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REGULAR ARTICLE

Antiangiogenic effect of silicate nanoparticles on corneal neo-vascularisation induced by vascular endothelial growth factor ☆

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KEYWORDS

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Abstract Corneal neo-vascularisation (NV) is a major sight-threatening condition and is caused by infections, degenerative disorders, inflammation and long-time contact lens wear. Corneal NV occurs when the balance between angiogenic and antiangiogenic factors is tipped towards angiogenic molecules. The abnormal vessels may decrease corneal clarity and vision, lead to inflammation and corneal scarring and worsen the prognosis of penetrating keratoplasty if needed.

There is no definite therapeutic approach for cornea NV. Medical and surgical therapies used to reduce corneal NV include corticosteroids and non-steroidal anti-inflammatory agents, laser photocoagulation and needle diathermy. Many of these therapies not only have demonstrated limited success but also have associated adverse effects. Therefore, it is very necessary to provide novel therapeutic approaches. Recently, anti-vascular endothelial growth factor (anti-VGEF) therapy has been introduced for the management of corneal NV.

Herein, we hypothesise the use of silicate nanoparticles (SiNPs) as a novel treatment for corneal NV. The penetration rate of SiNPs into the cornea is attributed to the size of nanoparticles. Therefore, different sizes of SiNPs (20–50 nm) would be prepared and loaded onto the tissue to determine corneal permeability towards them. In addition, SiNPs would be administered into the eye by topical, subconjunctival and corneal intrastromal injection and accumulate in newly formed vessels. This hypothesis has been developed by emphasising on the synthesis of SiNPs, characterisation of size-dependent properties and surface modification for the preparation of homogeneous

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nanocomposites, generated by a reverse micro-emulsion method. As the importance of concentration, shape and/or size of SiNPs could be key factors exerting their antiangiogenic effects, we suggest using 20–30-nm SiNPs to enhance their ability to penetrate into the corneal epithelium. We hypothesise that topical, subconjunctival and corneal intrastromal injections of SiNPs may effectively inhibit and treat corneal NV. Controlled experimental studies on rabbits are needed to test whether SiNPs are able to effectively inhibit VEGF-induced angiogenesis in every segment of the eye including anterior, middle (ciliary body and trabecular mesh work) and posterior segments.

Introduction

Nanotechnology has entered the field of medicine in recent decades and is used in different medical fields including diagnosis, biosensors and drug delivery and has thus provided novel nanomedicines and nanodevices [1]. Researchers are exploring nanotechnology as a drug delivery route for both systemic medications and ocular applications. Most of the conditions that affect the eye are treatable through the ocular surface [2–4]. Intravitreal administration of pharmacological agents has been applied for vitreoretinal diseases as drug delivery into the retina or the vitreous body is difficult to achieve through conventional methods in the presence of the blood–aqueous barrier and the inner and outer blood–retinal barriers [5]. For maximising the drug effect, the molecules of the drug need to reach specific locations within the target tissue. Because drug molecules typically cannot reach their site of action, there is a need for a technology that can efficiently deliver the required amount of the drug to its target site. Thus, many research studies on nano-sized drug carriers have been conducted in the field of ophthalmology [6,7]. Nanomedicine uses nanoscale technology for the treatment and prevention of disease that can pave the way for novel ophthalmologic therapeutic applications with an ultimate goal of improving quality of vision and finally the quality of life. Although new drugs have recently been developed within the field of ophthalmology, drugs administered systemically have poor access to the inside of the eye because of the cornea, which is an effective barrier to drug penetration by completely surrounding and effectively sealing the superficial epithelial cells. Conventional systems, such as eye drops, are inefficient, whereas systemic administration requires high doses resulting in significant toxicity. There is a need to develop novel drug delivery carriers capable of increasing ocular bioavailability and decreasing both local and systemic cytotoxicities. Nanotechnology is expected to revolutionise ocular drug delivery. Many nano-structured systems have been employed for ocular drug delivery and yielded some promising results.

