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Optical imaging of intracellular reactive oxygen species for the assessment of the cytotoxicity of nanoparticles

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ABSTRACT

The generation of intracellular reactive oxygen species (ROS) was optically monitored using ROS-sensitive gold nanoprobes in response to an exposure of nanoparticles (NPs). Fluorescent dye-labeled hyaluronic acid was grafted onto the surface of gold nanoparticles (HF-AuNPs) for imaging intracellular ROS. The ultrasensitive detection of intracellular ROS was utilized as a powerful analytical tool to assess early cellular toxicities of monodisperse polystyrene (PS) particles with different sizes and different functional groups on the surface. The effect of PEGylation on the surface of PS NPs was also investigated by evaluating intracellular ROS generation. For various PS NPs, the extent of intracellular ROS was well correlated with cellular uptake, apoptosis inducing activity, and cytotoxic effect of NPs. In addition to the nanoparticles, commonly used polymeric gene carriers such as linear and branched polyethylenimine (PEI) were tested to analyze their extent of intracellular ROS generation related to cellular toxicity. This study demonstrated that sensitive and optical detection of intracellular ROS generation can provide a valuable toxicity index value for a wide range of NPs as an early indicator for cellular responses.

1. Introduction

Over the past several decades, a wide range of organic and inorganic NPs have been utilized for electronic, medical, and cosmetic applications due to their unique physicochemical properties [1–5]. Recently, potential toxic effects of NPs have drawn much attention since the toxicity behavior of NPs is quite distinguished from bulk materials [6–11]. The different toxicological profiles between bulk materials and NPs mainly arise from a large surface-to-volume ratio of NPs and their unique characteristics such as surface charge, size, chemical composition, shape, solubility, and stability [12–16]. The nanotoxicity has been traditionally evaluated by determining the extent of cell survival after exposing NPs to cells. Upon contacting NPs with cells, they are mostly taken up by cells via an endocytic pathway and initiate disruption of various sub-cellular organelles. Generally, the cellular toxicity of NPs is assayed by measuring activity of mitochondrial enzyme released only from undamaged cells after all cellular toxic events of NPs are terminated. However, it is known that endocytosed NPs trigger an oxidative stress on cells by inducing the production of intracellular reactive oxygen species (ROS), which is the very first event of cellular toxicity cascade reactions [11,17–19]. ROS are oxygen containing molecules having unpaired electron or free radicals. They are highly reactive and elicit many physiological events within cells including nanotoxicity. While the intracellular ROS play a key role in initiating various cellular apoptotic pathways, they also have critical effects as essential signal molecules for cellular functions including proliferation. In normal conditions, the mitochondria generate and release moderate levels of ROS into the cytosol that may function as signaling molecules for cell survival. However, when intracellular NPs induce to generate excessive amount of ROS beyond the limit of natural antioxidant defense systems like reductive glutathione (GSH) and antioxidant enzymes, cells start to lose normal functions with consequently causing cell death [20,21]. Thus, it is highly important to monitor the intracellular level of ROS for evaluating nanotoxicity at the early stage. The detection and imaging of intracellular ROS would provide more useful information for assessing the safety of various NPs, as compared to the conventional cell death assays.

Previously, we have reported novel gold nanoprobes for *in situ* monitoring and imaging of intracellular ROS level [22]. The gold nanoprobes immobilized with fluorescence dye-labeled hyaluronic acid (HA) emitted a strong fluorescence signal in response to ROS. Fluorescent dyes conjugated on the HA chains were quenched by gold NPs through NP surface energy transfer (NSET), but recovered fluorescence signals by ROS-induced degradation of HA. The gold nanoprobes (HF-AuNPs) were used for *in vivo* imaging of over-expressed ROS in rheumatoid arthritis [23]. They showed extremely

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higher sensitivity to intracellular ROS generation than any commercialized ROS organic fluorescent probes that are unstable and easily photobleached under prolonged light exposure.

