Biomaterials 35 (2014) 7239–7247

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Multi-modal transfection agent based on monodisperse magnetic nanoparticles for stem cell gene delivery and tracking

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ARTICLE INFO

Article history:
Received 24 April 2014
Accepted 4 May 2014
Available online 29 May 2014

Keywords:
Multimodal imaging
Magnetic resonance imaging
Cell tracking
Stem cells
Catechol

ABSTRACT

Directing the controlled differentiation and tracking of stem cells is essential to achieve successful stem cell therapy. In this work, we describe a multi-modal (MR/optical) transfection agent (MTA) for efficient gene delivery and cell tracking of human mesenchymal stem cells (hMSCs). The MTA was synthesized through a facile two-step approach with 1) ligand exchange of a catechol-functionalized polypeptide (CFP) and 2) chemical immobilization of fluorescence labelled cationic polymer via aminolysis reaction. Cationic polymer-immobilized MTAs with size of ~40 nm exhibit greatly enhanced colloidal stability in aqueous solution. In addition, the MTAs were capable of binding DNA molecules for transfection. The MTA/pDNA complex showed relatively good transfection efficiency in hMSCs (compared to the commercial transfection agent, Lipofectamine) and good biocompatibility. MTA-treated hMSCs were successfully visualized after transplantation via MR and optical imaging system over 14 days. These studies highlight the challenges associated with the potential advantages of designing multi-modal nanostructured materials as tools for genetic materials delivery and cell-tracking in stem cell therapy.

1. Introduction

Stem cell therapies have had a certain degree of attention in the treatment of various human diseases, including ischemic brain injury, neurodegenerative disorders, cancer, and cardiovascular disease [1,2]. However, the multipotent differentiation of stem cells remains a problem and has limited the utilization of cell therapy and tissue engineering protocols [3]. Therefore, directing the controlled differentiation of stem cells in a lineage-specific manner is required. One feasible strategy is to deliver genes that encode appropriate differentiation factors into stem cells [4–7]. In addition, real-time tracking of the transplanted cells is crucial to determine whether cellular regeneration originated from an exogenous cell source without the requirement of sacrificing an animal for post-mortem histology.

In these regards, superparamagnetic iron oxide (SPIO) nanoparticles have been investigated for their use in the transfection of genetic materials (*e.g.*, pDNA and siRNA) into stem cells [8,9] as well as for tracking the genetically optimized stem cells with magnetic resonance (MR) imaging [10–13]. The major shortcoming of SPIO nanoparticles for labelling stem cells is their low intracellular labelling efficiency, which can be slightly improved by surface modification with polymeric coatings and delivery with transfection agents. Even with these improvements, however, they are still difficult to detect with clinical MR [14]. More recently, various bimetallic nanoparticles, including $CoFe_2O_4$, $MnFe_2O_4$, and $NiFe_2O_4$, have been investigated as potential MR cell tracking agents [15]. However, the wide bioapplications of these nanomaterials are currently delayed owing to their inherent cytotoxicity [16].

Recent developments in nano-technology, such as the 'Heat-up' method [17,18], enable fine control over size, crystallinity, uniformity, and superior biocompatibility of iron oxide nanoparticles, but such monodisperse iron oxide nanoparticles generally have very limited stability in water that in turn limits their use in bioapplications [19–21]. In order to impart their water solubility, it is necessary to exchange their initial capping agent with new ligand

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http://dx.doi.org/10.1016/j.biomaterials.2014.05.010

[22,23]. However, there is a challenge to design a ligand enabling not only functionalization (*e.g.,* conjugation with fluorescence dye or targeting ligand) but also colloidal stability.

Here, we describe a simple approach with which to obtain multi-modal transfection agent (MTA) with high transfection efficiency. This approach is based on monodisperse magnetic iron oxide nanoparticles that enable both MR and optical cell tracking (Fig. 1). This method involves 1) ligand exchange of a catechol-functionalized polypeptide (CFP) onto the surface of hydrophobic iron oxide nanoparticles (Cathechol functional group exhibits extremely strong affinity to diverse metal oxide surfaces through coordination bonding) [21] and 2) fluorescence labelling of a cationic polymer (*i.e.,* rhodamine-labelled polyethyleneimine, R-PEI) covalently immobilized onto surfaces through aminolysis between primary amine groups in R-PEI (nucleophiles) and the benzyl-ester groups in CFP [24,25]. The positively charged R-PEI moiety on iron oxide nanoparticles can electrostatically bind to negatively charged pDNA and mediate the transfection of stem cells, while the conjugated rhodamine dye enables tracking of the stem cells via optical imaging.