The human cornea is normally an avascular, transparent connective tissue that consists of three layers, epithelium, stroma and endothelium, and a mechanical barrier to inhibit transport of exogenous substances into the eye [8]. Each layer possesses a different polarity and a rate-limiting structure for drug permeation. The corneal epithelium is of a lipophilic nature, and tight junctions among cells are formed to restrict paracellular drug permeation from the tear film. The corneal epithelium is almost impermeable to any substance larger than 500 Da [9]. Most of the commonly used topical drugs are larger than that and are not able to cross the cornea. Instead, they permeate throughout the conjunctiva and the underlying sclera, known as 'non-productive passage'. Indeed, $< 5\%$ of topically administered drugs reach intra-ocular tissues [10]. The stroma is composed of an extracellular matrix of a lamellar arrangement of collagen fibrils. Drugs administered systemically because of the blood–aqueous and blood–retinal barriers and annular tight junctions, which make the cornea a major ocular barrier, have poor access to the retina and corneal stroma. Nanoparticle (NP)-mediated delivery not only overcomes the corneal epithelial barrier but can also prolong the resistance time of a drug in the pre-corneal tear-film layer. Therefore, preparing a topical nano-drug which is able to pass through ocular barriers is desirable [11].

New vessels, which sprout from the capillaries and venules of the pericorneal plexus, may block light, compromise visual acuity, worsen the prognosis of penetrating keratoplasty and lead to inflammation, corneal scarring and oedema [12]. In the clinical setting, topical corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) remain the principal primary treatment for corneal vessels [13]. However, in corneas in which vessels have been established, corticosteroid and NSAID treatments are ineffective. Although laser photocoagulation for corneal NV has been reported [14,15], this method achieves an inadequate effect because of the high incidence of recanalisation and thermal damage to adjacent tissue [16]. Other treatments including photodynamic therapy, fine needle diathermy and conjunctival, limbal and amniotic membrane transplantation [17–19] have limited clinical efficacy and also cause a multitude of undesirable side effects. Therapeutic NP technologies have the potential for parenteral, oral, ocular and trans-dermal applications as well as used in sustained release formulations and as a carrier for radionucleotides in nuclear medicine [20]. Sustained drug delivery systems can provide sustained drug levels to a particular tissue, thereby significantly reducing the dosing frequency and the associated complications. Several delivery systems including implants, scleral plugs and microparticles and NPs have been used for this purpose [21–23]. The particulate systems offer several advantages including ease of repeated injections and cellular entry [24]. NPs of various molecules, such as gold and silver, have been reported to have an antiangiogenic effect on pathological NV [25–27]. Silicate NPs (SiNPs) have been used in drug delivery, gene therapy, biolabelling and in combination with other treatment modalities [28–30]. Some characters of nano-sized silica which are size, size distribution and morphology are of great importance to its application. The large size is usually not effective for biomedical applications as cell uptake is limited. Another important requirement in the biomedical application of SiNPs is their aqueous suspensibility. Often, the high-temperature removal of the template poses problems in suspension in solution or destruction of encapsulated agents [31–33]. Many technologies have been explored to fabricate nanostructures and nanomaterials. In most of the preparation

methods, it is very difficult to control the size and shape of the particle except for the micro-emulsion method, in which we need to control one factor and that will control everything else for particle synthesis. A micro-emulsion is a thermodynamically stable dispersion of two immiscible fluids; the system is stabilised by added surfactant. In reverse micro-emulsion, the surfactant molecule dissolved in organic solvents forms spherical micelles. In the presence of water, the polar head groups organise themselves to form micro-cavities containing water, which are often called reverse micelles [34]. Besides effective control of the particle characteristics, simple operation and apparatus, mild reaction conditions which do not need high temperature and pressure are all inclusive [35,36]. One significant challenge for the successful development of therapeutic NPs is rapid clearance during systemic delivery [37]. Therefore, the factors that could affect the clearance and biodistribution of NPs, such as particle physicochemical properties and targeting legends [38], should be carefully considered for the optimal design of therapeutic NPs [39]. The fact that no direct toxicity of SiNPs was observed on retinal neuronal or endothelial cells, nor on the retinal tissue [40]. prompted us to hypothesise that topical, subconjunctival and corneal intrastromal injections of SiNPs would effectively inhibit corneal NV with minimal side effects.

