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Citraconylated exosomes for improved internalization into macrophages

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Abstract

Considering the close relation between macrophages and inflammatory diseases, the design of carriers for the delivery of drugs, genes, and small molecules into macrophages is crucial. In this study, the surface charge of exosome (EXO) was easily modified to highly negative charge by citraconylation. Prepared citraconylated EXO (cit-EXO) exhibited a significantly reduced surface charge down to − 50 from − 15 mV of EXO surface charge, despite similar hydrodynamic size. In the absence of serum proteins, both EXO and cit-EXO were similarly internalized into RAW264.7 cells and DC2.4 cells. However, cit-EXO exhibited superior intracellular uptake to that of EXO for RAW264.7 cells in the presence of serum proteins because of highly negative charges. However, there were no significant differences in intracellular uptake of EXO and cit-EXO for DC2.4 cells. Taken together, simple surface modification onto EXOs via citraconylation improved delivery of nanosized EXO (~ 50 nm) into macrophages, which could serve as a promising strategy for the development of carriers for efficient macrophage delivery.

Keywords: Macrophage, Exosome, Citraconic anhydride, Surface charge, Intracellular uptake

Introduction

Inflammatory processes have been considered crucial for the progression and pathogenesis of diseases, e.g., chronic obstructive pulmonary disease, asthma, atherosclerosis, cancer, tuberculosis, human immunodeficiency virus, and leishmaniasis [1–5]. Because the mononuclear phagocytic system (MPS) has pivotal roles during inflammation, the MPS has been examined as an important target for drug delivery to treat diseases [6]. In the MPS, monocytes, macrophages, and dendritic cells engulf and destroy apoptotic cells, pathogens, and other targets by phagocytosis via complement receptors, Fc-receptors, and an opsonin receptor-dependent mechanism [7]. Accordingly, the development of efficient delivery systems to target macrophages is crucial as a potential carrier to deliver anti-inflammatory drugs and lessen a wide range of inflammatory diseases.

Diverse particulate carriers, including liposomes, inorganic nanoparticles, and polymeric nanoparticles, have been fabricated to deliver small molecules, genes, and peptides to macrophages [8, 9]. However, several challenges, including particle stability, biocompatibility, and feasibility, still must be addressed [10]. Recently, biologically derived vesicles, e.g., cell-derived vesicles and microvesicles, have been intensively investigated as one of the alternatives for synthetic particulate systems. In particular, exosomes (EXOs) have been considered promising drug carriers because of their aqueous stability, homogeneous diameter, and biocompatibility [11–13]. In a previous study, serum derived EXOs had a narrow size distribution of approximately 50 nm as well as high production yield [14]. Accordingly, surface modification of EXOs has been examined to improve their delivery efficiency, targetability, and easy purification [15–18]. However, the effects of physicochemical properties of EXOs, such as surface charge, size, and shape, on intracellular uptake according to different types of cells have not been studied extensively.

In this study, the surface of EXO was modified with citraconic anhydride via Michael addition chemistry to examine the effects of surface charges on intracellular uptake of EXO for macrophages. EXOs were citraconylated at different citraconic anhydride/surface amine group molar ratios. Citraconylated EXO (cit-EXO) was

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analyzed by dynamic light scattering (DLS), zeta-sizer, and agarose gel retardation to confirm the change of surface charges. After labeling of EXO and cit-EXO with indocyanine green (ICG), the level of intracellular uptake was examined for two types of cell: macrophages (RAW264.7) and dendritic cells (DC2.4). To investigate the effects of serum proteins on cellular uptake to macrophages and dendritic cells, cellular uptake was performed not only for serum-free media but also for serum-containing media.

