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Research Paper

Cytotoxicity assessment of porous silicon microparticles for ocular drug delivery

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ABSTRACT

Porous silicon (PSi) is a promising material for the delivery and sustained release of therapeutic molecules in various tissues. Due to the constant rinsing of cornea by tear solution as well as the short half-life of intravitreal drugs, the eye is an attractive target for controlled drug delivery systems, such as PSi microparticles. Inherent barriers ensure that PSi particles are retained in the eye, releasing drugs at the desired speed until they slowly break down into harmless silicic acid. Here, we have examined the *in vitro* cytotoxicity of positively and negatively charged thermally oxidized (TOPSi) and thermally carbonized (TCPSi) porous silicon microparticles on human corneal epithelial (HCE) and retinal pigment epithelial (ARPE-19) cells. In addition to ocular assessment under an inverted microscope, cellular viability was evaluated using the CellTiter Blue™, CellTiter Fluor™, and lactate dehydrogenase (LDH) assays. CellTiter Fluor proved to be a suitable assay but due to non-specific and interfering responses, neither CellTiter Blue nor LDH assays should be used when evaluating PSi particles. Our results suggest that the toxicity of PSi particles is concentration-dependent, but at least at concentrations less than 200 μg/ml, both positively and negatively charged PSi particles are well tolerated by human corneal and retinal epithelial cells and therefore applicable for delivering drug molecules into ocular tissues.

1. Introduction

The eye is a challenging organ to treat. The cornea is constantly being washed by tear solution rinsing off topically applied therapeutic compounds from the eye. Retinal diseases, in turn, are challenging to treat since posterior parts of the eye are not easily reached by drugs. Age-related macular degeneration (AMD) and diabetic retinopathy (DR) are two chronic retinal eye diseases and in their most severe forms, they trigger excessive production of vascular endothelial growth factor (VEGF) by the retinal pigment epithelium (RPE) [1–3]. The RPE is a multifunctional single-cell layer at the back of the eye e.g. it protects the retina from excess light, contributes to the formation of the blood-retinal barrier, maintains the functionality of photoreceptors, as well as plays a principal role in the immune defense of macula [4]. Increased VEGF levels result in blood–retinal barrier breakdown and neovascularization *i.e.* the formation of abnormal new blood vessels that can burst and bleed causing edema and a rapid blurring of the vision [5,6]. Currently, the progression of wet AMD can be decelerated by intravitreous injections of VEGF antibodies [7–9]. However, there are many problems associated with this treatment not least the need for repeated injections over several months or even years, which is unpleasant for both the patients and the nursing staff and furthermore the treatment is associated with high financial costs. Moreover, continuous injections can damage the eye and trigger inflammatory responses [10,11].

Various options, such as biodegradable polymers, encapsulated cell technology, reservoir implants, gene therapy, medicated contact lenses, and nano- and micro-particles and films, have been developed in order to achieve a sustained release of ocular drugs and decrease the need for repeated intraocular injections [12–16]. Porous silicon (PSi) is a versatile material, which has found diverse technical applications; it has been incorporated into electrical or optical sensors for decades. Currently, the biomedical applications of PSi are an area of intensive research, such as in

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the delivery of hydrophobic drugs with low bioavailabilities [17–23]. The suitability of PSi-based materials for ocular medication has also been evaluated [24–36]. In addition to good general tolerability and ease of modification, PSi possesses a major advantage in that it has good biodegradability into harmless silicic acid ($Si[OH_4]$), a material that is also naturally present in tissues [18,25,32,37,38]. However, the high natural reactivity of PSi does require some surface treatments in order that it can be tolerated by cells [38]. For example, non-treated PSi particles killed human lens epithelial cells by producing reactive oxygen species (ROS), whereas the cells in contact with thermally oxidized PSi (TOPSi) microparticles remained viable [39]. Moreover, TOPSi membranes induced only a mild host response when implanted into the rat eye [25].

In addition to tolerability, surface modification contributes also to the degradation rate, cell adhesion, and drug release properties of PSi materials [40]. TOPSi was found to be more effective than either thermally hydrocarbonized (THCPSi) or undecylenic acid-treated THCPSi particles in its ability to release a peptide [41]. Collagen-coated and aminosilanized PSi has been shown to promote the adhesion of human lens epithelial cells, whereas three other forms, non-treated, ozone-oxidized, or polyethylene glycolsilanized PSi, were not so effective [39,40]. In a recent study, oxidized PSi particles additionally grafted with 8-hydrocarbon chains ($PSiO_2$-C8) displayed better carrier degradation, drug-loading capacity, and the release of active rapamycin than achieved with two other types of surface modifications (PSi-COOH and PSi-C12) [34]. Since the different drugs to be loaded may possess different charges, both positively and negatively charged surface modifications are needed. Positively charged, amino-grafted PSi particles have been shown to adsorb a negatively charged peptide more avidly than negatively charged TOPSi and TCPSi particles [42].