Hypothesis

According to the above-mentioned evidence and the antiangiogenic effects of SiNPs and the fact that SiNPs have no toxic effect on retinal tissue [40], it seems to be reasonable that topical, subconjunctival and corneal intrastromal injections of SiNPs can be a novel treatment for corneal NV with minimal side effects. As the importance of concentration, shape and size of SiNPs could be key factors exerting their antiangiogenic effects, we suggest using 20–30-nm SiNPs to enhance their ability to pass through the corneal tight junctions and reach the new vessels in the corneal stroma. Further studies can be performed on the efficacy of SiNPs on other ocular segments to control neo-vascular glaucoma and retinal NV due to diabetic retinopathy and vascular occlusive disorders (Figs. 1 and 2).

How to synthesise SiNPs

Generally, it is possible to produce particles in the nanometre size range by nucleation of NPs from supersaturated solution. Here, a two-phase nucleation method [42] has been suggested as a simple synthesis route for producing ultra-monodisperse SiNPs between 5 and 50 nm. This method is based on the very slow increase of the solution supersaturation and on the use of peptides for the silica condensation catalysis. Therefore, using tetraethylorthosilicate (TEOS) as an organic layer above the aqueous solution could limit the increase rate of silica monomer in the aqueous solution by controlling the TEOS hydrolysis rate. Dilution of TEOS in an inert oil further limits TEOS hydrolysis and allows the control of the water solution supersaturation increase rate. Indeed, existence of the interfacial area between the organic layer containing the TEOS molecules and the water phase is responsible for physical limits of TEOS hydrolysis.

In brief, different amounts of L-arginine as a catalyst are dissolved in 40 ml of water. Then, a certain volume of cyclohezane is added. To keep an aqueous phase undisturbed, TEOS is add subsequently [40]. The two-phase solution is left to warm for several minutes under agitation. The stirring rate is fixed such that the top organic layer is left almost undisturbed and the water phase is well mixed. By this method, we will be able to prepare spherical silica particles (5–50 nm) by controlling reaction factors. The size of silica displayed decreasing variation with the increase of L-arginine and decrease of temperature. The mean particle size could be determined by dynamic light scattering (DLS), Fig. 3.

The morphology of the NPs would be investigated by scanning electron micrography (SEM), Fig. 4, and transmission electron microscopy (TEM) [43].

Investigation of penetration of SiNPs into cornea

We should use *in vitro* permeability studies to determine whether NPs can penetrate into the cornea to gain access to the deep stroma and whether NPs can cross the cornea to gain access to the vitreous area.

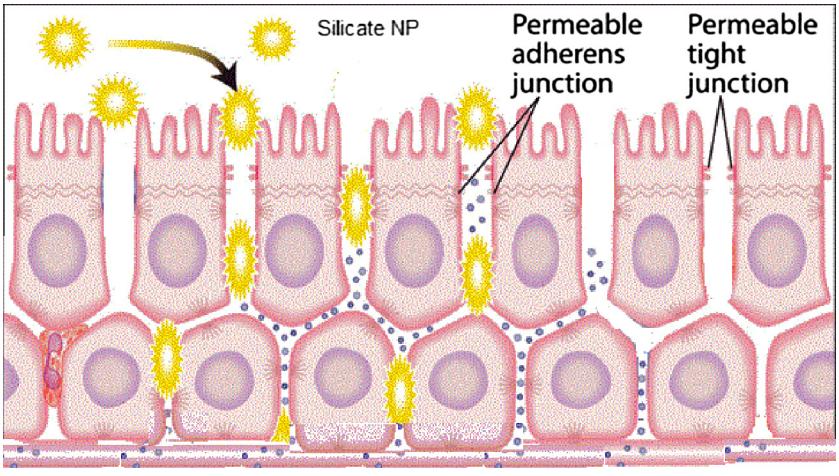

Figure 1 Structure of corneal epithelial tight junctions and possible way of SiNPs to penetrate this barrier.

