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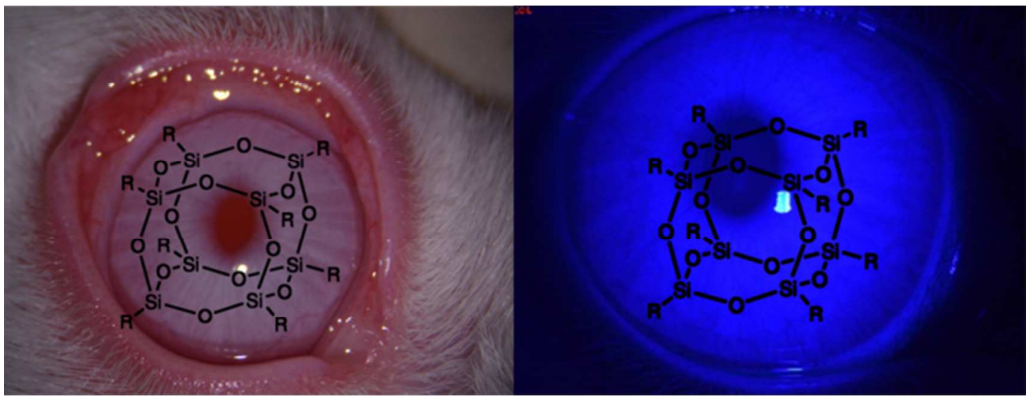
The ocular biocompatibility of polyhedral oligomeric silsesquioxanes (POSS) was systematically evaluated for ocular biomedical devices applications.



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Ocular biocompatibility evaluation of POSS nanomaterials for biomedical material applications

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The tremendous advancement of polyhedral oligomeric silsesquioxanes (POSS) has been focused on the field of biomaterial applications including tissue engineering, drug delivery, biomedical devices and biosensors. More recently, POSS have been used as components of ophthalmic biomedical devices, such as contact lenses and intraocular lenses due to their chemical inertness and transparency. The systematical biocompatibility evaluation of the POSS nanomaterials is thus essential. Herein, ocular biocompatibility and cytotoxicity of POSS nanomaterials were investigated both in vitro and in vivo. Three types of commercial POSS nanomaterials with different functional groups were utilized in this research, including Aminoethylaminopropylisobutyl-POSS (NH_2-POSS), Mercaptopropylisobutyl-POSS (SH-POSS) and Octahydroxypropyldimethylsilyl-POSS (HO-POSS). The cellular metabolic activity, membrane integrality, cell apoptosis and oxidative damage were tested on Human lens epithelial cells (HLEC) under different concentration of POSS nanomaterials exposure. The ocular irritation on rabbit eyes was measured as well. The results show that the studied POSS nanomaterials do not cause any significant toxicity to the cell growth and proliferation in most case except the NH_2-POSS, which decreases the cellular viability at high concentration. All the POSS nanomaterials slightly induced oxidative stress results in the increasing of Reactive Oxygen Species (ROS), whereas it doesn't generate cell apoptosis evidently. The animal experimental results also show that no acute ocular irritation can be detected after POSS nanomaterials administration. These results indicate the good ocular biocompatibility of the POSS nanomaterials in most case, which have great potential in ocular biomedical applications.

Introduction

Since their discovery in the 1964s by Scott, polyhedral oligomeric silsequioxanes (POSS) have gained an increasing attention due to their thermal, mechanical and electrical properties.[1, 2] POSS are a class of cage-shaped inorganic-organic hybrid molecular, which have grown dramatically in recent years. Their chemical structure is a formula unit between SiO_2 (silica) and R_2SiO (silicone). It refers to the typical formula $(RSiO_{3/2})_n$, where n can be an even integer 6, 8, 10 (denoted as T6, T8, T10). Among them, the general POSS molecules $(RSiO_{3/2})_8$ have been the most prevalent system investigated and broadly deem as the smallest silica at 1-3 nm. POSS are really hybrid inorganic-organic chemical materials that consist of an inorganic silica core (0.53 nm) and organic substituent groups, i.e., R-groups which possibly are organic functional groups such as an alkyl, acrylate, sulfonate, amine, hydrogen, hydroxyl, methyl, and so on. The presence of different R-groups subsequently realize to complicate and uncontrollable functions.[3-5]

