The uptake of nanoparticles by cells for labeling and tracking is a critical process for many biomedical therapeutic applications. However, nanoparticle labeling of porcine hematopoietic cells has not been demonstrated so far. In the present study, silica-coated nanoparticles conjugated with rhodamine B isothiocyanate (SR-RITC) were used to investigate the uptake of nanoparticles by porcine hematopoietic cells. Flow cytometric and confocal microscopic analyses reveled that the cells were efficiently internalized by the silica-coated nanoparticles. Furthermore, biocompatibility tests demonstrated that the SR nanoparticles were not cytotoxic, and they had no impact on proliferation. Our study demonstrates that silica-coated nanoparticles are taken up very rapidly and with high efficiency into porcine hematopoietic cells, with no apparent deleterious effects. Therefore, silica-coated nanoparticles appear to be a promising tool for tracking porcine hematopoietic cells.

(Key words : Silica-coated nanoparticles, Porcine hematopoietic cells, RITC, Biocompatibility, Confocal microscopy)

INTRODUCTION

Nanotechnology is currently receiving considerable attention in various fields of biotechnology and medicine, particularly those relating to gene therapy and drug delivery, imaging, disease diagnosis, and biomarker and biosensor applications (Moriguchi *et al.*, 2005; Iwasaki *et al.*, 2007; Ren *et al.*, 2008; Jain *et al.*, 2008; Wang, *et. al.*, 2008 ; Choi *et al.*, 2009). Nanoparticles, with their high surface-to-volume ratio and ultrasmall size, offer an alternative approach to obtaining biological information from within living cells, and are thus an excellent tool for biotechnology research. Numerous efforts have been devoted to developing new nanoparticles for biomedical applications, especially in regards to tracking cells (Huang *et al.*, 2005; Yang *et al.*, 2007). For example, protein transduction domains (PTDs), that promote the delivery of peptides or protein into cells by utilizing transfection agents, are often attached to nanoparticle surfaces to facilitate internalization (Heng *et al.*, 2005). Furthermore, surface modification with coating materials or chemical moieties has been shown to improve the likelihood of nanoparticle uptake (Bagwe *et al.*, 2006; Kamruzzaman *et al.*, 2007).

However, the efficiencies of cell labeling with nanoparticles, and their potential effects on biocompatibility, have not been investigated in porcine hematopoietic cells. For certain areas of biomedical research, including transplantation and human disease research, a large animal model has a number of advantages. Of the potentially suitable species, the pig has proven to be a particularly useful clinical xenograft donor (Ibrahim *et al.*, 2006; Cooper *et al.*, 2008) Advantages associated with the pig include its availability, blood characteristics, and its similarity to humans in terms of size, physiology, and immunology. Therefore, tracking of porcine hematopoietic cells, which are critical targets of tracking for xenotransplantation, is an important achievement. There have been numerous efforts to enhance the labeling efficiency of nanoparticles in cells for tracking (He *et al.*, 2004; Arbab *et al.*, 2005; Clement *et al.*, 2006; Chung *et al.*, 2007). While changes in the surface properties of nanoparticles can significantly increase uptake efficiency, they may also have deleterious effects on cellular function. Gupta *et al.* showed that non-toxic, pullulan-coated SPIO nanoparticles induced changes in the skeletal organization of dermal fibroblasts (Gupta *et al.*, 2004).

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Huang *et al.* have shown that silica-coated nanoparticles are efficiently internalized into human hematopoietic cells without inducing cytotoxic effect (Huang *et al.*, 2005). Moreover, the biocompatibility of nanoparticles makes them highly amenable to transplantation.

One aim of the present study was to determine how efficiently silica-coated nanoparticles were internalized by porcine hematopoietic cells. A second aim was to determine the biocompatibility of nanoparticles with these cells. To these ends we improved the versatility of silica-coated nanoparticles, with an organic fluorescent dye incorporated into the silica shell used to visualize and detect labeled porcine hematopoietic cells by flow cytometry or fluorescent imaging.

