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A plug-and-play ratiometric pH-sensing nanoprobe for high-throughput investigation of endosomal escape

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ABSTRACT

An important aspect in the design of nanomaterials for delivery is an understanding of its uptake and ultimate release to the cytosol of target cells. Real-time chemical sensing using a nanoparticle-based platform affords exquisite insight into the trafficking of materials and their cargo into cells. This versatile and tunable technology provides a powerful tool to probe the mechanism of cellular entry and cytosolic delivery of a variety of materials, allowing for a simple and convenient means to screen materials towards efficient delivery of therapeutics such as nucleic acids.

1. Introduction

Chemical sensing at a molecular level is fundamentally important to understanding the mechanisms governing a number of processes, including those of biological interest such as cell uptake, gene transfection, and tumor development and maturation [1–6]. A number of materials, including quantum dots [7], nanocrystals [8], and polymeric nanoparticles [9,10] have been developed for these purposes, using fluorescence as a means of probing cellular processes. These platforms have proven to be robust, highly fluorescent tags for labeling cellular compartments to facilitate studies utilizing fluorescence microscopy, as well as stable *in vivo* imaging systems.

Designing systems that can molecularly recognize and identify targets, or respond to the local environment, is an attractive "nanoparticle laboratory" concept – one that is being further developed with core-shell silica nanoparticle technology [11–13]. The purpose of these systems is to facilitate continued development of biomaterials for applications such as bioimaging or therapeutic delivery. Incorporation of these plug-and-play technologies offers useful insight into the trafficking of biomaterials in cells. Of particular interest to the community are more efficient modes of cytosolic delivery for therapeutics, such as nucleic acids, that require safe passage through endosomal/lysosomal compartments for biological effect [14–17].

Currently, the most common techniques used to assess endosomal escape use confocal microscopy [18–23]. In this approach, the biomaterial, the therapeutic and the intracellular organelles, such as endosomes and lysosomes, are directly labeled with fluorescent dyes, and endosomal escape is visually evaluated based on the temporal and spatial localization of fluorescence inside cells. While this does allow for direct visualization of the processes in question, this is a low-throughput, largely qualitative technique. What is currently lacking is the ability to easily perform a quantitative, high-throughput assay with commonly available equipment and techniques without the need to directly modify the cargo to be delivered. Here, we report the development of an easily adaptable assay for quantitative and high-throughput evaluation of endosomal escape. This was achieved through deployment of a ratiometric pH-sensing nanoprobe for real-time intracellular tracking of materials.

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2. Materials and methods

2.1. Materials

GFP-expressing plasmid DNA was purchased from Invitrogen. PLL (15 kDa and 50 kDa), poly-L-arginine (15 kDa and 50 kDa), poly-L-histidine (15 kDa), chitosan (15 kDa), linear PEI (2.5 kDa and 25 kDa) and branched PEI (25 kDa) were purchased from Sigma–Aldrich. Lipofectamine 2000 was purchased from Invitrogen. CCK-8 cell proliferation assay kit was purchased from Sigma–Aldrich. Human cancerous cell lines, including BT-20, A549, and MDA-MB-468 were purchased from ATCC and grown according to ATCC protocols. All cell culture media and reagents were purchased from Invitrogen. PLL, poly-L-arginine and PEI were dissolved in saline buffer at pH 7.4, containing 150 mM NaCl and 10 mM phosphate; whereas poly-L-histidine and chitosan were dissolved in saline buffer at pH 5.5, containing 150 mM NaCl and 10 mM acetate. Ultrapure water (18.2 MΩ cm) was generated with a Barnstead Nanopure water purification system. Absolute ethanol, ammonium hydroxide solution and Sodium hydroxide (NaOH, 98.0+%) were purchased from Fluka. 3-(triethoxysilyl)propyl isocyanate, 70 kDa poly-L-lysine, hydrochloric acid (HCl, 37%) and Orange II sodium salt (98.0+%) were purchased from Sigma–Aldrich. Atto647 succinimidyl ester was purchased from Atto Tec GmbH, Germany. 5(6)-Carboxyfluorescein, succinimidyl ester was purchased from Anaspec. Aminopropyltriethoxysilane (95%), PEG-silane (95%) and tetraethylorthosilicate (99%) were purchased from Gelest. pH of dye solution was measured on an Accumet Excel XL15 pH/mV/Temperature meter. Absorbance of solutions was recorded on a Varian Cary 5000 UV-Vis-NIR spectrophotometer.