A mass of researches have shown that POSS molecules incorporated as building blocks into polymers have been synthesized to enhance glass-transition temperatures, thermal stability, antimicrobial, mechanical stiffness and electrical properties.[4, 6, 7] The incorporating POSS improve the oxidation resistance, and reduce flammability, inflammatory reactions and oxygen permeability, which the main advantages for biological application.[8] Thus, it is not doubt that POSS polymers have been widely applied in various fields, especially biomedical application including tissue engineering, drug delivery, biomedical devices and biosensors.[5, 9] POSS nanomaterials integrated into poly (carbonate-urea) urethane (PCU) have been successfully used in the first synthetic trachea in the world, which known as the first-in-man implants.[10] A vascular grafts produced from POSS-PCU implanted into senescent sheep assessment with 64% patency rate.[11] A novel lacrimal drainage conduit constructed with POSS-PCU offer good compatibility to restore blockage of the tube in lacrimal surgery.[12] What's more, POSS are considered as an attractive candidates for ophthalmic applications such as contact lens and intraocular lens (IOL), in terms of their chemical inertness, transparency, biological properties.[3, 12] Previous research have found that POSS materials are able to form higher transparent materials that means have good light transmissions.[13, 14] The light transmission of POSS is suitable for optical materials especially contact lens and IOL.[15] In our previous study, POSS

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contained composite materials result in better transparency and good thermodynamic stability suggesting its priority as IOL materials.[14, 16]

The human eye is a sophisticated and sensitive organ. With increasing potential of POSS nanomaterials in ocular biomaterials,[14-16] the systematically ocular biocompatibility of POSS nanomaterials should be evaluated. Three kinds of POSS nanomaterials with different functional groups, including amino group, thiol group and hydroxyl group, were taken to investigate the ocular biocompatibility in this research. Human lens epithelial cell (HLEC) plays important roles in ocular physiological function. HLEC is located in the anterior surface of the crystalline lens, and is critical for lens formation, physiology and transparency, as well as the key reason for cataractogenesis.[17, 18] The dysfunction of HLEC may result in the posterior capsular opacification after the IOL implantation. So HLEC was used for cytotoxicity measurement of POSS nanomaterials. The toxicity, cellular membrane integrity, apoptosis and oxidative stress of HLEC, as well as ocular irritation on rabbit eye under POSS nanomaterials exposure were investigated for systematically evaluation of the ocular biocompatibility.

Experimental section

Materials

Octahydroxypropyldimethylsilyl-polyhedral oligomeric silsesquioxane (HO-POSS, Mw = 1482.60 g/mol), Aminoethylaminopropylisobutyl-polyhedral oligomeric silsesquioxane (NH_2-POSS, Mw = 917.65 g/mol), Mercaptopropylisobutyl-polyhedral oligomeric silsesquioxane (SH-POSS, Mw = 891.63 g/mol), and 2, 7-Dichlorodihydro-fluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (United States). Dulbecco's modified Eagle's medium (DMEM): F12 (1:1), Fetal Bovine Serum (FBS), Hank's Balanced Salt Solution (HBSS), 0.05% Trypsin-EDTA and penicillin-streptomycin were brought from Life technology (United States). Cell counting kit-8 (CCK-8), Lactate dehydrogenase release assay kit (LDH assay), and Hoechst staining kit were provided by Beyotime Institute of Biotechnology (China). Human lens epithelial cell line (HLE B3, CRL-11421™) was originated from American Type Culture Collection (ATCC). 200 mg/mL POSS nanomaterials stock solution were dissolved in tetrahydrofunan, and diluted by cell culture medium as used.