Herein, we used ROS-sensitive gold nanoprobes to evaluate the toxicity of various NPs by measuring the level of intracellular ROS. Polystyrene (PS) NPs with different sizes and surface functional groups were treated to macrophage cells, and their intracellular ROS inducing effects were analyzed. In addition, PS NPs with and without immobilization of poly(ethylene glycol) (PEG, MW 5000) were utilized to examine the effect of PEGylation on nanotoxicity. Lastly, common gene delivery carriers such as linear and branched poly(ethyleneimine) (PEI, MW 25,000) were used for evaluating their toxicities. PEI is a highly positive charged polymer and is known to have varying degree of cytotoxic effect to cells based on their structure and molecular weight [24,25]. The level of intracellular ROS detected by HF-AuNPs was compared with the cellular uptake extent of PS NPs or PEI polymers, their apoptosis inducing effect, and cell viability. To improve the detection sensitivity of intracellular ROS for other cells besides macrophages, cell penetrating peptides (CPP) were additionally conjugated to HF-AuNPs, and used as a general evaluation tool for cellular nanotoxicity.

2. Materials and methods

2.1. Materials

Sodium hyaluronic acid (MW 17,000 Da) was purchased from Lifecore Biomedical (Chaska, MN). 1-ethyl-3-[3-(dimethylamino)propylcarbodiimide] (EDC), sodium citrate, hydrogen tetrachloroaurate trihydrate ($HAuCl_4$), 3-hydroxytyramine hydrochloride (DOPA), 6′-aminofluorescein, and sodium cyanoborohydrate ($NaBH_3CN$) were all obtained from Sigma Aldrich Co. (St. Louis, MO). Various polystyrene particles (PS) were purchased from Polysciences (Warrington, PA). Magic Red caspase detection kit, caspace colorimetric assay kit, and Micro-BCA protein assay kit were obtained from Immunochemistry Tech. (Bloomington, MN), Promega Corp. (Madison, WI), and Pierce Biotech. (Rockford, IL), respectively. All other chemicals and reagents were of analytical grade.

2.2. Preparation of HF-AuNPs

Two hundred and twenty four microliter of FITC-labeled, DOPA end-functionalized HA (1000 M excess) was reacted with 10 ml of 6.4 nM gold nanoparticles (AuNPs) for 1 h in DDW. After reaction, the salt concentration was adjusted to 0.3 M NaCl in phosphate buffered saline (PBS) (pH 7.4) solution, followed by further incubation for 30 min. The final solution was purified by repeated centrifugation (14,000 rpm, 30 min) and re-dispersion with PBS solution 7 times.

2.3. Detection of intracellular ROS using HF-AuNPs

To evaluate the level of intracellular ROS which was generated by exposure of various PS NPs, macrophage cells (RAW 264.7) were used. Linear or branched PEI mediated intracellular ROS generation was evaluated using fibroblast cells (COS-7). HF-AuNPs (0.3 pMole) were treated to 3×10^5 cells pre-plated on a 6-well plate for 1 h (RAW 264.7 cells) or 3 h (COS-7 cells) in 10% serum condition. After treatment, the medium containing HF-AuNPs was discarded and the cells were washed with PBS solution, followed by adding 100 μg/ml of various PS NPs or 30 μg/ml of PEI (linear and branched PEI, MW 25 k) and incubating for 30 min. The exposed cells were finally detached with PBS solution containing 5 mM EDTA. The green fluorescence signal of detached cells was analyzed by flow cytometry. The mean fluorescence intensity of PS NPs or PEI exposed 10,000 cells was normalized by the mean fluorescence intensity of control 10,000 cells that were treated only with HF-AuNPs, but not exposed to PS NPs or PEI. For the confocal microscopy analysis, the cells were washed with PBS solution, fixed with 1 ml of 1% paraformaldehyde solution. 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to visualize the cell nucleus. The cells were imaged by an LSM510 confocal laser scanning microscope (Carl Zeiss, Germany).

2.4. Analysis of cell cytotoxic effect

Macrophage cells (RAW 264.7) and fibroblast cells (COS-7) was used to analyze the cytotoxic effect of various PS NPs and PEI (linear, branched PEI, MW 25 k), respectively. Various PS NPs (100 μg/ml) and linear or branched PEI (30 μg/ml) were exposed to 5×10^3 cells for 1 day. After exposure, the cytotoxic effect was assayed by using CCK-8 kit.

