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Sunflower-type nanogels carrying a quantum dot nanoprobe for both superior gene delivery efficacy and tracing of human mesenchymal stem cells

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ABSTRACT

Sunflower-type nanogels carrying the QD 655 nanoprobe can be used for both gene transfection and bioimaging of hMSCs. The entry of sunflower-type nanogels into hMSCs can be possibly controlled by changing the formation of QDs. The physico-chemical properties of sunflower-type nanogels internalized by hMSCs were confirmed by AFM, SEM, TEM, gel retardation, and ζ-potential analyses. The bioimaging capacity was confirmed by confocal laser microscopy, Kodak imaging, and Xenogen imaging. Specifically, we investigated the cytotoxicity of sunflower-type nanogels via SNP analysis. Internalization of sunflower-type nanogels does not cause malfunction of hMSCs.

1. Introduction

Nanotechnologies, including nanomedicine and nanocytotoxicity, have led to the design of nanostructured or nanofunctional particles that have been applied to trials in theragnostic fields such as managing disease and bioimaging of patients simultaneously [1,2]. Trials of theragnostics employing such nanotype particles have been widely applied in both basic and clinical research [3,4]. These types of particles, the functions of which can possibly be manipulated by modifying their surfaces during the primary fabrication stage, can modulate various properties of biological systems, such as cell signaling and tissue functions, following their binding [5]. Indeed, these nanostructures enter cells via membranes and are internalized by endocytosis; this is highly dependent on the sizes and morphologies of nanostructures [6–11]. After entering cells via endocytosis, nanostructures are removed from cells by exocytosis [12,13]. Thus, many researchers have focused on modifying nanostructure surfaces such that they can easily interact with cell membranes to trigger a cascade of events [14,15]. The removal of nanostructures from cells hinders their use for bioimaging.

In terms of the clinical use of nanostructures, modified nanotype particles have been specifically used as delivery vehicles and carriers due to their suitable optical characteristics and biological activities [16,17]. This has encouraged the study of inorganic nanoparticles (NPs) for applications in drug delivery and imaging systems [18,19]. Nano-sized semiconductor quantum dots (QDs), utilized as photoluminescent markers, can be employed for imaging in cells [20–22]. QDs that exist in the cytosol are useful imaging markers for tracing because they fluoresce at a specific wavelength of light according to their size [23,24]. Although QDs are thought to be critical molecular probes in cells and to have applications in diagnostics and targeted drug delivery, the use of QDs as carriers of several therapeutic biomolecules is impeded by problems related to cytotoxicity [25].

To overcome this obstacle, researchers have studied candidate QDs conjugated to a biocompatible polymer [26,27] or nanogels [28,29] in order to deliver specific drugs into cells without causing cytotoxicity. The encapsulation of a single QD by a carrier may result in high performance based on the fluorescence quantum yield and allow highly sensitive luminescence detection for bioimaging uses [30]. Recently, several trials, including the encapsulation of nanoprobes, have been conducted for biological applications [31]. These trials will have the opportunity in the

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clinical translation researches which can be easily used in bio-application. NPs with modified surfaces via ligand exchange reactions are of most use in biological applications [32].

Previously, we attempted to evaluate the transfection of human mesenchymal stem cells (hMSCs) with specific genes using multiple QD-bundled NPs as a multifunctional imaging platform for both gene delivery and cell tracking [23]. We also sought to enhance cellular uptake and transfection using different sizes of QDs [33]. In a previous study, we explored the use of poly(ethyleneimine) (PEI) to organize QDs into a larger bundled structure as a multifunctional imaging platform for translational research in stem cells. Although QDs showed the reasonable choice for gene transfection and bio-imaging, the cytotoxicity of QDs was not identified as a both gene carrier and cell tracing agent.

In this study, inspired by previous studies [23,33], we designed sunflower-type nanogels, in which QDs are complexed with PEI, as a multifunctional imaging platform for both gene delivery into and tracking of hMSCs. A few stem cell differentiation studies have employed nanogels complexed with QDs using the layer-by-layer system. QD NPs have only been used to trace stem cells transplanted into wound sites. The aims of this study were to complex NH_2-QD 655 (20 nm) with heparinized pluronic 127 nanogels via charge-charge interactions and then to coat these NH_2-QD-encapsulating heparinized nanogels with positively charged PEI for gene delivery. By modifying the surface charge of nanogels, they could be easily complexed with negatively charged plasmid DNA (pDNA). Finally, sunflower-type QD-carrying nanogels were fabricated for both gene delivery and cell tracing. This type of nanoparticles can be used in the fields of bioimaging, drug delivery system, gene delivery carriers, and stem cell engineering which can be tracking when they were implanted into bodies.

2. Materials and methods

2.1. Materials

Pluronic F127 (PEO-PPO-PEO; molecular weight, 12,600 Da) was purchased from BASF Corp. (Seoul, Korea). Heparin (165 IU/mg) was purchased from Acros (Pittsburgh, PA, USA). *Linear PEI (25,000 Da) was purchased from Polysciences* (Warrington, PA, USA). All chemicals were of analytical grade, obtained from commercial sources, and used without further purification. *655 ITK amino (PEG)* QDs, *3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), the* LIVE/DEAD® Fixable Green Dead Cell Stain Kit, *α-minimum essential medium* (*α-MEM), and fetal bovine serum (FBS)* were purchased from *Life Technologies (Thermo Scientific, USA)*.