Cell culture

HLEC was cultivated in DMEM:F12 (1:1) media which supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin solution. The cells were maintained in an incubator with a humidified air of 5% CO_2 at 37℃, displacing media every two days. When the cells were grown 80% confluence, 0.05% Trypsin-EDTA was utilized to cell passage.

Cell membrane integrality assay

Cell membrane integrality was measured by LDH leakage measurement, according to the kit protocol. Briefly, after exponentially growing cells were collected, 5×10^3 cells were seeded into 96-well plates and incubated at 37℃ over night. The next day, dose- and time- dependent surveys of LDH assay was carried out by treating with 5, 10, 50, 100, 150, 200 mg/L of NH_2-POSS, SH-POSS, HO-POSS for 24 h, 48 h, 72 h. One hour before the appointed time, 10% LDH cell lysis solutions were added in the cells without POSS, which were regarded as total releasing samples. The untreated cells (without lysis solution and POSS treating) were regarded as spontaneous releasing samples. The plates were then centrifuged at 300g for 5 minutes. Aliquots of Cell supernatants without cell were transferred to new 96-wells plates, mixed with LDH working solution at room temperature in the dark and the optical density (OD) was read by a Microplate Reader at 490 nm. The rate of LDH release (%) were calculated as follows: LDH release (%) = $(OD_{test}-OD_{spontaneity}) / (OD_{total}-OD_{spontaneity}) \times 100\%$.

Cell viability assay

Cell viability on the base of mitochondrial function was measured by CCK-8 assay. HLEC, was plated into 96-well plates at 5×10^3 cells per well and cultured overnight. Afterwards, the primary media were discarded and fresh media were added which contained different concentrations of POSS (NH_2-POSS, SH-POSS, HO-POSS, as shown in Fig. 1). The concentrations of POSS nanomaterials were 5, 10, 50, 100, 150, 200 mg/L, respectively. Cells without treatment were considered as control. At the end of the incubation time (24 h, 48 h, 72 h), the cells were washed twice with PBS and incubated with CCK-8 for 2 h at 37℃. The OD was monitored by a Microplate Reader at 450 nm. The cell viability (%) was calculated by the following equation: Cell viability (%) = $OD_{test}/OD_{control}\times100\%$.

ROS secretion

The intracellular ROS secretion was detected by applying fluorescence probes DCFH-DA as previous report.[19] HLEC was seeded a density of 5×10^4 cells/mL were plated on 6-well plates overnight. Then, the cells were incubated with 200 mg/L different POSS nanomaterials (NH_2-POSS, SH-POSS and HO-POSS, respectively) for 24 h, followed by DCFH-DA staining at 37℃ for 30 minutes. The fluorescent images were taken by fluorescence microscope.

Cell apoptosis

Apoptosis were often in fragmentation or condensation of nuclear, as a result that the normal cells showed blue, while the apoptosis cells showed uniform dispersion of fluorescent particles or brighter even whitish by Hoechst staining. Hoechst 33258 staining kit was used to determine HLEC apoptosis under POSS exposure. Briefly, 5×10^4 cells of HLEC were grown in 24-well plates for 24h. The final concentration of 200mg/L POSS media solutions were replaced and induced 24h. Cells were stained by Hoechst staining kit and visualized by fluorescence microscope.