Materials and methods

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin/streptomycin (P/S), MEM nonessential amino acids solution, and 2-mercaptoethanol were purchased from Gibco BRL (Grand Island, NY, USA). An ExoQuick-TC EXO precipitation kit was obtained from System Biosciences, Inc. (Mountain View, CA, USA). A micro-BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). Tetrabutylammonium iodide (TBAI), citraconic anhydride, and fluorescamine were obtained from Sigma Aldrich (St. Louis, MO, USA). Indocyanine green (ICG, $M_w = 775$ Da) was purchased from Dongindang Pharmaceutical (Siheung, Gyeonggi, Korea). A desalting column (MWCO = 40 kDa) and GelCode Blue Stain Reagent were purchased from Thermo Scientific (Rockford, IL, USA). Nanosep centrifugal devices with omega membranes (Nanosep, MWCO = 300 K) were purchased from Pall (Ann Arbor, MI, USA).

Cell culture

RAW264.7 cells (murine macrophage cell line) were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 [14]. DC2.4 cells were donated by Prof. Kenneth L. Rock [19]. DC2.4 cells (murine dendritic cell line) were maintained in RPMI-1640 supplemented with 10% FBS, 100-U/mL penicillin, 100-μg/mL streptomycin, 1% nonessential amino acid, and 270 nM 2-mercaptoethanol at 37 °C in a humidified atmosphere of 5% CO_2.

Preparation of citraconylated EXO

Serum-derived EXO was prepared according to a previous study with a slight modification [13]. Briefly speaking, FBS was centrifuged at 3000×*g* for 15 min. The supernatant of the centrifuged serum solution (0.95 mL) was mixed with the ExoQuick-TC EXO precipitation solution (190 μL) via an inverting tube. After incubation at 4 °C overnight, the mixture was centrifuged at 1500×*g* for 30 min. After additional centrifugation at 1500×*g* for 5 min, the resultant pellet was resuspended in phosphate-buffered saline (PBS) solution.

The amount of protein in the EXO was determined using the BCA protein assay kit according to the manufacturer's protocol. Briefly, after mixing reagent with each sample, the solution was incubated for 1 h at 37 °C. The absorbance was measured at a wavelength of 562 nm using a plate reader (SpectraMAX, Molecular Devices, Sunnyvale, CA, USA).

To calculate the amounts of existing amine groups, primary amine groups were quantitatively measured using a fluorescamine reagent and an amine reactive fluorescence indicator, as described previously. EXO in PBS solution was mixed with fluorescamine in acetone (2 mg/mL) at a sample/fluorescamine volume ratio of 10. The fluorescence intensities of the solutions were measured using a fluorospectrophotometer (Gemini EM microplate reader, Molecular Devices, CA, USA) at an excitation and emission wavelength of 390 and 475 nm, respectively. To prepare cit-EXO, amine groups (1 μmol) on the EXO in PBS solution were reacted with citraconic anhydride (0, 0.5, 1, and 2 μmol) in anhydrous dimethyl sulfoxide for 2 h at room temperature. After reaction, unreacted citraconic anhydride was removed via a 40 kDa desalting column [20].

Characterization of cit-EXO

EXO and cit-EXO in PBS solution at a final concentration of 0.5–1 mg/mL were analyzed using DLS (Nano-s, Malvern Instrument Ltd., Malvern, UK). The surface charge of cit-EXO in deionized water (DW) was measured using zeta sizer (ZEN 3690, Malvern Instrument Ltd.). For gel migration of EXO and cit-EXO, samples (18 μg) were loaded onto 0.5% agarose gel, and gel electrophoresis was performed for 40 min. After staining gels with Coomassie solution overnight, agarose gels were destained with DW and visualized by an illuminator.

