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Comparative study of nanosized cross-linked sodium-, linear sodium- and zinc-hyaluronate as potential ocular mucoadhesive drug delivery systems

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ABSTRACT

Hyaluronic acid (HA) and its derivatives play important roles in many fields of therapy, such as arthritis treatment, plastic surgery, dermatology, otology, ophthalmology, *etc.* With a view to increase the beneficial properties of HA in ocular drug delivery, many types of chemical structural modifications have been performed. In the course of our research work, we characterized nanosized cross-linked – (CLNaHA), linear sodium hyaluronate (NaHA) and zinc-hyaluronate (ZnHA), as potential ocular drug delivery systems. The aim was to determine the influence of the structure on biocompatibility, mucoadhesion and drug release. The structure was characterized by means of rheology. The cytotoxicity of the samples was determined on rabbit corneal epithelial cells (RCE) by the MTT test. Mucoadhesion measurements were made by a rheological method *in vitro* and by tensile tests *in vitro* and *ex vivo*. The release of sodium diclofenac, a frequently used non-steroidal anti-inflammatory drug with low bioavailability, from the gels was determined with a vertical Franz diffusion cell. The results demonstrated that all three derivatives have adequate mucoadhesive properties and their rapid drug release profiles are beneficial in ocular therapy. Thanks to these properties, the bioavailability of the ophthalmic preparations can be increased, especially with the application of CLNaHA.

1. Introduction

Hyaluronic acid (HA), a natural linear anionic polysaccharide (glycosaminoglycan), is the main component of the extracellular matrix of the connective tissue and has been proved to be biodegradable, biocompatible, non-toxic, non-immunogenic and non-inflammatory. Its structure is based on two disaccharide units, D-glucuronic acid and *N*-acetyl-d-glucosamine, polymerized into large macromolecules of over 30,000 repeating units. Under physiological conditions, HA is present in the form of its sodium salt (Ganguly et al., 2014; Lai and Tu, 2012; Mayol et al., 2008; Price et al., 2007; Vasi et al., 2014).

HA has a high capacity for lubrication, water binding and water retention, and in solution it has characteristic rheological properties (Lai and Tu, 2012; Saettone et al., 1991). Thanks to its unique properties, HA derivatives are used in many fields: osteoarthritis treatment, tissue engineering, otology and plastic surgery. Exogenously applied HA exerts a beneficial effect on several mechanisms of wound healing (Price et al., 2007).

In 1934, Karl Meyer and John Palmer, at the Columbia University, New York, isolated a new polysaccharide from the vitreous humour of cows and they called it "hyaluronic acid" (Meyer and Palmer, 1934). Over the following decades, Endre Balazs extracted hyaluronic acid from rooster combs and purified it for medical application in humans and suggested to use it in ophthalmic surgery (Balazs et al., 1972). During the ocular surgery, ophthalmic viscosurgical devices (OVD), containing hyaluronic acid, are able to maintain the deep chamber, to aid in tissue manipulation, to enhance visualization and to protect the corneal endothelium (Balazs and Stegmann, 1979).

As topical use, HA is applied in the treatment of dry eye and Sjögren's syndrome. In higher concentrations, with a gel-like

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structure, HA can be used to prevent the desiccation of the cornea and it can be utilized as a carrier for antibiotics to the eye, because a formulation with relatively high viscosity and mucoadhesive properties, prevents the drug from being washed out by the tears and the drug release is therefore prolonged. This is especially important in ocular therapy, because the bioavailability of the formulations, available on the market, is merely 2–10%, which could be increased by increasing the residence time on the eye (Ludwig, 2005; Price et al., 2007; Vasi et al., 2014).

Ocular mucoadhesion occurs when the polymer interacts with the mucin covering the conjunctiva and corneal surfaces of the eye. The ocular mucus has a turnover time of 15–20 h and plays a role in hydration, cleaning, lubrication and protection against pathogens and foreign substances. During the formulation of a mucoadhesive drug delivery system, eye movements and blinking have to be taken into consideration, because these create a shear force which may thin or dislodge the formulation. HA can serve as an appropriate vehicle thanks to its special viscoelastic rheological profile. During blinking, the HA molecules align with each other and spread over the surface of the cornea. Between blinks, the molecules form a tangled meshwork, resulting in a less elastic and more viscous solution that stabilizes the pre-corneal tear film and maximizes the residence time of the formulation on the surface (Robinson and Mlynek, 1995; Scheuer et al., 2010; Vogel et al., 2010).

Beside this viscoelastic property, interpenetration and secondary bond formation between the HA molecules and the mucin also play an important role in the process of mucoadhesion.

The goal of our work was to compare three types of HA derivatives, a nanosized cross-linked sodium salt (CLNaHA), a linear sodium salt (NaHA), present in living tissues, and a linear zinc salt (ZnHA), as potential ocular mucoadhesive drug delivery systems.

In earlier studies, nano-sized CLNaHA was prepared by a carbodiimide technique, based on covalent crosslinking *via* the carboxyl groups of the HA chains with a diamine in aqueous medium at room temperature. Through crosslinking of the HA molecules, the degradation time can be prolonged and the mechanical stability can be improved (Berkó et al., 2013; Bodnár et al., 2009; Kafedjiiski et al., 2007; Maroda et al., 2011; Vasi et al., 2014).