2. Materials and methods

2.1. Materials

3-(3,4-dihydroxyphenyl) propionic acid (DPA), β-benzyl L-aspartic acid (BLA), branched polyethylenimine (PEI, 25 kDa), rhodamine B isothiocyanate (RITC), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), hydrochloric acid, triphosgene, dimethylformamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO) and chloroform were purchased from Sigma (St. Louis, MO). Dialysis membrane (molecular weight cut-off (MWCO), 1 kDa) was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). VivaSpin 20 was purchased from Sartorius Stedim Biotech (Goettingen, Germany). Oleic acid-capped monodisperse iron oxide nanoparticles with a diameter of 15 nm were synthesized using a previously reported method [17,26]. α-methoxy-ω-amino-poly(ethylene glycol) (mPEG–NH_2) (Mw: 5 kDa) was purchased from Sunbio Corporation (Anyang, South Korea). β-benzyl L-aspartic acid N-carboxyanhydride (BLA-NCA) was synthesized as reported [27,28]. ^{1}H-NMR spectra were recorded using a Bruker NMR Spectrometer (300 MHz). The samples were prepared by adding aliquots of products (10 mg) into a deuterated solvent. ^{1}H-NMR chemical shifts are reported in ppm and calibrated against TMS (δ 0). GPC analysis was carried out using the following system: Waters 515 HPLC pump, Waters 410 differential refractometer, KF-805 and KF-804 columns (8.0 mm i.d. × 300 mm) (shodex, Japan). The eluent was DMF, and the flow rate was 1 ml/min. The system was calibrated with standard polyethylenglycol samples (shodex, Japan).

2.2. Preparation of CFP-functionalized iron oxide nanoparticles

CFP-functionalized iron oxide nanoparticles were prepared by ligand exchange [21]. Oleic acid-capped iron oxide nanoparticles (Fe, 2 mg) and CFP (30 mg) were mixed in $CHCl_3$ (5 mL). The solution was stirred at room temperature for 30 min. After the evaporation of the solvent, these nanoparticles were re-dispersed in water. The nanoparticle solution was filtered through a 0.2 μm syringe filter (Advantec, Japan). Excess CFP was removed by centrifugation or washed with a spin filter (MWCO: 100 kDa, 8000 × *g*, 20 min) 5 times. The resulting nanoparticles were re-dispersed in water for the further studies.

2.3. Rhodamine-conjugated PEI (R-PEI)

PEI (50 mg, 2 μmol) was dissolved in 5 mL DMSO and mixed with RITC (1.29 mg, 2.4 μmol) in DMSO, then stirred for 3 h in darkness at 25 °C. Unreacted RITC was removed by dialyzing the reaction mixture against deionized water (DW) using a dialysis tube (MWCO: 1 kDa).

2.4. Immobilization of R-PEI onto CFP-functionalized iron oxide nanoparticles

The immobilization of R-PEI onto CFP-functionalized iron oxide nanoparticles was carried out by aminolysis reaction [24,25]. R-PEI (50 mg, 2 μmol) was dissolved in DMSO (10 mL) to make an R-PEI solution. CFP-functionalized iron oxide nanoparticles (Fe, 2 mg) were added dropwise to the R-PEI solution, then stirred for 24 h at 25 °C. Excess R-PEI was removed by centrifugation or washed with a spin filter (100 kDa MWCO, 8000 × *g*, 20 min) 5 times. The R-PEI/iron oxide nanoparticles immobilization ratio was confirmed using an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin–Elmer Optima 4300 DV) for Fe, and fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan) for R-PEI (excitation 488 nm, and emission 530 nm). The R-PEI/Iron oxide nanoparticles immobilization ratio was approximately 24 (w/w).

2.5. Iron quantification

Iron (Fe) content of MTA was determined using an ICP-OES (Perkin–Elmer Optima 4300 DV) after digestion in 70% nitric acid.

2.6. Hysteresis loop measurement

The MTA was examined with an MPMS XL-7 superconducting quantum interference device (SQUID) magnetometer (Quantum Design, San Diego, CA, USA) at 37 °C. The measurements were normalized for the grams of iron in each sample; the measurements were then verified through ICP-OES.