Acute ocular irritation

The main text of the article should appear here with headings as appropriate. The approval of Laboratory Animal Ethics Committee of Wenzhou Medical University for animal study was obtained. Japanese White rabbits with weights from 2.0 kg-3.0 kg were obtained from Animal Administration Center of Wenzhou Medical University and raised at 22 ± 3℃, 40 to 70%

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humidity environments. The acute ocular irritation of POSS nanomaterials were carried out in accordance with the GBZ/T240.5-2011 and Organization for Economic Cooperation and Development (OECD) Test Guidelines 405. After the carefully health check, the rabbits' eyes were instilled with 100 μL POSS nanomaterials drops (200 μg/mL) on the right eyes. The left eyes were dropped with normal saline as control. The rabbits were weighted, and ophthalmological observations were performed on cornea, iris or conjunctiva at 0 h, 1 h, 6 h, 24 h, 48 h, and 72 h by fluorescein staining and slit lamp microscopes.

Statistical analysis

All data are presented as the average ± standard deviations (SD) ($n \geqslant 3$). Statistical significance ($p < 0.05$) was analyzed with Statistical Product and Service Solutions (SPSS) Software using one-way or two-way ANOVA analysis.

Results and discussion

The advances in nanomedicine have proposed novel therapeutics and diagnostics, which can potentially revolutionize current medical practice. POSS with a distinctive nano-cage structure consisting of an inner inorganic framework of silicon and oxygen atoms, and an outer shell of organic functional groups, are one of the most promising nanomaterials for medical applications.[20-23] Enhanced physicochemical properties and biocompatibility have resulted in the development of a wide range of nanocomposite POSS copolymers for biomedical applications, such as the development of biomedical devices, tissue engineering scaffolds, drug delivery systems, dental applications, and biological sensors in past few years.[12] More recently, POSS nanomaterials were found to be the prospective materials for ophthalmic applications such as contact lens and IOL due to their chemical inertness, transparency and oxygen permeability.[14-16] So it is necessary to investigate the ocular biocompatibility of the POSS nanomaterials deeply. Although the in vitro cytotoxicity assays including viability, cell membrane integrity, or apoptosis provide informative messages on the biocompatibility of the POSS nanomaterials, they can't reflect the in vivo response of the nanomaterials, as each of the test only presents one endpoint of cell response. A combination of several tests may provide more detailed information of the biocompatibility of the nanomaterials.

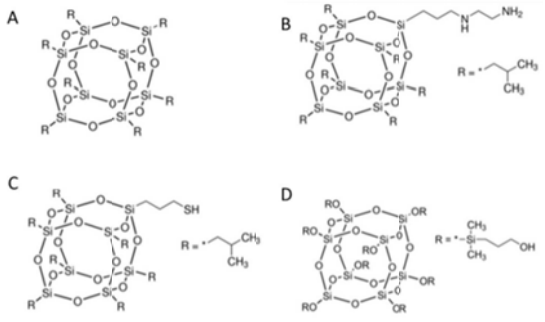

Fig. 1. The schematic structure of POSS (A), NH_2-POSS (B), SH-POSS (C) and HO-POSS (D).

Herein, the ocular biocompatibility of POSS nanomaterials with different functional groups, including amino, hydroxyl and thiol groups (as shown in Fig. 1), was systematically assessed by testing the damage to the ocular cell membrane, mitochondrial dehydrogenase viability or ROS secretion in cytoplasm and apoptosis via nucleus, as well as the animal ocular surface irritation.

The HLEC cell membrane integrity under POSS nanomaterials exposure was invested by LDH assay. When negative effects happened, the cells would result in membrane rupture, and for that reason cytoplasmic enzyme leaked out which including stable LDH.[24] The released LDH activity was determined by coupled enzymatic reaction via LDH kit. Fig. 2 shows that the LDH release rate against NH_2-POSS, SH-POSS and HO-POSS in different concentration and culture time. No time dependence on cell membrane integrity was observed. The LDH release rate is in the range of ± 5% even after incubation in 200 mg/mL POSS for 72 h, suggesting low cell membrane damage of POSS.