Intracellular uptake of cit-EXO

To visualize EXO and cit-EXO, the fluorescent dye indocyanine green (ICG) was incorporated, as previously reported [14]. For easy incorporation of ICG into EXO (ICG/EXO), hydrophobic ICG (700 μg) and TBAI (2 mg) were mixed to prepare ICG-TBAI complex. EXO or cit-EXO (500 μg) was mixed with ICG-TBAI (50 μg) (ICG/exosomal protein ratio of 0.1) at 37 °C for 3 h. After incubation, free ICG-TBAI was removed with 300 K Nanosep (MWCO = 300 kDa). To quantify the amount of ICG within EXO or cit-EXO, the fluorescent intensities of the ICG/EXO or ICG/cit-EXO solutions were measured with an IVIS instrument at excitation and emission wavelength of 780 and 831 nm, respectively.

RAW264.7 and DC2.4 cells were plated in six-well plates at densities of 6×10^5 cells/well 24 h prior to treatment, respectively. ICG/EXO or ICG/cit-EXO treated cells in each well at 5.8 μg/mL concentrations of ICG in the absence and in the presence of serum in media. After incubation for 4 h, cells were washed with 0.05% trypsin–EDTA in PBS solution once and PBS containing 5% FBS twice. After cell lysis by incubation with lysis buffer (1% Triton X-100 in PBS) for 10 min, intracellular ICG/EXO or ICG/cit-EXO was determined by measuring the fluorescence signals in the supernatants with the IVIS instrument at excitation and emission wavelengths of 780 and 831 nm, respectively.

Results and discussion

Synthesis and characterization of cit-EXO

Figure 1a shows the schematic illustration for the preparation of cit-EXO. Primary amine groups on an EXO surface were reacted with citraconic anhydride via Michael-type addition for 2 h. The resulting cit-EXO has a highly negative charge because of the substitution of primary amine groups in carboxyl groups. As shown in Fig. 1b, cit-EXO was treated on macrophages to assess intracellular delivery efficiency. It is well known that surface positive charge mediates easy interaction of particles with cells and efficient intracellular delivery [21–23]. However, recent studies reported that the effects of surface charge on intracellular uptake depend on the types of cell, including cancer cells and phagocytic cells. In particular, surface negative charge allowed efficient interaction of complement and antibodies in serum, which provided excellent phagocytic uptake of micro-sized particles. In this study, it was examined how surface charge modification of EXO to negative charge might enhance intracellular uptake into macrophages in serum-free and serum-containing media.

After chemical modification of EXO with citraconic anhydride at different citraconic anhydride/amine groups molar ratios (0, 0.5, 1, and 2), the size distribution of cit-EXO was analyzed by DLS. As shown in Fig. 2a, citraconylation of EXO did not result in changes in size distribution for all cit-EXO samples. As shown in Fig. 2b, the mean hydrodynamic size of cit-EXO at citraconic anhydride/amine group molar ratios (0, 0.5, 1, and 2) were 42.3 ± 3.6, 37 ± 3.3, 36.9 ± 1.9, and 46.3 ± 11.0 nm. The mean diameters of EXO and cit-EXO were similar. After citraconylation of EXO, the surface charges of cit-EXO at citraconic anhydride/

