Acta Biomaterialia 9 (2013) 7855–7864

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Chitosan microparticles for delivery of proteins to the retina

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ARTICLE INFO

Article history:
Received 12 December 2012
Received in revised form 27 March 2013
Accepted 15 April 2013
Available online 23 April 2013

Keywords:
Chitosan
Retina
Microparticles
Encapsulation
Biodegradation

ABSTRACT

Chitosan microparticles (CMPs) have previously been developed for topical applications to the eye, but their safety and efficacy in delivering proteins to the retina have not been adequately evaluated. This study examines the release kinetics of CMPs in vitro, and assesses their biocompatibility and cytotoxicity on retinal cells in vitro and in vivo. Two proteins were used in the encapsulation and release studies: BSA (bovine serum albumin) and tat-EGFP (enhanced green fluorescent protein fused to the transactivator of transcription peptide). Not surprisingly, the in vitro release kinetics were dependent on the protein encapsulated, with BSA showing higher release than tat-EGFP. CMPs containing encapsulated tat-EGFP were tested for cellular toxicity in photoreceptor-derived 661 W cells. They showed no signs of in vitro cell toxicity at a low concentration (up to 1 mg ml^{-1}), but at a higher concentration of 10 mg ml^{-1} they were associated with cytotoxic effects. In vivo, CMPs injected into the subretinal space were found beneath the photoreceptor layer of the retina, and persisted for at least 8 weeks. Similar to the in vitro studies, the lower concentration of CMPs was generally well tolerated, but the higher concentration resulted in cytotoxic effects and in reduced retinal function, as assessed by electroretinogram amplitudes. Overall, this study suggests that CMPs are effective long-term delivery agents to the retina, but the concentration of chitosan may affect cytotoxicity.

1. Introduction

Diseases of the retina, such as glaucoma, age-related macular degeneration and diabetic retinopathy, are major causes of irreversible blindness. The development of efficient delivery systems for ocular therapeutic agents is critical for the treatment of these diseases. Topical agents are generally used for corneal and anterior problems of the eye, but are mostly ineffective in targeting the retina at the back of the eye. For retinal disease, adeno-associated viruses (AAVs) are currently being used in clinical trials [1–6]. The viruses are safe and effective at delivering transgenes to the retina, and are long-lasting, making them ideal gene carriers for genetic forms of disease requiring long-term therapy. However, once AAV viruses are delivered to the retina, there is no way to disable the therapy if harmful side effects are discovered. Moreover, in cases such as retinal ischemia or retinal detachment, short-term treatment is essential, though, once the clinical problem is resolved, the therapeutic agent is no longer required. For this reason, there is still a vital need for safe, effective methods of short-term or sustained delivery of therapeutic agents to the retina. Biodegradable and biocompatible nano- and microparticles are ideal vehicles for such a purpose.

We have previously reported the use of polyethylene glycol–polylactic acid (PEG–PLA) microparticles as protein delivery vehicles to retinal cells [7]. The PEG–PLA microparticles offered the advantages of controlled, sustained protein release and biocompatibility. However, the amount of the protein released from PEG–PLA particles was somewhat low in comparison to AAV expression levels. In addition, the percentage of the encapsulated protein that was released was quite low, ranging from 1 to 5% over a period of several weeks, depending on the microparticle formulation. Although this may not be a concern in treatments where nanogram quantities of protein are sufficient for neuroprotection, PEG–PLA microparticles would not be useful when larger quantities of the therapeutic agent are required. In order to enhance the release profile of the particles, here we report the development and testing of chitosan microparticles (CMPs) for the delivery of enhanced green fluorescent protein fused to the transactivator of transcription peptide (tat-EGFP) to the retina.

Chitosan has been widely used in biomedical applications (e.g. for the development of scaffolds for artificial liver [8], for skin

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http://dx.doi.org/10.1016/j.actbio.2013.04.025

transplants [9], as a wound dressing [10] and as a drug delivery system [11]). Chitosan makes a good biomimetic component for extracellular matrices (ECM) due to its structural similarity to proteoglycans (one of the main constituents of ECM). This is mostly attributed to its reactive amino and hydroxyl groups that can be chemically modified, and also to the fact that it can be easily manipulated for different pore structures [12,13].

Chitosan is an amino polysaccharide (poly-1,4-D-glucoamine) derived from chitin by deacetylation. Chitin is one of the most abundant polysaccharides found in nature. It is almost as common as cellulose, and possesses many of the structural and chemical characteristics of cellulose. Chitin is an important structural component of the exoskeleton of a great number of organisms, such as insects and shellfish. It also serves as a cell wall component of fungi, and of numerous plankton and other small marine organisms. Due to the different biological requirements of these various species, chitin has proven to be an extremely versatile natural polymer. Chitin and its most important derivative, chitosan, have a number of useful physical and chemical properties, including strength, biodegradability and lack of cytotoxicity. Chitosan has been shown to be effective in transcellular transport [14,15] (for example, in enhancing the transport of drugs across the cell membrane [16,17]), and has been reported to be nontoxic on several types of cells [18].

