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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

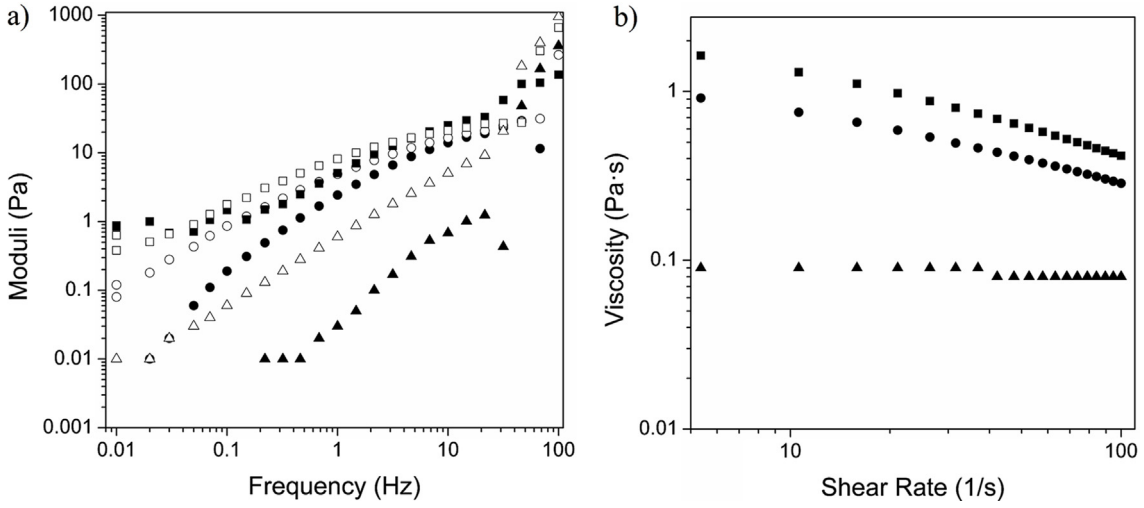

Fig. 1. (a) G' (solid symbols) and G'' (open symbols) values as a function of frequency of (◑) CLNaHA, (◨) NaHA and (◭) ZnHA; and (b) viscosity curves of (●) CLNaHA, (■) NaHA and (◭) ZnHA.

cells for 3 h. The samples were then removed and 50 μl MTT at 0.25 g ml^{-1} and 150 μl HBSS (Hank's Buffered Salt Solution, pH 7.4) was brought into contact with cells for 3 h. After the contact time, the reagent was removed from the wells and the cells were washed with HBSS to remove the samples and the unreacted MTT solution, followed by the addition of DMSO. The cell plate was shaken for 60 s and the absorbance was determined at 570 nm with the ELISA plate reader (ImarkAbsorbance Reader, Biorad, I), with the reference wavelength set at 690 nm. Cell viability was calculated as the % ratio of the absorbance of each sample and the absorbance of the cells kept in contact with HBSS (control). Eight replicates were performed for each sample (Sandri et al., 2012; Mori et al., 2014).

2.6. Tensile test

A TA.XT Plus (Texture analyser, ENCO, Spinea, I), equipped with a 1 kg load cell and a cylinder probe with a diameter of 1 cm, was used for mucoadhesion measurements. *In vitro* measurements were carried out with 50 μl 8% (w/w) mucin dispersion (prepared with simulated lacrimal fluid) (Horvát et al., 2015); *ex vivo* measurements were made with excised porcine conjunctiva and blank measurements with 50 μl simulated lacrimal fluid. The porcine conjunctiva, obtained from a slaughterhouse, was freshly detached from the connective tissue and stored at −20 °C until measurements. After complete thawing, the conjunctiva was placed on the previously wetted (with simulated lacrimal fluid) filter paper and fixed in the lower probe. 20 mg samples were attached to the cylinder probe, which was put in contact with the biological substrate at a preload of 2500 mN for 3 min at 35 ± 0.5 °C. The cylinder probe was moved upwards to separate the sample from the substrate at a prefixed speed of 2.5 mm min^{-1}. The work of adhesion (A, mN mm) was calculated as the area under the force *versus* distance curve (Sandri et al., 2006, 2012).