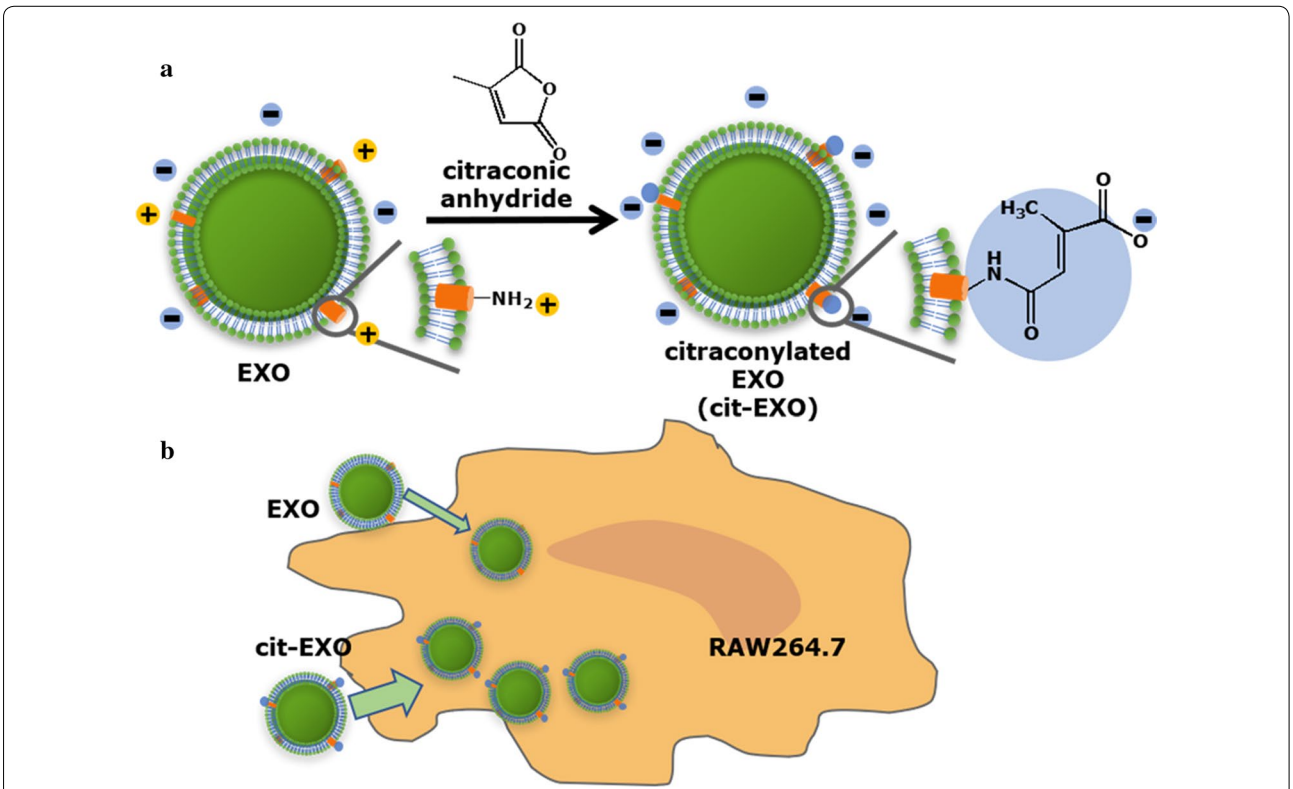

Fig. 1 **a** Schematic illustration for the preparation of citraconylated EXO (cit-EXO) and **b** intracellular uptake of cit-EXO for macrophages (RAW264.7 cells)