2.5. Analysis of apoptosis inducing effect

The apoptosis inducing effects of various PS NPs and PEI were determined by culturing cells in a 4-well culture plate at the density of 1×10^5 and a 6-well plate at the density of 3×10^5 for Magic Red assay and Annexin V/PI double staining assay, respectively. After cell attachment, various PS NPs (100 μg/ml) and PEI (linear or branched, 30 μg/ml) were treated to cells for 1 day. The apoptosis inducing effect was evaluated by using a Magic Red caspase detection kit or Annexin V/PI double staining protocol. For Magic Red caspase assay, the cell nuclei were stained with DAPI for 15 min, and the cells were fixed with 1% paraformaldehyde, and visualized by an LSM510 confocal laser scanning microscope (Carl Zeiss, Germany). For Annexin V/PI double staining analysis, the cells were detached with PBS solution containing 5 mM EDTA, and analyzed by flow cytometry.

2.6. Preparation of PS–NH_2/PEG

One hundred microliter of 2.54% (w/v) PS–NH_2 NPs was dissolved in 900 μl of PBS solution (pH 7.5). One milligram of succinimidyl derivative of PEG propionic acid (SPA-PEG, MW 5000 or MW 2000) was added to the above solution, reacted for 2 h, and centrifuged (14,000 rpm, 15 min). After washing with 100% ethanol, PEGylated PS–NH_2 NPs were re-dispersed in PBS solution. PS–NH_2 NPs were also reacted with rhodamine-isothiocyanate to label rhodamine dye. Briefly, 30 μL of rhodamine-isothiocyanate dissolved in DMSO (1 mg/ml) was added to 1 ml of PBS solution containing PS–NH_2 NPs (0.254% (w/v)). After 3 h, the solution was purified by repeated centrifugation (14,000 rpm, 15 min) and re-dispersion in 1 ml of 100% ethanol.

2.7. Preparation of HF-AuNPs/PEG-Hph

DOPA-PEG-maleimide (MW 5000) (2.7 mg) was reacted with 1 mg of Hph in borate buffer (pH 8.5) solution for 1 h. The reacted solution was purified by using an Amicon Ultra-4 membrane (MWCO 3000). DOPA-PEG-Hph (16 μg), DOPA-HA-FITC (224 μg), and AuNPs (64 pMole) were reacted for 1 h in deionized water. The solution was transferred into PBS solution containing 0.3 M NaCl. HF-AuNPs/PEG-Hph was separated by repeated centrifugation and re-dispersion in PBS solution.

3. Results and discussion

3.1. Cytotoxicity evaluation of various sized nanoparticles using ROS-sensitive gold nanoprobe

The fluorescent dye-labeled HA immobilized gold nanoprobes (HF-AuNPs) were utilized for sensitive detection of intracellular ROS generation. The gold nanoprobes emitted fluorescence signals by specific cleavage of grafted HA chains by ROS, resulting from reducing the extent of fluorescence quenching. The developed gold nanoprobes showed greater sensitivity and light-stability than commonly available organic ROS probes such as 2′,7′-dichlorodihydrofluorescein (DCFH) and 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF) [22,26]. In particular, the relative level of ROS could be directly visualized *in vitro* and *in vivo* through an optical imaging analysis, which has not been reported previously by using ROS probes based on the fluorescent organic dyes. In this study, ROS-sensitive gold nanoprobes (HF-AuNPs) were employed for evaluating the level of intracellular ROS that was the first event of cellular nanotoxicity. Macrophage cells (RAW 264.7) were incubated with 0.3 pMole of HF-AuNPs for 1 h for cellular uptake, and then various hydroxyl group functionalized PS particles with different sizes (59 nm, 480 nm, and 4.23 μm) were exposed to cells for 30 min. The fluorescence intensity within cells was quantified by flow cytometry as a function of particle size. As shown in Fig. 1A, 59-nm-PS particles induced a higher level of fluorescence signal than the other PS particles, indicating that the smallest PS particles generated the largest amount of intracellular ROS. To visualize the relative amount of intracellular ROS for the three different PS particles, the cells were observed by confocal microscopy (Fig. 1B). In the confocal images, the 59 nm-PS NPs emitted the brightest green fluorescence, followed by 480 nm- and 4.23 μm-PS NPs, which is consistent with the flow cytometry results. To prove the relationship between cytotoxicity and intracellular ROS generation, the cell viability was assayed after 1 day exposure of particles (100 μg/ml) by measuring mitochondrial