Another HA modification involves Zn(II)-HA complex formation by adding Zn(II) chloride to an aqueous NaHA solution at pH 5.5–6.5. Beside the typical HA effects, ZnHA has scavenger, bactericidal, bacteriostatic and fungicidal effects, which are useful in ocular therapy, because the traditional preservative may be omitted from the formulation (Illés et al., 2002; Nagy et al., 1998).

The mucoadhesive properties of CLNaHA, NaHA and ZnHA were demonstrated by rheological and tensile test methods *in vitro* and *ex vivo*. The cytotoxicity of the derivatives was determined by the MTT test on rabbit corneal epithelial cells. Besides these measurements, it was important to determine the drug release profiles, because of their potential application as drug delivery systems by instillation into the *cul-de-sac* or on the surface of the eye. Sodium diclofenac (SD), a drug generally used in ophthalmic practice, was used to investigate CLNaHA, NaHA and ZnHA as carrier molecules.

2. Materials and methods

2.1. Materials

NaHA (MW: 4350 kDa) and ZnHA (MW: 498 kDa) were purchased from Richter Gedeon Ltd. (Budapest, Hungary), and CLNaHA was prepared by BBS Biochemicals LLC (Budapest, Hungary). 2,2-(Ethylenedioxy)bis(ethylamine), 1-[3(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (CDI), mucin (porcine gastric mucin type II), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO) and sodium diclofenac (SD) were purchased from Sigma Aldrich (USA). A phosphate-buffered saline (PBS) solution of pH = 7.4 was prepared by dissolving 8 g dm^{-3} NaCl, 0.2 g dm^{-3} KCl, 1.44 g dm^{-3} $Na_2HPO_4{\cdot}2H_2O$ and 0.12 g dm^{-3} KH_2PO_4 in distilled water, the pH being adjusted with 0.1 M HCl. Lacrimal fluid of pH = 7.4 was prepared by dissolving 2.2 g dm^{-3} $NaHCO_3$, 6.26 g dm^{-3} NaCl, 1.79 g dm^{-3} KCl, 96.4 mg dm^{-3} $MgCl_2{\cdot}6H_2O$ and 73.5 mg dm^{-3} $CaCl_2{\cdot}H_2O$ in distilled water and the pH was adjusted with 1 M HCl.

2.2. Preparation of CLNaHA nanoparticles

The first step of CLNaHA nanoparticle preparation was to make a 1 mg ml^{-1} NaHA (MW: 4350 kDa) solution with pH adjusted to 5.5. Mixing of NaHA solution with diamine solution (1.0%, v/v) at room temperature for 30 min was followed by the dropwise addition of CDI solution, after which the reaction mixture was stirred for 24 h at room temperature. The aqueous system, containing CLNaHA nanoparticles was purified by dialysis for 7 days against distilled water and the system was finally freeze-dried. The final cross-linking ratio was 25% (Berkó et al., 2013; Bodnár et al., 2009; Maroda et al., 2011).

2.3. Gel formulation

In ophthalmic preparations, solvents buffered at pH 7.4 are often used. Gels of CLNaHA, NaHA and ZnHA were prepared in concentrations of 0.5, 1 and 2% (w/w). The samples were stored at 4 °C and were used for the measurements after 3 days. For cytotoxicity determination, formulations of 4% (w/w) were used in 20-fold dilution. For drug release determination, 1% (w/w) formulations of CLNaHA, NaHA or ZnHA were prepared containing 0.1% (w/w) SD. First SD was dissolved in PBS followed by the addition of CLNaHA, NaHA or ZnHA to the solution and the samples were stored for 3 days.

2.4. Rheology

Measurements were carried out with CLNaHA, NaHA and ZnHA gels and their mixtures with mucin dispersion for the mucoadhesive investigation (the mucin concentration in the mixture was 5%, w/w) (Horvát et al., 2015). A Physica MCR 101 rheometer (Anton Paar, Austria) with a cone-plate measuring device (CP-50, Anton Paar, Austria; cone angle = 1°; the gap height in the middle of the cone 0.046 mm) was used for rheological measurements. Flow curves were determined at 35 ± 0.1 °C by increasing the shear rate from 0.1 to 100 s^{-1} and then decreasing it from 100 to 0.1 s^{-1} (Gratieri et al., 2010). Frequency sweep tests were performed to determine the viscoelastic character. Measurements were made over the frequency range from 0.01 to 100 Hz, whereby the storage modulus (G'), loss modulus (G'') and viscosity (η) were determined. The strain value (1%) used in the measurements was in the range of the linear viscoelasticity of the gels.

2.5. Cytotoxicity

For the cytotoxicity measurements, the RCE cell line (rabbit corneal epithelial cells) was used, obtained from the European Cell Culture Collection (No 95081046, ECACC, Salisbury, UK). For the cytotoxicity determination, the MTT test was performed, which is based on the conversion of MTT in formazan by the mitochondrial dehydrogenases of the vital cells. The RCE cell suspension was seeded in wells at a density of 7500 cells/well and was kept at 37 °C in an atmosphere of 95% air and 5% CO_2 and 95% relative humidity for 24 h to ensure attachment of the cells to the wells.

CLNaHA, NaHA and ZnHA gels were prepared in 4% w/w concentration and, after 20-fold dilution, were brought into contact with