Despite the fact that chitosan is widely used as a drug delivery vehicle for various medical applications and has been extensively studied for topical application of drugs to the eye (reviewed in Ref. [19]; see also Refs. [20,21]), its safety and efficacy have not been fully evaluated for intraocular ophthalmic drug delivery applications. To our knowledge, only a limited number of studies have examined the effects of intraocular delivery of chitosan [22–25]. In one study, chitosan was used as in intravitreal tamponade material [25]. The authors concluded that there were no significant effects on the histology of the eye, with only slight increases in inflammatory factors. In a second study [22], intravitreal delivery of chitosan particles was part of a larger analysis examining several types of nanoparticles. The authors reported a significant inflammatory response following the injection of CMPs into rabbit eyes. However, cytotoxicity can be influenced by the material purity and source, the fabrication process, the particle size, the morphology and concentration, the type of target cell and the mode of administration of the particles (e.g. intravitreal vs. subretinal). The authors of the study acknowledged that it was possible that contaminants of the material were responsible for the immune response following intravitreal delivery [22]. Two recent studies examined the use of oligochitosans for gene delivery to the retina [23,24]. They showed that oligochitosan polyplexes, at low concentrations, are efficient at delivering plasmid DNA to the retinal pigment epithelium and the ganglion cells of the retina. However, in vivo toxicity issues and retinal function were not assessed.

None of the above studies examined protein release kinetics from chitosan particles. Furthermore, the different outcomes seen in these studies with regard to retinal toxicity suggest that a more detailed evaluation of ocular applications for chitosan particles is warranted. In the current study, we have developed CMPs for the encapsulation and release of tat-EGFP to the retina. We evaluate the release kinetics of these particles in vitro and assess their toxicity on retinal cells in vitro, and in vivo following subretinal delivery into the eye.

2. Methods

2.1. Materials

Low-molecular-weight water-soluble chitosan powder was purchased from Jinan Haidebei Marine Bioengineering Co. Ltd (60 MESH, with a viscosity of 260 Pa.s). tat-EGFP was synthesized as we previously reported [7]. Sodium triphosphate pentabasic (TPP; Cat# 72061) and bovine serum albumin (BSA; Cat# A-3912) were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Preparation of CMPs and protein encapsulation

Chitosan microparticles loaded with BSA or tat-EGFP proteins were prepared by a modified ionotropic gelification technique previously developed by Calvo et al. [26] and de la Fuente et al. [27]. Briefly, 12 mg of BSA or 2.5 mg of tat-EGFP (in 1 ml of ddH_2O) were mixed with 4 ml of a 0.7% mg ml^{-1} solution of TPP. The protein/TPP solution was then added to 15 ml of a 1 mg ml^{-1} chitosan in water solution under magnetic stirring using a dropwise technique and a syringe pump (Baxter PCAIII) at a rate of 15 ml h^{-1}. The weight ratios of protein to chitosan were lower for the tat-eGFP (0.17) than for the BSA (0.80) because it was difficult for us to synthesize high concentrations of soluble tat-eGFP protein. Agitation was maintained for 15 min to allow the complete formation of the particles. The solidified microparticles were collected by centrifugation, washed three times with sterile phosphate-buffered saline (PBS), frozen overnight, lyophilized for 6 h and stored at 4 °C until use.

The encapsulation efficiency (*EE%*) was calculated using the following formula:

$$EE\% = (W_t/W_i) \times 100\%$$

where W_t is the total amount of protein in the wash supernatants from the particles and W_i is the total amount of protein used in the initial preparation of the particles.

2.3. Scanning electron microscopy (SEM)

SEM was used to assess the morphology and size of freeze-dried CMPs (Model S-2250N, Hitachi, Japan). In preparation for SEM, the particles were mounted on metal holders using conductive double-sided adhesive tape, and sputter coated with a gold layer for 60 s at 0.1 bar vacuum pressure (Cressington Sputter Coater, 108).

2.4. Transmission electron microscopy (TEM)

The morphology and size of the particles were further characterized by TEM using a Tecnai G2 20 S-TWIN model transmission electron microscope with 200 kV acceleration voltage. For this, 10 mg of the sample particles was dispersed in 10 ml of ddH_2O in an ultrasonic bath. Approximately 200 μl of the dispersed particle suspension was placed on a copper grid coated with a carbon film.

2.5. In vitro release from CMPs

Microparticles were dispersed in high glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Thermo Scientific, Waltham, MA), supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 10000 units ml^{-1} penicillin,10 mg ml^{-1} streptomycin, 2 mM L-glutamine (Gibco, Invitrogen Life Technologies, Carlsbad, CA) and 1 mM sodium pyruvate (Sigma). To follow protein release over time, dispersed particles were aliquoted into 2 ml microcentrifuge tubes and incubated at 37 °C with agitation.

2.5.1. In vitro release of BSA from CMPs

Protein release from BSA-loaded microparticles was assessed using two methods. In Method 1, microparticles were aliquoted into three identical tubes. At each time point, the tubes were centrifuged at 16,000 g in a microfuge for 10 min, then the supernatant was collected and frozen at −20 °C. The microparticles were then resuspended in fresh medium and returned to 37 °C with