2.7. Drug release

A vertical Franz diffusion cell system (Microette Plus, Hanson, USA) was used to determine the SD release profile. 0.3 g samples containing 0.1% (w/w) SD were placed as donor phase on the previously impregnated (in pH 7.4 PBS) Porafil® membrane filter (Macherey–Nagel GmbH & Co., Germany; pore size 0.45 μm). The acceptor phase was PBS (pH = 7.4), thermostated at 35 °C. Measurements were performed for 6 h. 0.8 ml samples were taken from the acceptor phase by the autosampler and replaced with fresh PBS. The amount of SD released was quantified by UV spectrophotometry at 275 nm (Berkó et al., 2013; Csizmazia et al., 2011).

2.8. Statistical analysis

The results were evaluated and analyzed statistically with GraphPad Prism version 5 software. Two-way ANOVA analysis was applied with Bonferroni post-tests (Patterson et al., 2010). The values are expressed as means ± standard deviation (SD). A level of $p \leq 0.05$ was taken as significant, $p \leq 0.01$ as very significant, and $p \leq 0.001$ as highly significant.

3. Results and discussion

3.1. Rheology of the gels

The viscoelastic characters of the CLNaHA, NaHA and ZnHA were determined by frequency sweep testing in the frequency range from 0.01 Hz to 100 Hz. G' corresponds to the elastic and G'' to the viscous behaviour of the measured samples. The cross-over points of these curves show the transition from viscous to elastic behaviour (Berkó et al., 2013; Cowman and Matsuoka, 2005). Fig. 1 shows (a) the frequency sweep test and (b) viscosity results on the measured samples at 1% (w/w) polymer concentration.

The highest viscosity was observed for NaHA, which corresponds to its long linear structure. CLNaHA exhibited a lower viscosity, because it contains intrachain cross-linking, which produces nanoparticles with a particle size <110 nm (Maroda et al., 2011), and ZnHA had the lowest viscosity. The structure of the ZnHA molecules in the formulation probably involves fewer entanglements, and this causes lower viscosity.

CLNaHA and NaHA displayed viscoelastic behaviour, acting as viscous solutions in the lower frequency range, and demonstrating elastic properties at higher frequency. The cross-over point for NaHA was seen at lower frequency than that for CLNaHA, from which it can be concluded that CLNaHA showed less elastic behaviour. In contrast with CLNaHA and NaHA, ZnHA behaved as a viscous fluid; G'' predominated over G', and no cross-over point could be detected.

The shear thinning and frequency dependent (time-dependent) behaviour of HA have been noted by several publications (Balazs and Denlinger, 1985; Milas et al., 2001). The polymer solutions

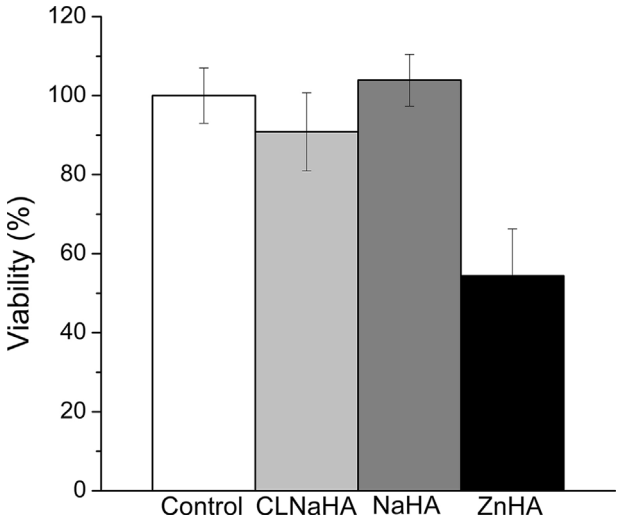

Fig. 2. Biocompatibility of CLNaHA, NaHA and ZnHA.

showed a Newtonian plateau with decreasing shear rate. This plateau viscosity is known as zero-shear viscosity. Increasing the shear rate over the rate at which HA chains can relax, the chains remain distorted and the changes of viscosity refer to shear-thinning behaviour. Both shear-thinning and time-dependent behaviour, where elastic dominance ($G' > G''$) can be seen at high frequencies but viscous dominance ($G'' > G'$) can be observed at low frequencies, are related to the relaxation time of HA. In case of semi-dilute HA solutions the relaxation time depends on the concentration, the solution conditions and the molecular weight of HAs (Cowman and Matsuoka, 2005).