MATERIALS AND METHODS

Nanoparticles

Fifty-nm size nanoparticles labeled with RITC (Sigma-Aldrich) were synthesized according to a previously described method (Yoon *et al.*, 2005; Kim *et al.*, 2006). Trimethoxysilane modified by rhodamine B isothiocyanate (RITC) was prepared from 3-aminopropyltriethoxysilane (APS) and rhodamine B isothiocyanate. In a reaction vessel, 0.0938 g of the coupling agent APS (4.25×10^{-4} mol) was added to 0.1178 g of the fluorophore RITC (2.20×10^{-4} mol) with 10 ml of anhydrous ethanol. The reaction was performed under nitrogen, and by stirring for 17 h in the dark. The organosilica cores of the SR modified nanoparticles were synthesized under nitrogen in 40 ml of ethanol and 2.1 ml of ammonia (4.2% vol). Reagent concentrations were chosen to prepare spheres with a radius of approximately 50 nm. The reagent TEOS (0.75 ml, 0.0036 mol) was added by a funnel under the solution surface accompanied by strong mechanical stirring. The solution became turbid after a few minutes, and the synthesized nanoparticles were dispersed in ethanol and precipitated by ultra-centrifugation (18,000 rpm) for 3 h. The supernatant solution was then removed and the precipitated particles were redispersed in PBS.

Porcine Hematopoietic Cells

Pigs (7~12 weeks old), which were breeding at the National Institute of Animal Science, were used in this study. Porcine hematopoietic cells derived from peripheral blood of pigs were isolated following density gradient centrifugation at 2,000 rpm for 30 min on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). The cells were washed twice with PBS and suspended at a concentration of 10×10^6 cells/ml in PBS.

Flow Cytometry Analysis

The investigation of nanoparticle uptake efficiency was performed by flow cytometry analysis. Cells were incubated with 100 μg/ml of SR-RITC suspension in PBS for 1 h. The cells were washed three times with PBS and resuspended in staining medium containing 2% fetal bovine serum (FBS) and 0.1% sodium azide in PBS. Dead cells were excluded from the flow cytometric analysis by using the 5 μg/ml propidium iodide (PI) stain. The red emitting rhodamine B dye component of SR-RITC served as a marker for quantitatively determining cellular uptake, and was analyzed using FACS Vantage SE (Becton Dickinson, CA, USA). Flow cytometry data were analyzed using the CellQuest software.

Cell Viability and Proliferation Assay

We investigated the biological effects of nanoparticles on pig hematopoietic cells by assessing cellular viability with an MTT assay. Cells were incubated with 100 μg /ml SR-RITC suspension in PBS for 1 h, and then washed three times with a PBS-free solution. Potential cytotoxic effects were assessed by adding 10 μl of MTT solution to each well, incubating at 37℃ for 4 h, and then adding 100 μl of solubilization solution. We investigated proliferation as follows: Cells were incubated with 100 μg/ml SR-RITC for 1 h, grown in DMEM with a high-glucose medium(GIBCO), supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 24 h; they were then incubated with the MTT reagent. The dark blue formazan dye generated by living cells was used as a measurement of cell proliferation and activity, and was analyzed on a microplate reader (Bio-Rad Model 680, Richmond, CA, USA). All cultures were kept in an atmosphere of 5% CO_2 at 37℃.

Cellular Distribution of SR Nanoparticles

We investigated the intracellular distribution of SR-RITC. Cells were preincubated with SR-RITC for 1 h, washed three times with PBS, and visualized in serum-free media. Images were acquired with an LSM-519 META confocal microscope (Carl Zeiss, Germany).

Statistics Analysis

Results are expressed as mean±SD. All statistical analyses were preformed using students paired *t*-test. A value of $p<0.05$ is considered significant in comparison with the respective control groups.

RESULTS

Determination of SR Nanoparticle Uptake by Porcine Hematopoietic Cells

Porcine hematopoietic cells were exposed to biofunctional silica-shell nanoparticles that were modified with

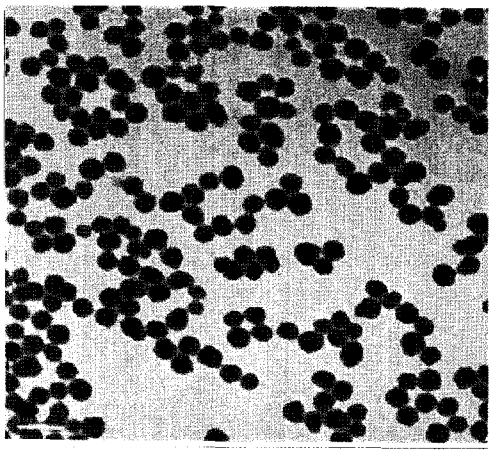


Fig. 1. TEM image of SR-RITC nanoparticles with an average size of 50 nm.

rhodamine B isothiocyanate (SR) and synthesized with an average core diameter of 50 nm (Fig. 1). Porcine hematopoietic cells derived from peripheral blood include immune cells that are used in xenotransplantation. Mononuclear cells were isolated, and incubated with 100 μg/ml of SR nanoparticles in serum-free, low-glucose, Dulbecco's modified Eagles's medium (DMEM) for varying periods of time, i.e., 0.5, 1, or 2 h. The cells were washed, and their efficiency of nanoparticle uptake analyzed by flow cytometry. Incubation of cells with SR nanoparticles for 1 h revealed a homogenous labeling of more than 67% of the porcine hematopoietic cells (Fig. 2A and B).