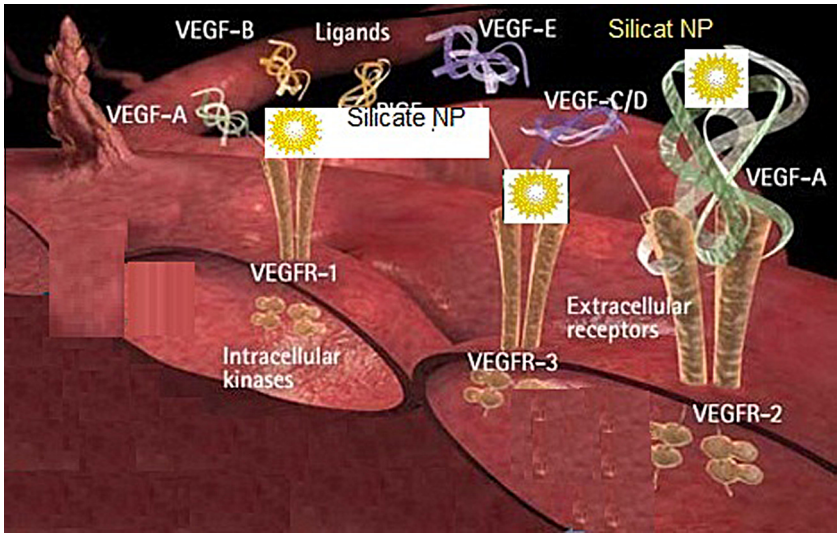

Figure 2 Ligands for different VEGF receptors. SiNP molecule can not only block the VEGF1 receptors but also can effectively inhibit VEGF 2 and VEGF 3 receptors, simultaneously.

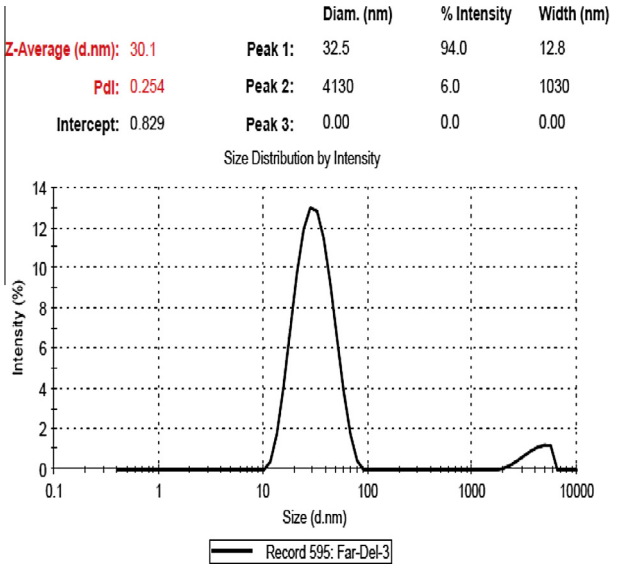

Figure 3 Scattered intensity of the nanoparticles in the aqueous phase after 16 h of reaction times in a two-phase reactor at 60 °C and different R and L-Arginine concentration by Dynamic Light Scattering DLS.

To visualise the transport and diffusion of NPs through the tissue, SEM was used. After performing the transport across the tissues as described above, the part of the tissue exposed to the NP suspension was cut off from the rest of the tissue and embedded in an optimal cutting temperature (OCT) medium for frozen sectioning. The eyes were kept at −80 °C before sectioning. Sections around 10 μm in thickness were cut and visualised using SEM.

Evaluation of hypothesis

An animal (rabbit) model experimental study is suggested to test this novel hypothesis. A study with two arms is suggested. One group would receive topical, subconjunctival and corneal intrastromal injections of SiNPs before corneal NV, which is induced by vascular endothelial growth factor (VEGF) to evaluate inhibition of SiNPs towards cornea NV. The other group would receive topical, subconjunctival, and corneal intrastromal injections of SiNPs after corneal NV to evaluate treatment of SiNPs towards corneal NV. In addition, the level of NV in the corneas of all rabbits would be monitored and quantified with a micrometer-calibrated stereomicroscope equipped with a digital camera.