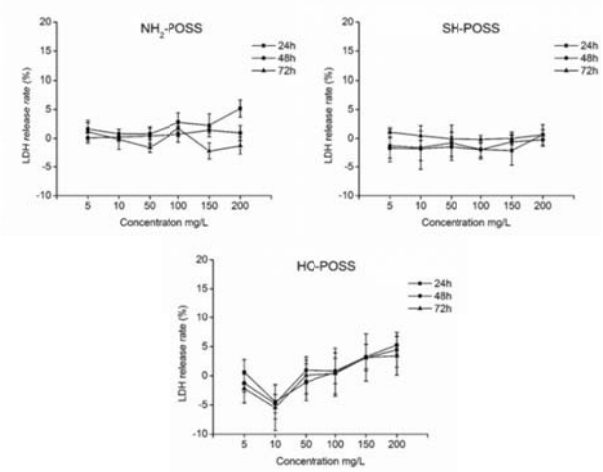

Fig. 2. LDH release rate of NH_2-POSS, SH-POSS and HO-POSS (5-200mg/mL) on HLECs at 24h, 48h and 72h measured by LDH assay.

The HELC viability in POSS-incorporated culture medium was measured by CCK-8 assay. The same as the MTT assay,[25, 26] CCK-8 assay is based on the reduction of water-soluble tetrazolium salt to formazan by mitochondrial dehydrogenase. As shown in Fig. 3, the cell viability of SH-POSS and HO-POSS were higher than 90% for three days in the detecting concentrations, indicating the high cell viability of HLEC under SH-POSS and HO-POSS exposure. However, the NH_2-POSS have different effects on the cells. The cell viability of HLEC under NH_2-POSS exposure was above 100% when the nanomaterials concentration was under 10 mg/L in observation periods. Whereas the cell viability significant decreases to 47.6%, 44.0%, 35.8% and 24.0% in concentrations of 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L at 24h. The prolonged culture time induced the lower cell viability. The viability decreased to 12% in concentration of 200 mg/L when cultured for 72 h. These results demonstrated that the SH-POSS, HO-POSS or low-dose of NH_2-POSS ($\leqslant$10 mg/L) presents low cytotoxicity on HELC, whereas high-dose of NH_2-POSS ($\geqslant$50 mg/L) have noticeable cytotoxicity on HLEC.

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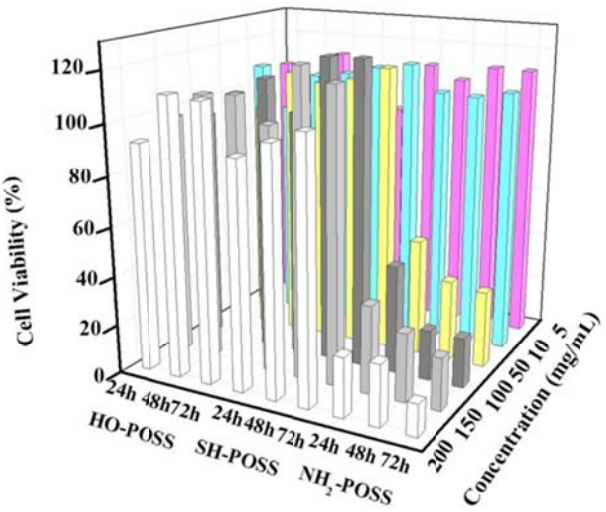

Fig. 3. Cell viability of NH_2-POSS, SH-POSS and HO-POSS on HLECs.

ROS are chemically reactive molecules containing oxygen, including oxygen ions, superoxide anion, peroxides and hydrogen peroxide. ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis.[27] However, during times of environmental stress, ROS levels can increase dramatically, which may result in significant damage to cell structures.[27] This is known as oxidative stress. Herein, the POSS nanomaterials induced oxidative stress on HLEC is elucidated via DCFH-DA staining assay.[28] The method determined on the basis of the oxidization of cell permeable DCFH-DA without any fluorescence to the highly fluorescent DCF upon reaction with cellular ROS.[29] The cells were incubated with POSS nanomaterials with different functional groups for 24 h, followed by DCFH-DA staining and fluorescence microscope observation. As shown in Fig. 4 A, low level of ROS secretion is presented in native cells, whereas the POSS nanomaterials exposure induced the ROS up-regulation in the cytoplasm (Fig. 4 B, C, and D). It can be observed that the cells exposed with HO-POSS have less ROS secretion, compared with which exposed with NH_2-POSS or SH-POSS.

