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The mechanisms of surface chemistry effects of mesoporous silicon nanoparticles on immunotoxicity and biocompatibility

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ABSTRACT

Despite steadily increasing insights on the biocompatibility of PSi nanoparticles (NPs), an extensive biosafety study on the immune and red blood cells (RBCs) is still lacking. Herein, we evaluated the impact of the PSi NPs' surface chemistry on immune cells and human RBCs both in vitro and in vivo. Negatively charged hydrophilic and hydrophobic PSi NPs caused less ATP depletion and genotoxicity than the positively charged amine modified hydrophilic PSi NPs, demonstrating the main role of PSi NPs' surface charge on the immunocompatibility profile. Furthermore, cells with lower metabolic activity, longer doubling time, and shorter half-life were more sensitive to the concentration- and time-dependent toxicity in the following order: T-cells ≈ monocytes > macrophages ≈ B-cells. RBC hemolysis and imaging assay revealed a significant correlation between the surface chemistry, the amount of the PSi NPs adsorbed on the cell surface and the extent of morphological changes. The in vivo results showed that despite mild renal steatosis, glomerular degeneration, hepatic central vein dilation and white pulp shrinkage in spleen, no notable changes were observed in the serum level of biochemical and hematological factors. This study is a comprehensive demonstration of the mechanistic direct and indirect genotoxicity effects of PSi NPs, elucidating the most influencing properties for the PSi NPs' design.

1. Introduction

Parallel to rapidly growing advances of nanoparticulate drug delivery systems [1–4], nanotoxicology studies are increasing with the aim to evaluate the biosafety of developed nanomaterials via case-by-case investigation of their effects on healthy tissues [5,6]. It is now acknowledged that due to the differences in the surface properties and markedly enhanced surface-to-volume ratio, nano-sized materials are chemically more reactive than the corresponding bulk materials. Therefore, the increasing applications of the nanomaterials in healthcare-related industries can presumably lead to unforeseeable toxicity effects [5,7,8].

Despite many promising applications currently foreseen for porous silicon nanoparticles (PSi NPs), including drug delivery, immunotherapy, and theranostics [9,10], before being applied in a clinical setting, adequate investigations regarding the NPs' interactions with the immune and red blood cells (RBCs) are needed. Although there is now abundant information regarding the biocompatibility and genotoxicity profiles of many types of NPs [11–13], the research on PSi NPs is still in its infancy [1,14–16], and thus, little is still known about their biosafety and mechanisms involved in their genotoxicity, particularly in immune cells and erythrocytes. Accordingly, since NPs made of the same parent substance can display completely different behaviors owing to the differences in the surface functional groups, surface area, pore size, shape, aggregation behavior, charge and size [17], we hypothesize that the surface chemistry of the PSi NPs may have a potential to change the cellular interactions of the particles or dictate different biological responses [18,19].

Therefore, designing a proper experimental setup can be useful to scrutinize direct or indirect DNA damage mechanism(s) of PSi NPs via analyzing different tiers of nanotoxicity, including cell membrane integrity, cell morphology, reactive oxygen and nitrogen oxide species (ROS and RNOS, respectively), tumor necrosis factor alpha (TNF-α) production, ATP depletion, and DNA proliferation [18,20,21]. For instance, the NPs may capture electrons, leading to

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ROS generation due to Fenton chemistry and dismutation reaction and, consequently, ATP content reduction, which results in indirect DNA damage as well as apoptosis activation [7,22]. Generally, the toxicity mechanisms elucidated so far include: (1) interactions with cell membranes, which cause hole formation or instability in ion transport; (2) direct interactions with cell mitochondria and ATP depletion; (3) interference with anti-oxidant defenses and ROS and/or RNOS production; (4) induction of pro-inflammatory responses; and (5) binding to nucleus and direct DNA damage [23]. It is worth pointing out that the loss of membrane integrity, oxidative stress and pro-inflammatory responses, all act via decreasing of the ATP content that finally results in indirect DNA damage [23,24].

