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Effects of chitosan and chitosan N-acetylcysteine solutions on conjunctival epithelial cells

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ABSTRACT

Aim of the study: Chitosan, a partially deacetylated polysaccharide derived from chitin, and chitosan-N-acetylcysteine (C-NAC), a thiolated chitosan, both show enhanced retention times on the ocular surface when compared to other polymers commonly used in eye drops. To evaluate these compounds as adjuvants for ocular drug delivery or uptake of topically administered conjunctival vaccines, biochemical characteristics of both polymers were investigated *in vitro* and *in vivo*.

Methods: Human conjunctival epithelial (HCjE) cells were used to investigate biocompatibility of buffered chitosan and C-NAC containing formulations. Cellular uptake was studied using fluorescein-isothiocyanate (FITC)-labelled polymers. Transepithelial electrical resistance (TEER) measurements were performed to determine effects of chitosan on tight junctions of stratified HCjE cells *in vitro*. *In vivo* uptake of topically applied chitosan into conjunctival epithelial cells was investigated in guinea pigs.

Results: Minimal effects on cell viability were seen with both compounds after application for 30 min in a concentration of 0.1%. *In vitro* uptake into HCjE cells was only observed with the chitosan containing solution. An effect on tight junctions was demonstrated by significantly ($P < .05$) decreasing TEER levels 60 min after incubation with chitosan. *In vivo*, FITC-labelled chitosan was detected within guinea pig conjunctival epithelial cells 120 min after topical administration. No adverse effects were observed.

Conclusions: Results demonstrated that chitosan is taken up into conjunctival epithelial cells *in vitro* and *in vivo*, therefore fulfilling some of the requirements of an adjuvant for conjunctival mucosal vaccines. C-NAC is also well tolerated but not taken up by cells, making it a good candidate for ocular formulations supposed to persist on the ocular surface for an extended period of time.

Introduction

Efficacy of current topically administered ocular medication is often restricted due to limited retention times of the active compound at the ocular surface. In addition, since the eye is physiologically built to protect itself from harmful particles by the conjunctival and corneal epithelia and the mucosal tear film, topical drug delivery is challenging. Manufacturers try to overcome these problems by including mucoadhesive components and increasing viscosity of the formulation. This should avoid the inconvenience of frequent instillation and help to retain the required drug concentration at the site of action [1]. Ocular formulations are provided in liquid form, which tends to be most comfortable for patients, semi-solid as gels and ointments, or solid, as inserts [2]. Liquid formulations aimed towards being mucoadhesive may include acrylates, polyethylene glycol, hyaluronan and other polysaccharides [2]. This last group also includes chitosan, a positively

Abbreviations: C-NAC, chitosan-N-acetylcysteine; HCjE, human conjunctival epithelial; TEER, Transepithelial electrical resistance; BAB, boric acid buffer; FITC, fluorescein-isothiocyanate; LDH, lactate dehydrogenase; DAPI, 4′,6-Diamidin-2-phenylindole; PBS, phosphate buffered saline; H&E, hematoxylin and eosin; SD, standard deviation; EDTA, ethylenediamine-tetra-acetic acid; DES, dry eye syndrome; Mw, molecular weight

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charged partially deacetylated polysaccharide derived from chitin, which shows great potential in drug delivery applications [3].

Chitosan has been shown to be highly biocompatible, antimicrobial, and enhancing the retention time of topically co-administered drugs at the ocular surface. Thus chitosan-containing solutions were shown to inhibit the growth of *E. coli* and to kill *S. aureus* [4]. Moreover, 0.5% chitosan when added to formulations significantly enhances the retention time on the precorneal surface and significantly delays elimination of these formulations from the precorneal area [5].

Chitosan is also described as a potential enhancer of transmucosal drug delivery. Several studies with the intestinal Caco-2 cell line [6] or co-cultures of intestinal and epithelial cells (HT-29/B6) [7] indicated that chitosan increases cell permeability by affecting paracellular and intracellular pathways of epithelial cells, in a reversible manner. These results suggest that opening of the tight junction barrier is caused by the binding of positively charged chitosan to the cell membrane, thereby activating the chloride-bicarbonate exchanger which increases permeability for molecules up to 10 kDa [8].

In vitro uptake and biocompatibility of chitosan has been studied in A546 cells, a human lung carcinoma cell line, and was found to be independent of its molecular weight (10 kDa–213 kDa) with no significant impact on cell viability below a concentration of 0.741 mg/ml [9]. HEK 293 cells showed uptake of chitosan at various conditions, but only cells cultured with acidified media showed chitosan uptake in the absence of serum [10]. This ability to penetrate cells makes chitosan a good candidate as an enhancer of drug delivery.

The safety and efficacy of chitosan as an adjuvant in mucosal vaccines has been demonstrated in multiple animal models [11–13]. Furthermore, clinical trials with vaccine formulations containing chitosan were found to be safe, able to induce T cell responses [14], and effective in inducing protective antibody production [15]. Chitosan also shows pseudoplastic and viscoelastic properties [16,17] and is currently considered in various ophthalmic nanotechnological applications [18].