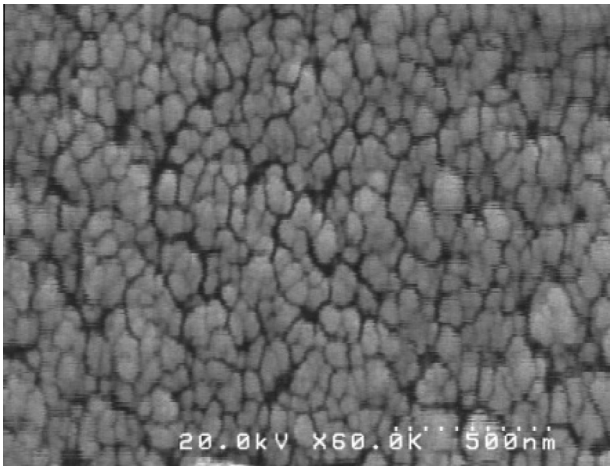

Figure 4 The SEM image of spherical silica nanoparticle. [L-Arginine] = 6 mM/L, reaction time: 24 h, $T = 26 \pm 1$ °C, at low agitation rate.

Discussion

Corneal NV occurs as a result of disequilibrium between angiogenic and antiangiogenic stimuli. Angiogenesis is the process of new blood vessel growth from pre-existing vascular

structures. Corneal angiogenesis occurs in several pathological conditions and brings about a variety of unwanted consequences. Corneal angiogenic factors include VEGF. This factor promotes vascular endothelial cell proliferation, migration and tube formation [41]. It also increases vascular leakage and promotes monocyte chemotaxis and b-cell production in mice, indicating the key role of VEGF in inflammation [42]. VEGF binds to two members of a receptor tyrosine kinase family, VEGF receptor (VEGFR)-1 and VEGFR-2, also known as Flt-1 and kinase insert domain receptor (KDR), respectively. VEGFR-2 is considered the main VEGF receptor and mediates the proliferative effects of VEGF on vascular endothelial cells. VEGF binding to VEGFR-2 induces the dimerisation and subsequent autophosphorylation of receptors by intracellular kinase domains, which leads to a mitogenic and proliferative signal [43]. VEGF and VEGF-dependent signalling pathways are known to be involved in pathological NV. In particular, VEGFR-2 is considered to play a pivotal role in developmental angiogenesis [44]. This theory assumes that SiNPs effectively suppressed phosphorylation of VEGFR-2. This result suggested that the mechanism of antiangiogenic effects of SiNPs was also based on inhibition of VEGFR-2 activation. Among downstream pathways of VEGFR-2 signalling, protein kinase C/MARK (protein kinase C–MAP/microtubule affinity-regulating kinase) and phosphoinositide 3-kinase/AKT (PI3K/AKT) pathways are known to be involved in processes of angiogenesis [45,46].

The requirement of VEGF for corneal NV was first demonstrated in a rat model where NV was later subsequently blocked by anti-VEGF antibodies [47,48]. Indeed, anti-VEGF antibodies have shown initial therapeutic success. Bevacizumab is a humanised murine monoclonal antibody that recognises all isoforms of VEGF. Bevacizumab was initially approved to treat metastatic colon cancer [49] but has also shown efficacy in partial reduction of corneal NV through topical, subconjunctival and intra-ocular applications of bevacizumab [50–53]. NPs of antibiotics have also been suggested for treatment of ocular infections. The initial antibiotic regimens should be changed from time to time to prevent multidrug resistance. Therefore, resistance to antibiotics such as chloramphenicol, cefazolin and trimethoprim towards *Pseudomonas aeruginosa* (PA), which is the leading cause of contact lens-induced keratitis and corneal ulcers, is very common and these antibiotics should not be considered. Previously, we reported that PA was highly sensitive to ceftazidime, ciprofloxacin and amikacin [54]. The authentic value of an anti-*Pseudomonas* NP such as ceftazidime in decreasing PA keratitis/endophthalmitis morbidities can be evaluated in well-designed experimental animal models [55].