This viscoelastic behaviour of the derivatives is very beneficial for purposes of ocular therapy because they can easily spread over the eye surface during blinking and prolong the residence time of the drug delivery system.

3.2. Cytotoxicity

Fig. 2 shows the results of the biocompatibility determination of CLNaHA, NaHA and ZnHA on RCE cells by the MTT test. As control, HBSS was used.

CLNaHA and NaHA are biocompatible: the cell viability was $90.84 \pm 9.90\%$ in case of CLNaHA and $103.90 \pm 6.56\%$ in the case of NaHA; ZnHA displayed lower biocompatibility (cell viability was $54.39 \pm 11.91\%$) after a 3 h contact time.

Zinc is an essential metal, with important roles in the regulations and structure and as a cofactor for many enzymes. Under *in vivo* conditions, it is non-toxic, thanks to the homeostatic regulatory mechanisms. The maintenance of homeostasis in cell lines is difficult, which leads to a decrease in cell viability. It was established earlier that tolerance to zinc can be dependent on the rate of zinc uptake and the capacity of the protective mechanism (Borovansky and Riley, 1989; Bozym et al., 2010; Mehr, 2011; Ugarte and Osborne, 2001; Ugarte et al., 2013).

Our results demonstrated that CLNaHA and NaHA are biocompatible. Although ZnHA exhibits lower biocompatibility in the RCE cell line, under *in vivo* conditions it may have better biocompatibility thanks to the *in vivo* homeostatic mechanisms.

3.3. Mucoadhesion

3.3.1. Rheology

The mucoadhesive nature of a sample can be determined by the rheological method developed by Hassan and Gallo. During this measurement, the sample is mixed with mucin dispersion and the synergistic increase in rheological parameters is determined, which is caused by chemical and physical bond formation between the mucin and the bioadhesive component. This synergism parameter (bioadhesive viscosity component, η_b) can be calculated from the following formula (Caramella et al., 1999; Hassan and Gallo, 1990; Madsen et al., 1998):

$$\eta_b = \eta_t - \eta_m - \eta_p \quad (1)$$

where η_t is the viscosity of the mucin-polymer solution system, and η_m and η_p are the viscosity components of the mucin and polymer solution, respectively.

Measurements were performed at three different concentrations; 0.5, 1 and 2% w/w. Flow curves of the CLNaHA, NaHA and ZnHA formulations and their mixtures with mucin are presented in Fig. 3.

The measured derivatives and their mixtures with mucin displayed shear-thinning behaviour, with the shear viscosity dependent on the degree of shear load and the flow curve exhibiting a decreasing slope, which is typical for polymer systems. At the beginning of the test, where the shear values are low, the macromolecules are in the state of the lowest level of energy consumption looking like a coil. Each coil is entangled with neighbouring macromolecules. Increasing the shear values, the macromolecules partly disentangle, orient in the shear direction, which lowers their flow resistance. In the third part of the test, where the shear values reduce, fast gel structure regeneration can be observed (Mezger, 2002).

Mucoadhesive behaviour was observed for all formulations at all three concentrations. The shear stress values of the mixtures (gel and mucin) were higher than those of the HA derivatives without mucin. These results correspond to the phenomenon that interactions can occur between the polymers and the mucin. Mucin has a gel-strengthening effect, because more network links are created by entanglements and secondary bond (hydrogen-bond) formation. The calculated synergism parameters of viscosity at a shear rate of $100\,s^{-1}$ are illustrated in Fig. 4.

The calculated values revealed that the mucoadhesive behaviour increased with the increase of the polymer concentration. At higher concentration, an adequate gel structure is probably formed, which can easily interpenetrate and form secondary bonds with the mucin. CLNaHA is a nanoparticulate system which contains intrachain cross-linking, enabling the CLNaHA molecules to interpenetrate more easily than the other two derivatives at all three concentrations. At 0.5% (w/w), CLNaHA exhibited more marked mucoadhesion than those of NaHA and ZnHA, which is very beneficial in the case of eye drops for instillation. ZnHA at lower concentrations has a liquid-like structure, which causes difficulty in interpenetration, while at higher concentration (2%, w/w) it has a gel-like structure and its mucoadhesive behaviour is similar to those of the other derivatives. At 1 and 2% (w/w), there is no significant difference in the mucoadhesivity of CLNaHA and NaHA.