2.2. Synthesis of core-shell silica nanoparticles

The core-shell silica nanoparticles were synthesized according to the previously established method [9] (Supporting Fig. 1). Briefly, the reference dye, Atto647 succinimidyl ester was conjugated to the silica precursor aminopropyltriethoxysilane in an anhydrous nitrogen environment. This conjugate was then hydrolyzed in basic ethanolic solution with a pure silica precursor, tetraethylorthosilicate, catalyzed by concentrated aqueous ammonia. After the synthesis of these particles containing the reference dyes, the sensor dye, fluorescein (FITC), in the form of fluorescein succinimidyl ester, was conjugated with 3-aminopropyltriethoxysilane under similar conditions. The sensor dye precursor was then hydrolyzed with further tetraethyl orthosilicate to form the sensor layer. Following synthesis, the particles were centrifuged and resuspended repeatedly in ethanol and finally deionized water. We functionalized the surface of the pH sensor particles with a molar mixture of 95:5 PEG-silane and Poly-L-lysine where the latter was first silanized with 3-(triethoxysilyl)propyl isocyanate. The functionalized pH sensor particles were purified via centrifugation washes using 100 k MWCO spin filters to remove unbound PEG or poly-L-lysine.

2.3. Nanoparticle characterization

Primary amine groups from poly-L-lysine conjugated to pH sensor particles' surface were quantified by Orange II method reported by Noel et al. [24]. One milligram pH sensor particles was immersed in 1.5 ml of dye solution (14 mg/ml) in acidic solution (pH = 3) for 30 min at 40 °C. The particles were then washed thrice using the acidic solution (pH = 3) through spin filter (100 kDa MWCO) at 5000 rpm for 10 min each to remove unbound dye. The particles were air-dried and then immersed in 3 ml of alkaline solution (ultrapure water adjusted to pH 12 with a 1 M NaOH solution) for 2 h to release the bound dye from particle surface into the solution. The pH of the solution containing the desorbed dye was adjusted to pH 3 by adding 37% HCl. The absorbance at 484 nm of the solution was then measure. The concentration of primary amine group can be calculated using reported extinction coefficient of Orange II at 482 nm ($\varepsilon = 18{,}200\ M^{-1}\ cm^{-1}$). It is estimated that 45 ± 10 primary amine groups present on a 30 nm pH sensor particle.

2.4. Intracellular pH sensing assays

To assess the ability to sense pH intracellularly, silica nanoparticles were incubated with cells at different pH and cell-associated fluorescence was quantitated by flow cytometry. Briefly, cells were seeded in a 24-well plate at density of 1×10^5 cells per well and grown for 24 h. After changing into fresh the media, cells were treated with the silica nanoparticles at the concentration of 100 μg/ml for 2 h at 37 °C. At the end of incubation, free nanoparticles were washed off twice with fresh media, and the cells were trypsinized and pelleted. Cells were then resuspended in each of different cellular pH clamping buffer (pH 4, 5, 6, 6.6 and 7.4) that were made by mixing of 50 mM HEPES (pH 7.4) and 50 mM MES (pH 4.0) buffers, each containing 50 mM NaCl, 30 mM ammonium acetate and 40 mM sodium azide [25].

2.5. In vitro cell trafficking – flow cytometry

To screen the intracellular trafficking of gene delivery vehicles, the cells were firstly seeded in a 96-well plate at density of 2×10^4 cells per well for 24 h, and co-treated with each of the transfection agents and silica nanoparticles at various concentrations and incubation times. By the end of the incubation, cells were washed in fresh media and resuspended with trypsin. The cell-associated FITC:Atto647 fluorescent ratio was determined by flow cytometry and the ratios were converted to the average intracellular pH using a calibration curve.