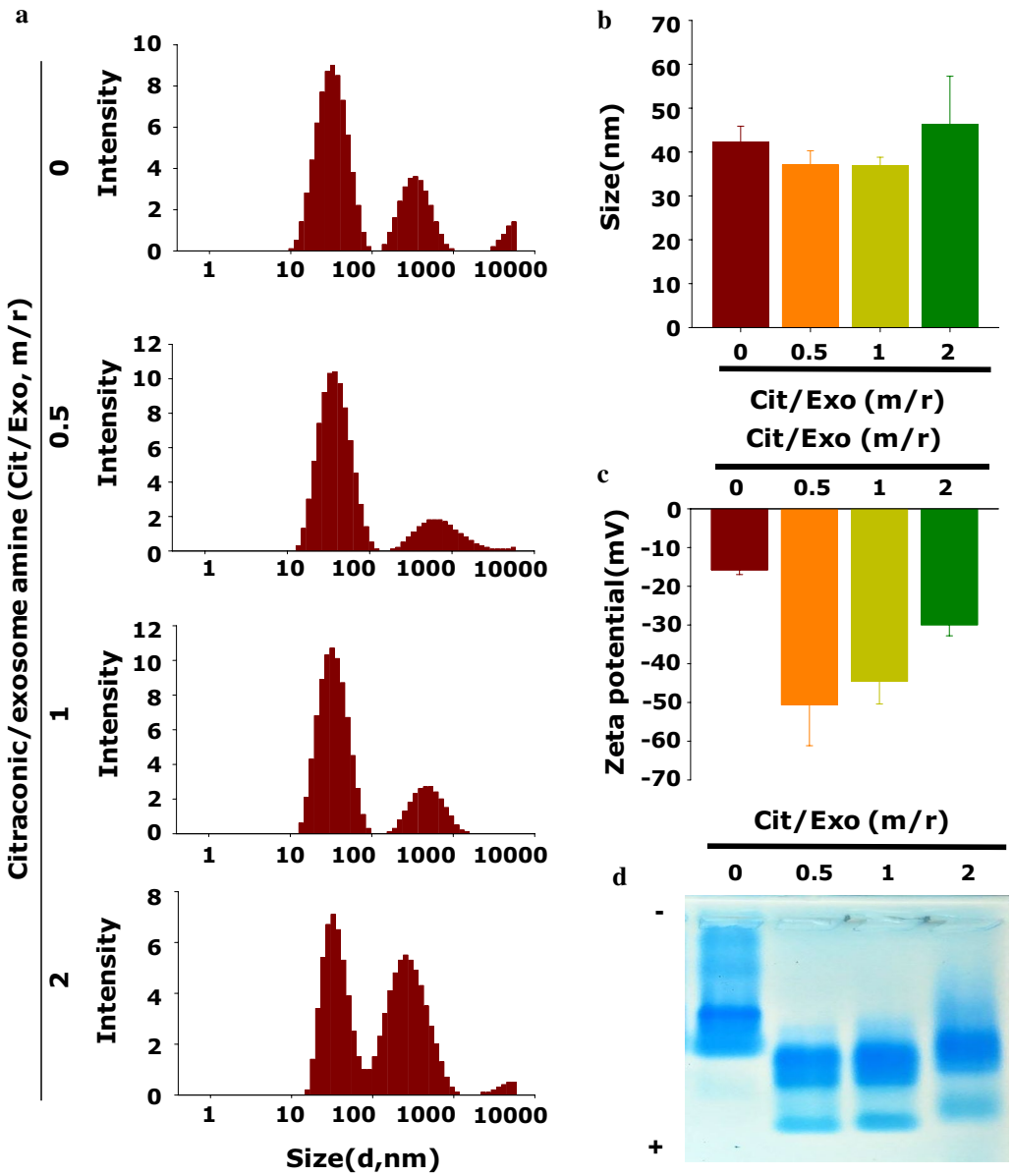

Fig. 2 **a** Size distribution, **b** mean diameter, and **c** surface charge of cit-EXO at different citraconic anhydride/amine groups of EXO molar ratios (m/r; 0, 0.5, 1, and 2). **d** Agarose gel electrophoresis of cit-EXOs and Coomassie staining

EXO amine molar ratios of 0.5, 1, and 2 were reduced to − 50.8 ± 10.4, − 44.5 ± 5.9, and − 29.9 ± 2.9 mV due to adopted carboxylic acid groups, respectively, while that of EXO was − 15.7 ± 1.3 mV (Fig. 2c). The surface charge of EXO was consistent with data in a previous study [24]. To confirm the surface charge of EXO, EXO and cit-EXOs were loaded onto 0.5% agarose gels for 40 min, and proteins of each sample were stained with Coomassie blue dye. Figure 2d shows noticeably fast migration of cit-EXO at citraconic anhydride/EXO amine molar ratios of 0.5, compared with EXO. These results demonstrated that citraconylation of EXO was successfully performed and significantly reduced the surface charge of EXO in physiological condition. Interestingly, the surface charge of cit-EXO at citraconic anhydride/EXO amine molar ratios of 0.5 was a significantly lower surface charge and faster migration in zeta potential analysis and agarose gel electrophoresis than that of cit-EXO at citraconic anhydride/EXO amine molar ratios of 2.