In this paper, we aimed to demonstrate the effect of the surface chemistry and surface charge of five different types of PSi NPs, namely thermally oxidized PSi (TOPSi), thermally carbonized PSi (TCPSi), (3-Aminopropyl) triethoxysilane functionalized thermally carbonized PSi (APSTCPSi), thermally hydrocarbonized PSi (THCPSi) and undecylenic acid functionalized THPSi (UnTHCPSi), with similar size, surface area and pore volume, on the biosafety of immune cells and RBCs. In this way, we evaluated the genotoxicity mechanisms of the above-mentioned PSi NPs in Raji (B-cell), Jurkat (T-cell), U937 (monocyte) and RAW 264.7 (macrophage) cells as a function of the exposure time and NP concentration. While B-cells are the only antibody producing cells of the immune system, T cells are responsible for the immune response regulation or even direct attack to the infected or cancerous cells; monocytes are produced in very small amounts by bone marrow as the only source of macrophage generation; and macrophages play a crucial role in immune cells activation and various antigen-destroying chemicals secretion [25]. Therefore, any toxicity on the immune cells tested in this study may induce different unwanted physiological alterations and cause immunomodulation. Here, we have also measured the hematological and biochemical parameters of the PSi NPs in vivo following intravenous administration to rats. This is a comprehensive report in which the effects of the surface chemistry and charge of PSi NPs on the cytotoxicity is systematically investigated on immune cells and RBCs both in vitro and in vivo.

2. Materials and methods

2.1. Preparation and physicochemical characterization of the PSi NPs

The PSi NPs were prepared by the electrochemical anodization method as described elsewhere [2,10,14]. Details of the PSi NPs' preparation and characterization can be found in the Supporting Information.

2.2. Cell cultures

For the in vitro studies, B lymphocytes (Raji), T-cells (Jurkat), monocytes (U937) and RAW 264.7 macrophage cells were cultured according to the protocols described in detail in the Supporting Information.

2.3. SEM imaging

The cell membrane integrity as well as the morphology of the immune cells and RBCs in the presence of the PSi NPs was analyzed using SEM. Briefly, all the cells (4×10^5 cells/well) were seeded in 6-well plates in 1 mL of HBSS (pH 7.4) buffer and then mixed with 1 mL of various PSi NPs (100 μg/mL) to reach a final concentration of 50 μg/mL. Afterwards, treated cells and controls were cultured at 37 °C for 24 h. The cells were then fixed in 2.5% glutaraldehyde for 1.5 h and post-fixed in 1% osmium tetraoxide in 0.1 M NaCac buffer (pH 7.4) for 1 h. The cells were then dehydrated in increasing concentrations of 50, 70, 96, and 100% of ethanol for 5, 10, 20 and 15 min, respectively [26]. Finally, the cell suspensions were dropped onto plastic coverslips, dried, and sputter coated with platinum before being observed under SEM (Zeiss DSM 962).

2.4. ROS, RNOS and TNF-α determination

The protocols performed to determine the oxidative stress and pro-inflammatory responses have been described elsewhere [10,14,15,17], and are comprehensively described in Supporting Information.

2.5. ATP activity

To determine the ATP activity of the cells as an indicator of metabolically active cells [14,17], CellTiter-Glo® reagent (Promega Corporation, Fitchburg, USA) was used according to the manufacturer's protocol as described in Supporting Information.