Having determined an appropriate time of incubation for labeling porcine hematopoietic cells, we investigated how nanoparticle concentration influenced uptake. Cells

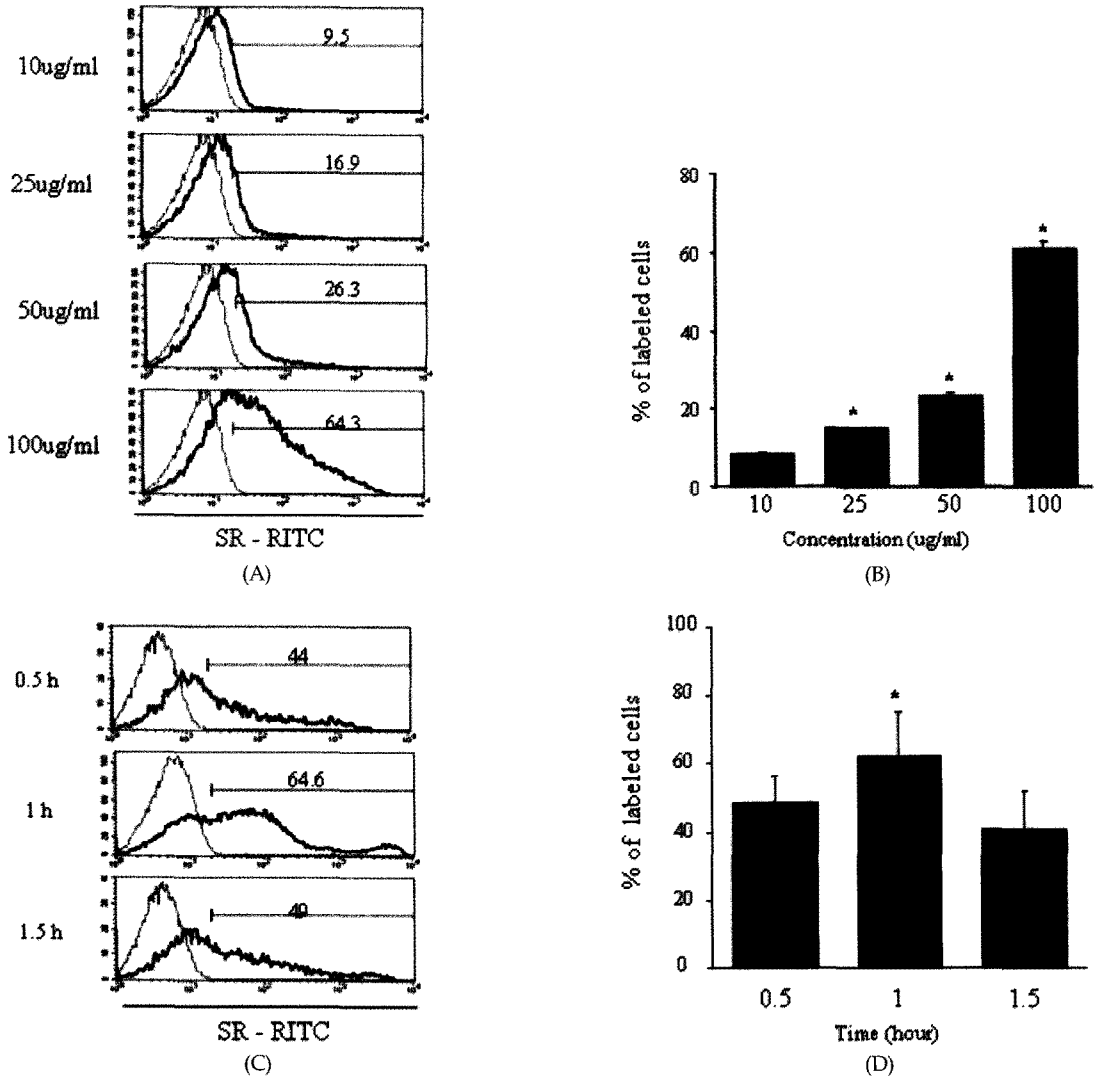
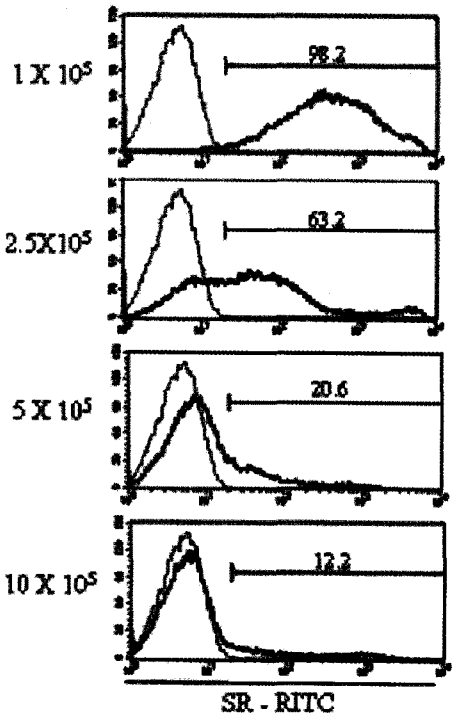