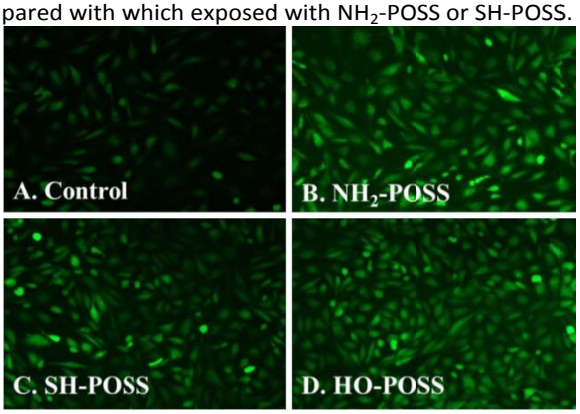

Fig. 4. Fluorescence micrographs of DCFH-DA stained HLEC cells (A), and cells incubated with 200 mg/mL NH_2-POSS (B), HO-POSS (C) or SH-POSS (D), for indicating the intracellular ROS level.

To further investigate the cytotoxicity of the POSS nanomaterials, the cell nucleus damage is investigated. The nucleus damage of the cells may cause cell apoptosis, which can be defined in the light of nuclear morphology by Hoechst 33258 staining. As shown in Fig. 5, the regular cells have regular contours and homogenously nucleus showing normal blue stained, but the apoptotic cells exhibit nuclear pyknosis, condensed chromatin and fragmented nucleus, which shows enhanced brightly blue signal (arrows in Fig. 5). The control cells without POSS exposure exhibit rarely hyper-condensed and shrinkage nucleus with apoptosis rate of 2.3%, suggesting the basic level of apoptosis. When treated with 200 mg/L POSS nanomaterials for 24 h, slight increase of the apoptotic cells is found. The apoptosis rates are in the range of 3.1% to 5.9%, which are at low cell apoptosis level. This result indicates that although the POSS nanomaterials incubation may slight increase the nucleus damage, the cell apoptosis rates of different functional groups are in the low level (< 5%).

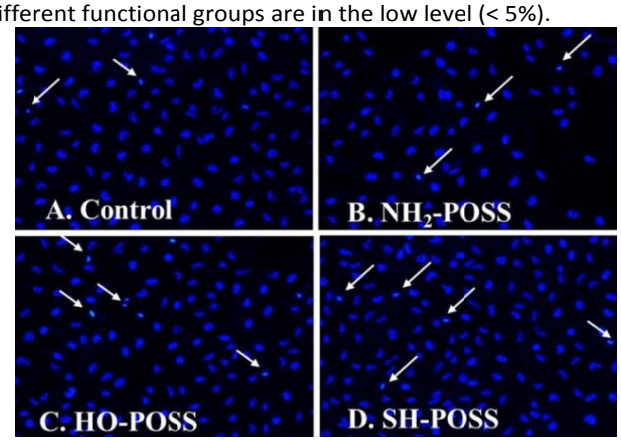

Fig. 5. Fluorescence micrograph of Hoechst 33258 staining of HLEC (A) and cells incubated with 200mg/L NH_2-POSS (B), HO-POSS (C) and SH-POSS (D) for 24h. (White arrows indicate cell apoptosis). (200×)

Table 1. Summary of the damage of NH2-POSS, SH-POSS and HO-POSS to HELC.*

Group	Cell membrane integrity Damage	Mitochondrial dehydrogenase viability	Oxidative stress	Nucleus damage
NH_2-POSS	-	++	+	-
SH-POSS	-	-	+	-
HO-POSS	-	-	+	-

*(+) indicates the POSS with significant responses, while (-) indicates the POSS without significant harmful responses.