The distinct mucoadhesive characteristics of chitosan were previously shown to be enhanced after introduction of thiol groups facilitating interaction with integral cell membrane proteins and proteins of the mucus layer [19]. The thiolated chitosan derivative C-NAC is synthesized by conjugation of chitosan with N-acetylcysteine [20]. Excellent tolerability of topically administered eye drops containing the C-NAC polymer was shown in two phase-1 clinical trials for both single and two-times-daily instillations of various concentrations of C-NAC [21,22], as well as in a randomized double blinded study for the treatment of dry eye syndrome (DES) [23]. Within these studies, it was shown that a single instillation of C-NAC eye drops significantly increased the mean tear film thickness in patients with DES as early as 10 min after instillation and lasted for 24 h.

The aim of the current study was to investigate the effects of chitosan and C-NAC on conjunctival epithelial cells in terms of their applicability as components of topical ocular drugs and/or as an adjuvant for ocular mucosal vaccines.

Materials and methods

Ethics statement

All experiments were approved by the "Ethics Committee for the Welfare of Experimental Animals" at the Institute of Virology, Vaccines and Sera–Torlak and conformed to the Serbian laws and European regulations on animal welfare (Approval No. 011-00-00510/2011-05/2). All animals were handled in strict accordance with good animal practice as defined by the Serbian code of practice (published in Službeni Glasnik No. 41/9) for the care and use of animals for scientific purposes, the Guide for the Care and Use of Laboratory Animals of the Torlak Institute (2133/1, 21.04.2011) and a Basel declaration that is committed to the 3R principle (Replace, Reduce, Refine) as well as the EU Directive 2010/63/EU for animal experiments. Finally, animal testing was planned and carried out with extreme care.

Materials

Chitosan (Mw 190 kDa) and C-NAC (Mw 190 kDa) were obtained from Croma Pharma. Both were dissolved in 100 mM boric acid buffer (BAB) with an osmolarity of 294 mosmol/L and a pH of 6.67. 100 mM BAB with the same osmolarity and pH was always added as a control. Croma Pharma also kindly provided fluorescein isothiocyanate (FITC)-labelled chitosan and FITC-labelled C-NAC. Biocompatibility of BAB used to dissolve the respective polymers was demonstrated previously [24].

Cell culture

Human conjunctival epithelial (HCjE) cells, kindly provided by Ilene Gipson (Schepens Eye Research Institute, Harvard Medical School, Boston, MA, USA), were maintained in serum-free keratinocyte growth medium (Life Technologies, Paisley, UK) at 37 °C/5% CO_2/95% humidity. The medium was changed every second day, and the cells were passaged at 70% confluence. Cells were harvested by trypsinization (0.05% Trypsin/0.02% EDTA in phosphate buffered saline (PBS); PAA Laboratories GmbH, Pasching, Austria), and seeded as described below within passage numbers 5–20.

Viability assay

For the assessment of possible effects of chitosan and C-NAC containing solutions on cell viability, HCjE cells were seeded with a density of 40,000 cells/well in 96-well plates. 24 h later, buffered 0.1% chitosan or 0.1% C-NAC containing solutions were applied, while BAB was used as a negative control. Concentrations were chosen according to ongoing clinical studies evaluating C-NAC as an active ingredient in an ophthalmic medical device designed for the treatment of DES [23] and to allow sterile filtration (0.2 µm) of the applied polymer solutions.

Cells were then incubated at standard conditions for 5, 15, and 30 min and supernatants collected. Cells treated for 30 min were washed with cell culture medium and incubated for further 24 h for detection of possible delayed cell death. Cell viability was evaluated by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium upon damage of the plasma membrane (LDH Cytotoxicity Assay Kit II; BioVision, Milpitas, CA).

Staining for epifluorescence microscopy

To visualize polymer uptake, 100,000 cells/well were seeded in four-chamber slides; three days later, 0.1% FITC-chitosan or 0.1% FITC-C-NAC dissolved in BAB were added to separate wells containing HCjE cells and incubated for 15 min. Cells were washed and subsequently covered with 0.4% trypan blue for 5 min to quench non-specific green fluorescence. Cells were then stained with CellMask plasma membrane stain (2.5 µg/ml; Molecular Probes, Inc., Eugene, OR) and 6-diamidino-2-phenylindole (DAPI, 1 µg/ml; Sigma Aldrich, St. Louis, MO, USA) for 5 min, at 37 °C.

Then cells were fixed with 4% *p*-formaldehyde (PFA), mounted and examined by epifluorescence microscopy (Zeiss AxioObserver and TissueFaxS; Carl Zeiss GmbH, Vienna, Austria).

Scanning electron microscopy

The interactions of chitosan and C-NAC with the surface of HCjE cells were investigated by scanning electron microscopy. Therefore, cells were seeded at 1×10^5 cells/well into 24-well plates filled with 11 mm ACLAR discs. When cells had reached confluence, they were treated with buffered 0.1% chitosan or 0.1% C-NAC for 15 min, while BAB was applied to control wells. Upon the removal of polymer-