Fig. 2. Efficiency of cellular uptake of SR-RITC nanoparticles by porcine hematopoietic cells. (A) Flow cytometry data with 10, 25, 50 or 100 μg/ml dose of SR, (B) Percentage of positively labeled cells. (C) Flow cytometry data of time dependent uptake with 0.5, 1, 1.5 h, (D) Percentage of positively labeled cells.

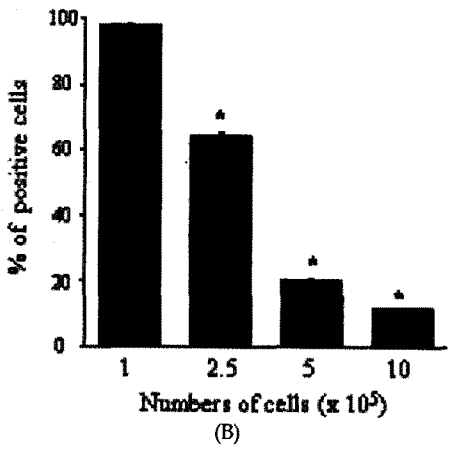
were incubated at 37℃ for 1 h in one of the following SR nanoparticle concentrations: 10, 25, 50, or 100 μg /ml. Analysis of uptake frequency and fluorescence intensity by flow cytometry revealed that the efficiency of SR nanoparticle uptake increase by dose dependent manner (Fig. 2A and B).

To further refine the conditions for optimal labeling we investigated effects associated with the number of cells exposed. Different concentrations of cells ranging from $1\sim10\times10^5$ were incubated with SR nanoparticles (100 μg/ml) for 1 h at 37℃. Flow cytometry analysis revealed that uptake efficiency increased as cell numbers decreased (Fig. 3A and B). The RITC-labeled porcine hematopoietic cells were readily detected by fluorescence microscopy.

Our initial results showed that the efficiency of nanoparticle uptake was both time- and concentration-dependent, with the optimal conditions being an incubation time of 1 h and a nanoparticle concentration of 100 μg/ml. These conditions were used for our subsequent experiments.



(A)



(B)

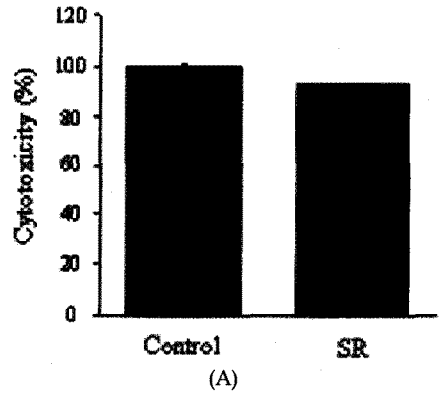
Fig. 3. Cell number dependent cellular uptake of SR nanoparticles. (A) Cellular uptake efficiency of SR with $1\sim10\times10^5$ porcine hematopoietic cells. (B) Percentage of SR positive cells.

Cytotoxicity and Effects of Nanoparticles

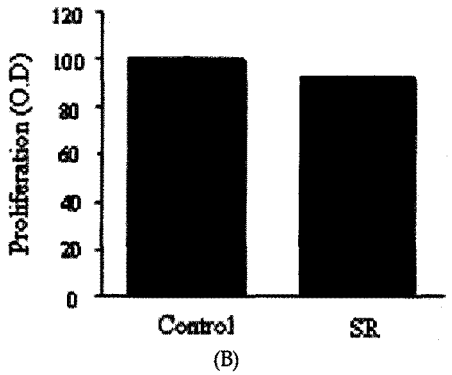
An essential characteristic of nanoparticles for labeling purposes, in addition to cellular uptake efficiency, is biocompatibility. Accordingly, we assessed whether labeling with SR nanoparticles influenced the bioactivity of porcine hematopoietic cells. Cells were incubated at 37℃ by treatment with SR nanoparticles (100 μg/ml) for 1 h and then determine cell viability after 24 h from incubation. MTT assay results revealed no apparent cytotoxicity or reduction in proliferation (Fig. 4A and B).