In the present study, we have examined the tolerability of negatively charged TOPSi and TCPSi microparticles, as well as their positively charged amino-grafted counterparts NH_2-TOPSi and NH_2-TCPSi by human corneal epithelial (HCE) and retinal pigment epithelial (ARPE-19) cells. In particular, there is very little known about the suitability of positively charged PSi particles for ocular drug delivery. Since PSi may cause spontaneous redox reactions or adsorb assay dyes or reporter enzymes [40,43,44], we have measured cellular viability using three different assays.

2. Experimental section

2.1. Cell cultures

The human corneal epithelial (HCE) cell line has been kindly donated by Dr. Hitoshi Watanabe (Osaka University, Osaka, Japan) [45]. The cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C in Dulbecco's Modified Eagle Medium/Nutrient mixture F12 (1:1) growth medium (DMEM/F12; Life Technologies, Paisley, UK) containing 15% inactivated fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies), 5 μg/ml insulin (Sigma–Aldrich, Steinheim, Germany), 0.1 μg/ml cholera toxin (Calbiochem; San Diego, CA, USA), 10 ng/ml epidermal growth factor (EGF; Sigma), 0.5% dimethyl sulfoxide (DMSO; Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Cells from passages 32 to 49 were used in the viability assays.

Human retinal pigment epithelial (ARPE-19) cells were purchased from the American Type Culture Collection (ATCC). The cells were cultured in DMEM/F12 (1:1) growth medium (Life Technologies) containing 10% inactivated FBS (Hyclone, Logan, UT, USA), 2 mM L-glutamine (Lonza, Walkersville, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (both from Lonza) at 37 °C in humidified conditions with 5% CO_2. Cells from passages 6 to 19 were used in the viability assays.

2.2. Preparation of mesoporous silicon microparticles

Porous silicon was etched on a silicon wafer (Okmetic Oyj, Vantaa, Finland) (0.01–0.02 Ω cm) in a 1:1 mixture of HF (39%) and ethanol (99.5%) at a current density of 50 mA/cm^2 for 40 min. A high current pulse was applied at the end of the etching to detach the porous film. Porous films were milled into microparticles and sieved into either 25–53 or 53–75 μm size fractions.

The microparticles were oxidized at 700 °C for 7.5 min in order to coat the TOPSi particles with an oxide surface [46]. The particles were then further oxidized in the liquid phase [SC1 (standard clean) and SC2] to produce a high density of –OH groups on the surface [46]. Thermally carbonized particles were prepared by quickly treating the sieved particles with a 1:1 mixture of HF (39%) and then thermally carbonizing the particles with acetylene in a two-step process as described elsewhere [47].

Amine functionalization was performed by silanizing the TOPSi and TCPSi particles with propyldimethoxymethylsilane (AEAPMS) as described elsewhere [6]. TCPSi particles were also oxidized with SC1 and SC2 solutions before amine functionalization. The particles were sterilized with 70% ethanol and dried at 65 °C for 1 h before the cell experiments.

Nitrogen sorption measurements (Tristar II, Micromeritics, USA) were performed to determine pore size, pore volume and surface area of the particles by applying BJH-theory, single point measurement and BET theory, respectively. The surface chemistry of the samples was characterized by Fourier-transform infrared spectroscopy (FT-IR, Thermo Scientific Nicolet 8700).

2.3. Exposure of cells to microparticles

In the CellTiter-Blue and CellTiter-Fluor assays, HCE cells were seeded in the growth medium at a density of 1×10^4 cells per well and ARPE-19 cells at a density of 1.5×10^4 cells per well on 96-well culture plates (Corning Inc., Corning, NY, USA). In the evaluation of the release of lactate dehydrogenase (LDH) enzyme, 2×10^5 HCE or ARPE-19 cells were seeded per well in the growth medium on 12-well culture plates (Corning Inc., Corning, NY, USA). After a 24 h (96-well plates) or 48 h (12-well plates) incubation in a humidified incubator at 37 °C, the cells were washed once, resuspended in the culture medium containing all of the supplements except serum and incubated for another 24 h. Thereafter, the serum-free exposure medium also lacking cholera toxin and DMSO for HCE cells was replaced with fresh medium containing the mesoporous silicon microparticles at final concentrations of 5, 50, 100, and 200 μg/ml. The cells were exposed to microparticles in a humidified cell incubator at 37 °C for 24 h. In order to determine the possible interference of mesoporous silicon particles with the assay reagents, cell-free particle incubations were also performed on the plates.

2.4. Cellular viability assays

Just prior to collection of medium samples, cellular viability was observed and photographed under an inverted microscope (Zeiss Axio, Germany). Medium samples were collected into microtubes and centrifuged at 380*g* for 10 min. Supernatants were transferred into clean tubes, and stored at −70 °C. The LDH activity was measured from fresh samples prior to freezing.

CellTiter-Blue™ and CellTiter-Fluor™ assays (Promega Corporation, Madison, WI, USA) were performed according to the manufacturer's protocols. Fluorescence was measured using a multilabel reader (EnVision 2104 Multilabel Reader, Perkin Elmer) with exci-