Cellular uptake

In a previous study, the intracellular uptake of particles was significantly different in serum-free and serum-containing media because of proteins, e.g., complement and albumin in serum [25, 26]. To examine intracellular uptake of EXO and cit-EXO for phagocytic cells comparatively, samples were treated with two types of cell, dendritic cells (DC2.4 cell) and macrophages (RAW264.7 cell), in serum-free media. Figure 3a shows that the extents of intracellular uptake of EXO and cit-EXO for RAW264.7 cells and DC2.4 cells were similar. To investigate the effects of serum proteins for intracellular uptake of EXO and cit-EXO, samples were also treated with two types of phagocytic cells in serum-containing media (Fig. 3b). Over all, the extents of internalized particles were significantly lower in the presence of serum

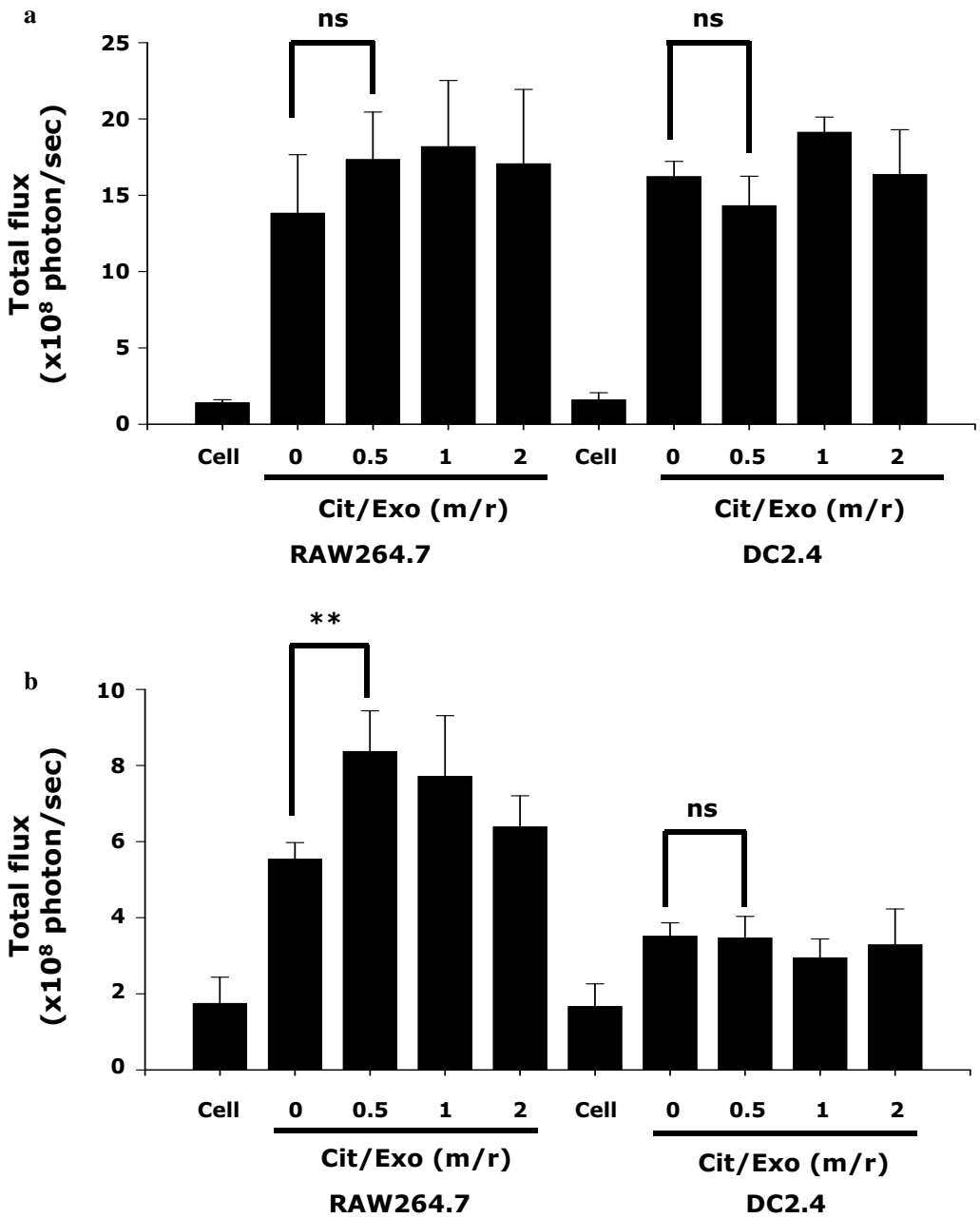

Fig. 3 Intracellular uptake of ICG incorporated EXOs and cit-EXOs **a** in serum-free media and **b** in serum-containing media for RAW264.7 cells and for DC2.4 cells. $^{**}p < 0.01$. *ns* not siginificant

proteins than those in the absence of serum protein for both RAW264.7 cells and DC2.4 cells. Interestingly, cit-EXOs showed significantly improved intracellular uptake for RAW264.7 cells compared with EXOs. The relative fluorescence intensities of EXO and cit-EXO (citraconic anhydride/EXO amine = molar ratio of 0.5) were 5.5 ± 0.4 and 8.4 ± 1.0 ($\times 10^8$), respectively. However, the extents of intracellular fluorescence intensity for DC2.4 cells were similar after treatment with EXO and cit-EXOs. DC2.4 cells showed significantly poor particle uptake compared with RAW264.7 cells. In previous studies, surface charges of micro-/nano-particles determined types of surface corona protein, which affect intracellular uptake of particles in macrophages [27, 28]. In addition, negatively charged nanoparticles with a size of over 100 nm exhibited superior intracellular uptake into macrophages compared with positively charged or slightly negative nanoparticles due to different corona protein e.g., complement C [29, 30]. In this