2.6. Genotoxicity analysis

The detection of genetoxicity by the PSi NPs was made using the BrdU (5-bromo-2′-deoxyuridine) ELISA based assay kit (Millipore, Corporation, MA, USA). For this assay, 100 μL of the cells were plated and cultured at a concentration of 2×10^5 cell/mL in 96-well plates. Various concentrations (25, 50 and 100 μg/mL) of all five different types of PSi NPs in HBSS (pH 7.4) were then added to each well. According to the manufacturer's protocol, the cells were further supplemented with 20 μL of the BrdU reagent and incubated for 6 and 24 h at 37 °C. At certain time points, the cells were fixed with a fixative solution and the DNA was denatured in one-step by adding 200 μL/well of the fixative solution and incubation at room temperature for 30 min. After three-time washings, 100 μL/well of anti-BrdU monoclonal antibody was added to bind to the BrdU in the newly synthesized cellular DNA. Then, peroxidase labeled goat anti-Mouse IgG was added to make immune complexes detectable after 30 min incubation with 100 μL/well of TMB peroxidase substrate at room temperature in the dark. Finally, the reaction product was quantified by the addition of 2.5 N sulfuric acid stop solution and measuring the absorbance using a microplate reader (Varioskan Flash) at a wavelength of 450 nm. HBSS and 1% Triton X-100 treated cells were used as negative and positive controls, respectively. Each experiment was carried out in triplicate for each PSi NP concentration and each cell-type tested.

2.7. Hemolysis and PSi NPs–RBC membrane interactions

Heparin-stabilized fresh human blood was obtained from anonymous donors from the Finnish Red Cross Blood Service and used within 2 h. A 5-mL sample of the whole blood was mixed gently with 10 mL of Dulbecco's phosphate-buffered saline (D-PBS) before isolating the RBCs from serum by centrifugation at 3000 rpm for 6 min. The RBCs were then washed further for five times with sterile D-PBS solution. After washing, 2 mL of the RBCs was diluted to 40 mL by adding D-PBS (5% hematocrit) [27,28]. Next, 0.1 mL of the diluted RBC suspension was added to 0.4 mL of the PSi NP suspensions in D-PBS to a final concentration of 25, 50, 100, and 200 μg/mL. The suspension obtained was gently vortexed before incubating at static condition at room temperature for 1, 4, 8, 24, and 48 h. Afterwards, the samples were gently vortexed again and centrifuged at 13,000 rpm for 3 min. 100 μL of the supernatant was transferred to a new 96-well plate to measure the absorbance values of hemoglobin at 577 nm with a reference wavelength of 655 nm using a microplate reader. D-PBS and water (0.4 mL) were used as negative and positive controls, respectively.

To evaluate the morphological changes and also the PSi NP–RBC interactions, the diluted RBC suspension (5% hematocrit, 0.1 mL) was mixed with PSi NPs in PBS (0.4 mL) at the final concentration of 100 μg/mL and incubated at room temperature for 4 h. The samples were then fixed with 2.5% glutaraldehyde and further incubated at 37 °C for 1 h, followed by post-fixation using 0.5% osmium tetroxide in PBS for 1.5 h. Prior to SEM imaging, the samples were prepared as described in Section 2.3.

2.8. In vivo biochemical and hematological analyses

For these experiments, 18 adult male Sprague–Dawley rats weighting between 250 and 275 g were cared according to the standard guidelines and the whole animal study protocol was reviewed and approved by the Ethics Committee of Zanjan University of Medical Sciences. 5 days before the study, all animals were kept at ventilated temperature-controlled animal room (20 ± 2 °C), with relative humidity of 60 ± 10%, and a 12-h light/dark daily cycle. During this period, the animals were housed in standard polycarbonate stainless steel wire-topped cages with free access to rat chow and water ad libitum. The rats were randomly divided to 6 groups of 3 animals: control group (treated with normal 0.9% sodium saline) and five experimental groups to receive a single injection of all five types of PSi NPs (700 μg/kg) via tail vein. After 24 h, the rats were anesthetized and the plasma samples were collected by cardiac puncture and analysed for kidney, liver and spleen function by measuring the serum level of different biochemical and hematological parameters. The blood counts were measured within 4 h post-collection, and the plasma biochemistry determinations were made on the same day of the blood collection.

2.9. Histopathological experiments

For the histological analyses, tissue specimens from the liver, kidney and spleen of the rats were fixed in 10% solution of formalin in PBS and then processed routinely by embedding in paraffin. Afterwards, 5 μm sliced tissues were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus BH-2, Tokyo, Japan).