2.6. Cell transfection

DNA-polymer complexes were firstly prepared by mixing 1 μg/ml GFP plasmid DNA with 10 μg/ml polymeric vehicles or 3 μl/ml Lipofectamine 2000 in serum-free OptiMEM for 30 min at room temperature. The siRNA-loaded lipid-based nanoparticles were prepared according to the previously described methods using microfluidic device. The cells, seeded in a 96-well plate at 2×10^4 cells per well for 24 h, were treated with the transfecting complexes at various concentrations. After 48 h of incubation, the GFP expression was examined for DNA transfection or for siRNA silencing using flow cytometry.

2.7. Flow cytometry

Flow cytometry was performed using a FACS LSR II coupled with a high-throughput system for the 96-well plate format (BD biosciences, San Jose, CA). FITC were excited at 488 nm and detected at 530/30 nm; where Atto647 were detected at 635 nm and detected at 660/20 nm. Cells were gated by forward and side scatter and 5000 events were collected for each sample. The geometric means of fluorescence intensities were recorded to determine the FITC:Atto647 fluorescent ratios. The cellular uptake of the silica nanoparticles were assessed using the geometric means of cell-associated Atto647 fluorescence that is insensitive to pH change.

2.8. Cytotoxicity assay

Cytotoxicity assays were performed in parallel with the intracellular pH sensing assays to assess the cytotoxicity of the silica nanoparticles and transfection agents. Briefly, cells were firstly seeded in a 96-well plate at density of 2×10^4 cells per well for 24 h and treated with the nanoparticles or transfection agents at various concentrations. Over different incubation times, the cells were replaced in fresh serum-free OptiMEM media containing 10% v/v of the CCK-8 proliferation reagent. After 2 h incubation at 37 °C, the absorbance at 450 nm was measured by a plate reader. Cell viabilities were normalized to an untreated control and calculated using a standard curve.

2.9. Confocal microscopy

Confocal microscopic images were taken using a Nikon A1R Ultra-Fast Spectral Scanning Confocal Microscope (Nikon instruments Inc., Melville, NY). Cells were seeded in CELLview glass bottom dish (Greiner Bio-One GmbH, Germany) at 1×10^5 cells per well and grown overnight. Cells were treated with 3 μg/ml of branched PEI or 3 μl of Lipofectamine 2000 in the presence of 100 μg/ml nanoparticles, or with 100 μg/ml nanoparticles along for 4 h. Cells were then washed three times and stained with DAPI for 15 min at 37 °C, followed by three more washes and microscopic imaging at DAPI, FITC and Atto647 channels.

2.10. Statistical analysis

Experiments were performed in triplicates, or otherwise indicated. Data were analyzed using descriptive statistics, single-factor analysis of variance (ANOVA), and presented as mean values ± standard deviation (SD) from three to eight independent measurements. Statistical comparisons between different treatments were assessed by two-tailed t tests or one-way ANOVA assuming significance at p-value < 0.05.

3. Results and discussion

The ratiometric pH-sensing nanoprobes were developed based on a core-shell silica nanoparticle architecture, and were synthesized via a modified Stöber method whereby a reference dye was covalently bound within the silica core initially, followed by deposition of a sensor dye-containing silica layer on the surface [12,13] (Supporting Fig. 1). The exterior silica layer can be further modified with silane-functionalized ligands through the surface tetraethyl orthosilicate [13]. Herein, nanoprobes of 30 ± 2.6 nm in diameter were specifically synthesized (Supporting Fig. 2A). The nanoparticles were modified with a molar mixture of 95:5 polyethylene glycol (PEG)-silane and silanized poly-L-lysine (PLL) to create a slightly positively charged surface with a zeta potential of 5 ± 1.2 mV when measured in PBS at pH 7.4 (Supporting Fig. 2B). The nanoparticles were uniform spheres (Fig. 1A and Supporting Fig. 2C) with a polydispersity index of 0.13. A pH-insensitive reference dye, Atto647, was covalently incorporated deep within the organosilica core during particle synthesis and is thus protected