The results of rheological measurements indicated that CLNaHA, NaHA and ZnHA are mucoadhesive, especially at higher polymer concentration. The pronounced mucoadhesive nature of CLNaHA at 0.5% (w/w) is very advantageous in ocular therapy, because the washing-out from the eye by lacrimation after instillation demands more effort as compared with formulations without mucoadhesive polymers. Thanks to the mucoadhesive and viscoelastic behaviour of CLNaHA, NaHA and ZnHA, they are able to prolong the residence time on the ocular surfaces.

3.3.2. Tensile test

Tensile test involves measurement of the force of detachment and the total work of adhesion needed to separate the surfaces,

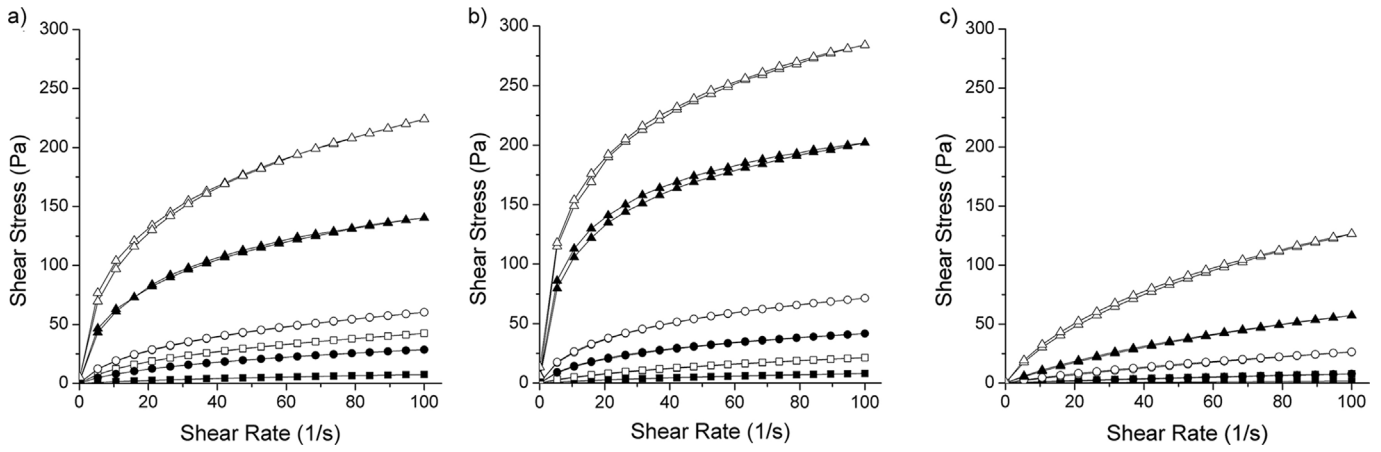

Fig. 3. Flow curves of CLNaHA (a), NaHA (b) and ZnHA (c) at: (◨) 0.5% (w/w), (◑) 1% (w/w) and (◭) 2% (w/w), with mucin (open symbols) or without mucin (solid symbols).

which results from the area under the force–distance curve (Woertz et al., 2013). Many factors influence the phenomena of mucoadhesion, *e.g.* physiological factors (mucin turnover, diseases, *etc.*), environment-related factors (pH, contact time, *etc.*) and polymer-related factors (the molecular weight, the flexibility of the polymer chains and the concentration of the polymer, *etc.*). The studies by Park and Munday established the dependence of the adhesive force of chemical bond formation between the polymers and mucin, whereas the work of adhesion is dependent not only on chemical bond formation, but also on physical mechanisms (entanglements and interpenetration) (Park and Munday, 2002; Vasir et al., 2003).