The results of the cytotoxicity assessments are summarized in Table 1. When nanomaterials are exposed to cells and cause damage, the first step is the damage to the cell membrane. The nanomaterials may enter cells via endocytosis, and causing some damage to the cell membrane integrity. The LDH releasing assay is a sensitive target for membrane integrity. The damage of the membrane may cause the release of LDH

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into the cell culture medium. As shown in Fig. 2, very slight damage to the cell membrane is detected when POSS nanomaterials are incubated with HLEC. No matter the POSS with functional groups of amino group, thiol group, or hydroxyl group, the LDH release rate is lower than 5%, which indicates the non-cytotoxicity to the cell membrane. However, after endocytosis, the POSS may cause some damage to cellular pathways in cytoplasm. The induced ROS secretion is up-regulated after POSS incubation, which may further induce the cell dysfunction. The generation of ROS has been reported as one of the key molecular mechanism of cellular responses and a main factor in determining toxicity.[30] Oxidative stress induced by in vitro exposure nanoparticles leading to the production of ROS.[31] However, if the oxidative stress beyond the level of protective mechanisms, cell death will be programmed via apoptosis, autophagy or necrosis.[32] As expected, the ROS level of HLEC on the three POSS is increased evidently. The secretion of ROS when cells are exposed with nanomaterials may due to the cell self-protection.[33] As a result, although all of the three kinds of POSS nanomaterials induce the ROS up-regulation, not all of them cause strong cytotoxicity. Only the amino group ended POSS show evident decrease to the cell viability. The cytotoxicity of the NH_2-POSS on HLEC shows time depended or concentration depended manner. The low concentration does not show evident toxicity on HLEC, whereas the high concentration of NH_2-POSS renders strong cytotoxicity. The cytotoxicity increase with the incubation time increasing. The activity of mitochondrial dehydrogenase decreases to 12% when incubated with 200 mg/L NH_2-POSS and cultured for 72 h. However, the viability of the HLEC is as high as 90% to 110% of the control when exposed with HO-POSS or SH-POSS. Surface groups can make different functions containing hydrophilic or hydrophobic, cationic or anionic.[34] Stronger cytotoxicity of amino-terminated materials is also presented in other particles.[35] In aqueous solution, amino group shows a higher degree cationic charge than thiol group, while hydroxyl groups is neutral. Positive charge constructs show stronger cytotoxicity than negative ones.[36] On one hand, positive groups electrostatically attracted to the negatively plasma membrane, thereby interfere with cellular natural activity.[37] On the other hand, positive ones are often easy taken up but higher uptake related to greater toxic effects.[38] Although NH_2-POSS is found to have strong cytotoxicity on cell viability, it does not show greater damage to the cell nucleus, compared with the HO-POSS and SH-POSS at same concentration. According to the Hoechst staining, it is observed that significant apoptosis were found neither in the POSS group nor in the control group. Only slight increase of nuclear pyknosis is observed in all of three kinds of POSS. The induced cell apoptosis rate is below 6% in all the cases.

In present study, notable up-regulation of ROS is found when POSS nanomaterials exposure whereas only NH_2-POSS at high concentration is found to have strong cytotoxicity in cell viability, let alone that almost no damage to the cell membrane and the nucleus is detected in all the investigated POSS nanomaterials. Considering that the toxic pathway of the nanoparticles may attribute to the cell autophagy or necrosis rather than apoptosis,[39, 40] the cytotoxicity of POSS on HLEC maybe cause cell death through autophagy or necrotic instead of apoptosis.[26] The cytotoxicity mechanism of POSS that ROS is rising under oxidative stress as an initiating response which maybe not result in apoptosis but rather protect cells from damage and further studies required to verify the mechanism.[27]