Intracellular Uptake of Nanoparticles

We observed highly efficient labeling of porcine hematopoietic cells with SR nanoparticles. We used CLSM to determine whether this labeling occurred via staining



(A)



(B)

Fig. 4. Effects of SR-RITC nanoparticles on porcine hematopoietic cells. Proliferation. (A) and cytotoxicity (B) were investigated with MTT assay after incubation with 100 μg/ml of SR nanoparticles for 24 h and 1 h, respectively.

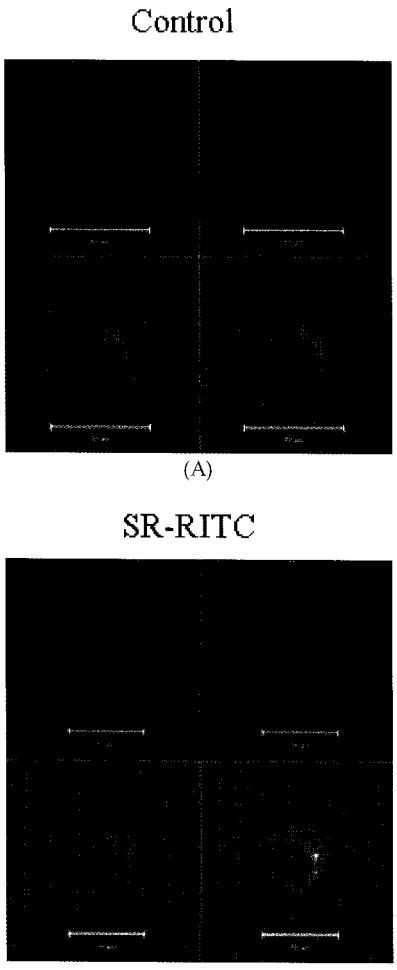


Fig. 5. Localization analysis of SR-RITC nanoparticles in porcine hematopoietic cells with confocal laser scanning microscopy.

of the cell membrane or entry into cells. Fig. 5 showed representative confocal images of labeled cells with DAPI nuclear counterstaining. Such data indicate that SR nanoparticles were internalized by porcine hematopoietic cells, primarily into the cytoplasm.

DISCUSSION

Nanoparticles have been used to successfully label and track cells in many species, including mice, humans, and non-human primates. We recently investigated the uptake efficiency of murine hematopoietic stem/progenitor cells for silanol-coated nanoparticles conjugated with RITC (SR) with an ionic surface charge to generate sol-gel silica. In previous study, we observed that SR nanoparticles were internalized by more than 97% of murine hematopoietic stem/progenitor cells under certain conditions, and were determined to be the most effective and biocompatible of the modified nanoparticles for cell labeling purposes.

However, there is no previous report on the labeling of porcine hematopoietic cells by nanoparticles that are efficiently internalized and biocompatible. In the present study, porcine hematopoietic cells were successfully labeled with silica-coated nanoparticles conjugated with the fluorescent rhodamine B isothiocyanate dye (SR). SR nanoparticles were effectively internalized into porcine hematopoietic cells in a concentration- and time-dependant manner. The internalization of SR nanoparticles occurred over very short time periods: 44% of labeling occurred within the first 30 min of incubation, with maximal labeling achieved after 1 h. Confocal microscopic analysis demonstrated that labeling involved internalization into the cytoplasm rather than cell surface staining. Moreover, the uptake of SR nanoparticles by porcine hematopoietic cells appeared to have no cytotoxic effects or impact on growth. These results, and the relatively simple methods involved, suggest that SR nanoparticles could be a powerful and safe tool for tracking porcine hematopoietic cells.

Taken together, SR nanoparticles used in this study may a powerful material for tracking of porcine hematopoietic cells *in vivo* in virtue of a very efficient and simple method without any adverse effects on cellular proliferation or cytotoxicity.

In summary, the present study demonstrated that SR nanoparticles were efficiently internalized by porcine hematopoietic cells over short incubation times. Under appropriate conditions, most porcine hematopoietic cells in a sample could be labeled. Furthermore, SR nanoparticles were not cytotoxic nor did they affect proliferation.

Our results suggest that SR nanoparticles could be used to effectively label porcine hematopoietic cells for imaging or tracking *in vivo*.

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