study, nanosized and negatively charged cit-EXO with a size of approximately 50 nm showed higher intracellular uptake than EXOs with less negative charge. Previously, it was reported that not dendritic cells but macrophages express several complement C receptors, which could facilitate the phagocytic uptake of particles for macrophages [31, 32]. Accordingly, it is likely that serum proteins, e.g., complement C, might provide improved intracellular uptake of highly negatively charged cit-EXO compared with EXO. Citraconic anhydride has been used for reversible modification of amine groups because of the easy reversibility of the amide bond [33]. It is considered that cit-EXO could readily reverse the surface charge for an acidic phagosome after intracellular uptake and following biological pathways similar to EXO.

In this study, the surface charge of EXO was easily modified by citraconylation. The prepared cit-EXO exhibited a significantly reduced surface charge down to − 50 mV to EXO despite similar hydrodynamic size. Although EXO and cit-EXO showed similar intracellular uptake in the absence of serum, cit-EXO exhibited superior intracellular uptake to that of RAW264.7 cells in the presence of serum proteins. However, there were no significant differences in the intracellular uptake of EXO and cit-EXO for DC2.4 cells. It is expected that the citraconylated EXOs with protein coronas including complement C might be favorably delivered to RAW264.7 cells in serum media, compared to EXOs. Probably due to high expression of surface receptors e.g. complement C receptors onto RAW264.7 cells, RAW264.7 cells showed higher uptake of cit-EXO than DC2.4 cells. Simple surface modification onto EXOs via citraconylation improved the delivery of nanosized EXO (~ 50 nm) into macrophages, which could be harnessed as a promising strategy for the development of carriers for targeted macrophage delivery.

Authors' contributions

YK carried out experiments. YK and HM designed all of the experiments and drafted the manuscript. Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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