For ocular biomedical application purposes, the acute ocular irritation of POSS nanomaterials on rabbits is further carried out. Typically, the rabbits' eyes were instilled with 100 μL POSS drops (200 μg/mL) on the right eyes. The left eyes were dropped with normal saline as control. The rabbits were weighted, and ophthalmological observations were performed on cornea, iris or conjunctiva at 0 h, 1 h, 6 h, 24 h, 48 h, and 72 h by fluorescein staining and slit lamp microscopes. Fig. 6 shows the weight changes after the POSS nanomaterials administration. It is obviously that the weights of the animals do not lighten in the experimental periods. The slit lamp observation results (Fig. 7) show that neither acute inflammation nor cornea epithelial lesion is detected after POSS nanomaterials exposure for three days. No significant irritation on the cornea, iris or conjunctiva is found in all the experimental animal eyes when administrated by all the three kinds of POSS nanomaterials. These results show that the POSS nanomaterials have excellent in vivo biocompatibility to ocular tissues.

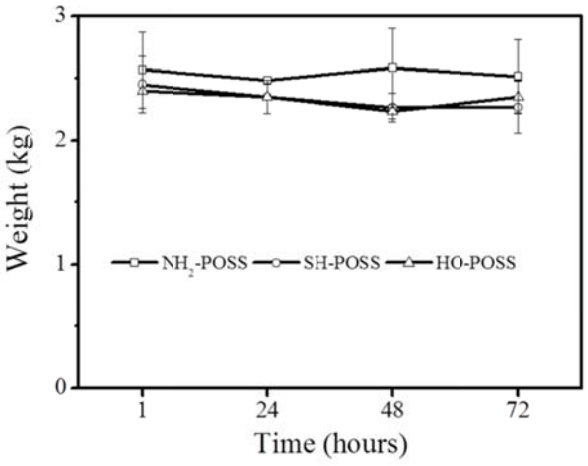

Fig. 6: The weight changes of rabbits after ocular irritation.

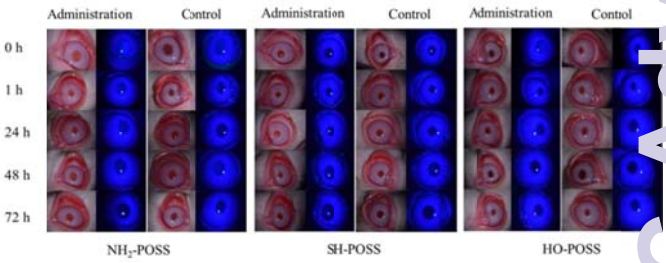

Fig. 7: Slit lamp images of the rabbit eyes with POSS or normal saline drop ocular surface administration and fluorescein stained at 0, 1, 24, 48 and72 hours.

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Conclusions

The ocular biocompatibility of POSS was systematically assessed by testing the POSS nanomaterials damage to the ocular cell membrane, mitochondrial dehydrogenase viability or ROS secretion in cytoplasm and apoptosis via nucleus, as well as the animal ocular surface irritation. The results show that the POSS nanomaterials do not cause damage to the HLEC cell membrane and nucleus, whereas they will notably up-regulate the secretion of ROS. The up-regulation of ROS does not directly relate with the cell viability or apoptosis. Only high concentration of NH_2-POSS presents cytotoxicity to cell viability, whereas the HO-POSS and SH-POSS render good cell viability even in high concentration and incubation for lone time. The in vivo results show that neither acute inflammation nor cornea epithelial lesion is detected after POSS nanomaterials exposure. No significant irritation on the cornea, iris or conjunctiva is found in all the experimental animal eyes. These results show the good ocular biocompatibility of the POSS nanomaterials except NH_2-POSS in high concentration, indicating that POSS nanomaterials may a good alternative of ocular biomedical materials in most cases.

Acknowledgements

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