2.2. Preparation of QD-carrying heparinized nanogels

To synthesize heparin-pluronic nanogels, the required amount of heparin was added to pluronic F127 solution. Pluronic F127 was dissolved in 0.1 M MES buffer, and 1-Ethyl-3-(3-dimethylamino propyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were added as coupling agents. Then, heparin dissolved in dioxane was poured into the mixture and stirred at room temperature for 24 h. The solution was filtered and dialyzed against de-ionized water for 3 days (molecular weight cut-off, 50 kDa) and lyophilized. Briefly, heparin-pluronic F127 (100 mg) was dissolved in 10 mL of de-ionized water with vigorous stirring and stored at 4 °C.

QD-encapsulating heparinized self-assembled nanogels (1:0.25, 1:0.5, 1:1, and 1:2) were prepared by dissolving 10 mg of heparin-pluronic nanogels (10 mg/ml) in 1 ml of de-ionized water, adding 0.25, 0.5, 1, or 2 mL of QDs, and stirring. To prepare sunflower-type nanogels, QD-encapsulating heparinized nanogels were coated with PEI. Typically, heparin-pluronic nanogels (10 mg) was first dissolved in 1 mL of de-ionized water. QDs (0.25, 0.5, 1, and 2 mL) were added whilst the mixture was vigorously stirred, and the suspension was continuously stirred in air for an additional 5 min. Then, the mixture (QD-encapsulating heparinized nanogels) was coated with PEI. The size of sunflower-type nanogels was dependent on the amounts of QDs and heparin-pluronic nanogel used. When the ratio of heparin-pluronic nanogels to QDs was 1:0.5 (as it was when coating with PEI), sunflower-type nanogels were in a single layer. An increased heparin-pluronic nanogel:QD ratio (1:1 and 1:2) resulted in multi-layered nanogels. Sunflower-type nanogels were formed by mixing 0.25–2 mL of QD solution with various concentrations of heparin-pluronic nanogels.

2.3. Assessment of the cytotoxicity of nanogels

The cytotoxicity of heparin-pluronic nanogels, PEI, QDs, QD-encapsulating heparinized nanogels, and sunflower-type nanogels were assessed using the LIVE/DEAD® Fixable Green Dead Cell Kit. hMSCs were seeded at a density of 2×10^5 cells/well in a 6-well plate and incubated overnight in a 5% CO_2 incubator. hMSCs were treated with heparin-pluronic nanogels, PEI, QDs, QD-encapsulating heparinized nanogels, and sunflower-type nanogels for 6 h. The medium was then replaced with 2 mL of fresh medium containing 10% FBS and 1% antibiotics from each nanoplex solution. After incubation for 48 h, cells were rinsed with PBS, trypsinized, centrifuged for 3 min at 1300 rpm, and resuspended in 1 mL of medium containing reconstituted florescent reactive dye. Cell suspensions were incubated at room temperature, with protection from light, washed once with 1 mL of PBS, and resuspended in 900 μL of PBS, after which 100 μL of 37% PFA was added and cells were incubated for 15 min. Cells were washed twice with cold PBS containing 1% bovine serum albumin and analyzed using the Guava EasyCyte System equipped with a 488/642 nm excitation laser. Data show the mean fluorescence signals of 5000–10,000 cells.

2.4. CNV (copy number variation) and SNP (single nucleotide polymorphism) analysis

For analysis of CNVs and SNPs, genomic DNA was extracted from cell cultures using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) and analyzed using an Affymetrix CytoScan® High-Density array, which contains 2.6 million combined SNP and CNV markers with a median inter-marker distance of 500–600 bases. All procedures, including DNA labeling, hybridization, washing, and scanning of chips, were performed according to the manufacturer's recommendation (Affymetrix Inc., Santa Clara, CA, USA). In brief, 250 ng of genomic DNA was digested with the restriction endonuclease *Nsp1*. Fragmented DNA was ligated with primer/adaptors and amplified by PCR. The amplicons were labeled with biotinylated nucleotides using terminal deoxynucleotide transferase. Finally, 200 ng of labeled DNA was hybridized with a pre-equilibrated array chip at 50 °C for 16–18 h. *De novo* copy number changes and loss of heterozygosity were analyzed using Chromosome Analysis Suite 2.1.0.16 (Affymetrix Inc.) with respect to a reference set of samples. Genotyping of the CytoScan® High-Density array was accomplished using the BRLMM-P algorithm described in a previous study [34].

2.5. Gene delivery vehicles for hMSCs

For transfection, hMSCs were plated in a 6-well plate (2×10^5 cells/well) and cultured overnight. The culture medium was replaced with 2 mL of Opti-MEM medium and cells were treated with PEI, QDs, QD-encapsulating heparinized nanogels, and sunflower-type nanogels (1:0.25, 1:0.5, 1:1, and 1:2) for 6 h.