The bioavailability of an instilled conventional drug onto the ocular surface is usually low. Most of it is lost due to physiological mechanisms, such as tear drainage and blinking, a few seconds after instillation [56]. Therefore, there is limited absorption of the drug and limited access to intra-ocular tissues through the conjunctival–scleral pathway [57]. Indeed, NPs were considered to offer the possibility of more facile delivery and transport across tissues.

The intrinsic capacity of NPs to adhere to the ocular surface and interact with the epithelium has stimulated researchers to find applications for them in ophthalmology. The possibility of the controlled release of drugs that surpass the ocular barriers and effectively reach the target is the ability that makes them applicable to treat eye diseases. Nanoparticulate systems improve the delivery of poorly water-soluble drugs while significantly reducing toxicity compared to the free drug [58]. There are several different modalities for ocular drug administration. The most common include liquids topically applied onto the front of the eye in the form of eye drops, subconjunctival or sub-Tenon's injection in the conjunctival tissue or below the Tenon's capsule and intravitreal injection.

Dong and his co-workers demonstrated that SiNPs had negligible acute toxicity to retinal neuronal cells, retinal endothelial cells and the retinal tissue at concentrations 100 times the effective therapeutic dosage. In addition, they reported that SiNPs had antiangiogenic effects [40].

We want to note that reports of other groups suggested the antiangiogenic effects of 50 nm gold (AuNPs) and silver NPs [39,27]. These results suggest that the concentration, shape or size of NPs could be key factors exerting their antiangiogenic effects. According to tight junctions between corneal epithelial cells, the small size of NPs (20–50 nm) will be effective for increasing drug penetration into deep corneal stromal layers that warrants NP efficacy. The physicochemical characteristics of SiNPs are critical for cellular uptake, intracellular trafficking and interaction with plasma proteins. Nonetheless, the issue of biodistribution of NPs should be addressed in their biomedical application. Therefore, biodegradation and biodistribution of NPs might be investigated before their clinical application.

We believe that SiNPs as well as AuNPs are able to inhibit the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK 1/2), a signalling molecule on the MARK pathway, not that of AKT. Furthermore, mass-produced SiNPs may be more feasible and cost-effective than monoclonal antibodies such as avastin or bevacizumab. In addition, SiNPs could have modifiable size and concentration. Using topical, subconjunctival and corneal intrastromal injections of SiNPs as an anti-VEGF therapy seems effective in suppressing new vessel formation and vascular leakage, which can improve visual function.

In conclusion, we suggest that SiNPs, because of their small size, can have acceptable permeability through the corneal epithelial tight junctions and can be safely considered an effective modality in the prevention and treatment of corneal NV. Further studies can be performed on the efficacy of SiNPs on other ocular segments to control neo-vascular glaucoma and retinal NV due to diabetic retinopathy and vascular occlusive disorders.

Conflict of interest statement

None declared.

Disclosure

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The authors have no financial or proprietary interest in a product, method or material described herein.

Overview Box

First Question: What do we already know about the subject?
Corneal NV is a serious condition that can lead to a profound decline in vision. The abnormal vessels block light, compromise visual acuity, cause corneal scarring and may lead to oedema.

Second Question: What does your proposed theory add to the current knowledge available, and what benefits does it have?
This theory assumes that topical, subconjunctival and corneal intrastromal injection of SiNPs is a novel therapeutic approach for the treatment of cornea NV, which is more feasible and cost-effective than monoclonal antibodies such as avastin. Further, SiNPs could have modifiable size and concentration. There is no adverse reported side effect in intravitreal injection of SiNPs. Therefore, it is reasonable to hypothesise that subconjunctival and corneal intrastromal injections of SiNPs would not have any direct toxicity.

Third question: Among numerous available studies, what special further study is proposed for testing the idea?
Preliminary experimental studies on animal models where corneal NV is induced by VEGF are suggested.

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