2.7. Cell culture

Human mesenchymal stem cells (hMSCs) were obtained from Lonza (Lonza, Walkersville, MD) and cultured in α-minimum essential medium (α-MEM, HyClone, Logan, UT) containing 10% foetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.8. Preparation of MTA/plasmid DNA (pDNA, pEGFP) complex

MTA/pDNA (encoding an enhanced green fluorescent protein reporter gene) complexes were prepared by mixing MTA and pDNA in HEPES-buffered saline with various *N*/*P* ratios (ratio of nitrogen in R-PEI to phosphate in pDNA). The MTA/pDNA complexes were incubated for 15 min at room temperature. For the gel retardation assay, 3 μL of a gel loading dye was added to each MTA/pDNA complex solution, and 20 μL of the resulting polyplex solution was loaded to an agarose gel (1%, w/v) containing 1 μg/mL ethidium bromide.

2.9. DNase I protection assay

DNase I protection assay was performed using the method reported by Sawant et al. [29]. The MTA/pDNA complexes (*N*/*P* ratio = 10) prepared as mentioned above were incubated with of DNase I (1 unit/μg of DNA, Promega Corp., Madison, WI) for 30 min at 37 °C. The digestion was stopped by the addition of EGTA and EDTA to a final concentration of 5 mM. To dissociate the DNA molecules from complexes, heparin (50 units/μg of DNA) was added, the mixtures were further incubated for 30 min at 37 °C, and the final products were analyzed on an agarose gel (1%, w/v).

2.10. Cellular uptake of MTA/pDNA complex

The hMSCs (2 × 10^5 cells/well) were seeded in a 6-well plate and cultured at 37 °C and 5% CO_2, after which they were rinsed twice and pre-incubated for 1 h with 2 mL Opti-MEM medium at 37 °C. MTA/pEGFP complexes were added to the cells and incubated for 30 min to 12 h at 37 °C. The cells were then washed 3 times with 1 mL phosphate-buffered saline (PBS, 100 mM, pH 7.4) to remove any free MTA/pEGFP complexes, suspended in PBS, and subsequently introduced into a flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 554 nm argon laser. The data shown are the mean fluorescent signals for 10,000 cells. For confocal microscopy, the cells were fixed with 4% paraformaldehyde. The cells were mounted in mounting medium (Dado, Glostrup, Denmark) and visualized using a confocal laser-scanning microscope (LSM 510 Meta; Zeiss, Germany). The fluorescence was monitored in a RITC channel (excitation 554 nm, emission 576 nm). For the transmission electron microscopy (TEM), the cells were subject to dehydration using graded ethanol (20, 40, 60, 80, and 100%) and embedded in Epon 812 resin. The resin blocks were sectioned using a microtome and imaged with JEOL 200CX (80 kV).

2.11. Transfection efficiency of MTA/pDNA complex

The hMSCs (2 × 10^5 cells/well) were seeded in a 6-well plate and cultured at 37 °C and 5% CO_2, after which they were rinsed twice and pre-incubated for 1 h with 2 mL Opti-MEM medium at 37 °C. MTA/pEGFP complexes (*N*/*P* ratio = 10) or comparison complexes (lipofectamine/pEGFP and PEI/pEGFP complexes) were added to the cells and incubated for 4 h at 37 °C. The hMSCs were then washed 3 times with 1 mL PBS (100 mM, pH 7.4) to remove any free gene complexes, suspended in PBS, and treated cells were then incubated for 12–72 h. To determine transfection efficiency, the cells were harvested and analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 508 nm argon laser. The data shown are the mean fluorescent signals for 10,000 cells. For the confocal microscopy, the cells were fixed with 4% paraformaldehyde. The cells were mounted in mounting medium (Dado, Glostrup, Denmark) and visualized using a confocal laser scanning microscope (LSM 510 Meta; Zeiss, Germany). Fluorescence was monitored in the EGFP (excitation 488 nm, emission 530 nm) and DAPI channels (4′,6-diamidino-2-phenylindole; excitation 358 nm, emission 461 nm).

2.12. Western blot analysis

Total protein was extracted from cells in accordance with the standard protocol. The protein-extracted lysates were resuspended in 100 and 200 μL of RIPA buffer, respectively, after which they were analyzed via electrophoresis on 10% (w/v) acrylamide SDS-PAGE gels. Western blot analyses were conducted using a semidry system. Blots were incubated overnight at room temperature with a monoclonal antibody diluted to 1:1000 and binding was detected using HRP-conjugated goat