The adhesive force (F) and the work of adhesion (A) of CLNaHA, NaHA and ZnHA were determined in contact with mucin (Fig. 5).

The values of F for all three derivatives did not increase with increase in concentration. Their potential for chemical bond formation had reached the maximum and the adhesive force could not increase. The values of A increased with increase of the polymer concentration thanks to the physical mechanisms between the polymer and the mucin. These results correspond with the phenomena described by Park and Munday. There was no significant difference between the values of F and A in the cases of CLNaHA and NaHA. ZnHA does not have a gel-like structure at 0.5% (w/w) which would enable it to interpenetrate and form entanglements in the same way as for the other two derivatives. At higher ZnHA concentrations, F and A increased because of the gel-like structure, but not so strongly as for the other two derivatives.

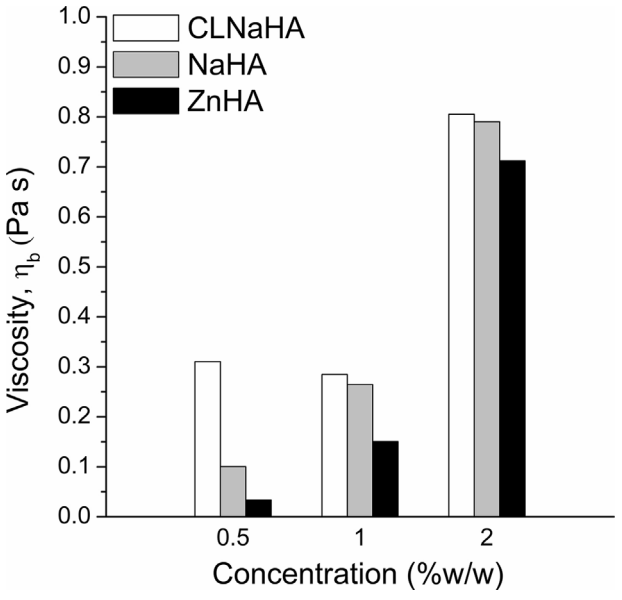

Fig. 4. Calculated synergism parameter values of viscosity at a shear rate of 100 s^{-1}.

The tensile test results correlated with the results of the rheological measurements. In both cases, CLNaHA and NaHA showed the highest capability for mucoadhesive bond formation, and ZnHA the lowest.

Ex vivo measurements were also performed. Gels were placed in contact with excised porcine conjunctiva (Fig. 6). These measurements related to conditions closer to the real mucoadhesive circumstances of the eye.

The values of A were at least twice as high in the *ex vivo* measurements as those measured with mucin in the case of the *in vitro* measurements. This is beneficial for ophthalmic therapy, because it can be predicted that the mucoadhesion of the gels will be higher on the surface of the eye. In these measurements, CLNaHA gave significantly higher A values than those of the other two derivatives. Its nanosized structure leads to easier and deeper interpenetration and easier chemical bond formation with the mucus layer of the eye. The pronounced mucoadhesive behaviour of CLNaHA at 0.5% (w/w) was also seen in the *ex vivo* measurements, proving the possibility of prolonging the residence time on the eye surface even at low CLNaHA concentration. NaHA and ZnHA under *ex vivo* circumstances were probably not able to interpenetrate to the same extent as CLNaHA, but they showed increase in mucoadhesion and no significant difference was observed between them.

3.4. Drug release

The drug release from CLNaHA, NaHA and ZnHA at 1% (w/w) polymer concentration containing 0.1% (w/w) SD was measured with a vertical Franz diffusion cell. Fig. 7 shows the amount of drug released (% w/w) during time (h).

In the first hour of measurements, a rapid diffusion of SD was observed from all three formulations, but their release profiles then separated. Statistical analysis showed that there was no significant difference between CLNaHA and NaHA in the first hour, but CLNaHA later released a higher amount of SD as compared with NaHA. This can be explained by the easier diffusion of SD from the CLNaHA gels, due to the smaller particle size and lower viscosity. NaHA has a linear structure and SD probably cannot diffuse to such an extent as in the case of CLNaHA. ZnHA released a significantly lower amount of SD, even in the first hour, possibly because interactions may occur between SD and ZnHA. This needs to be investigated, but did not constitute part of the present research work.

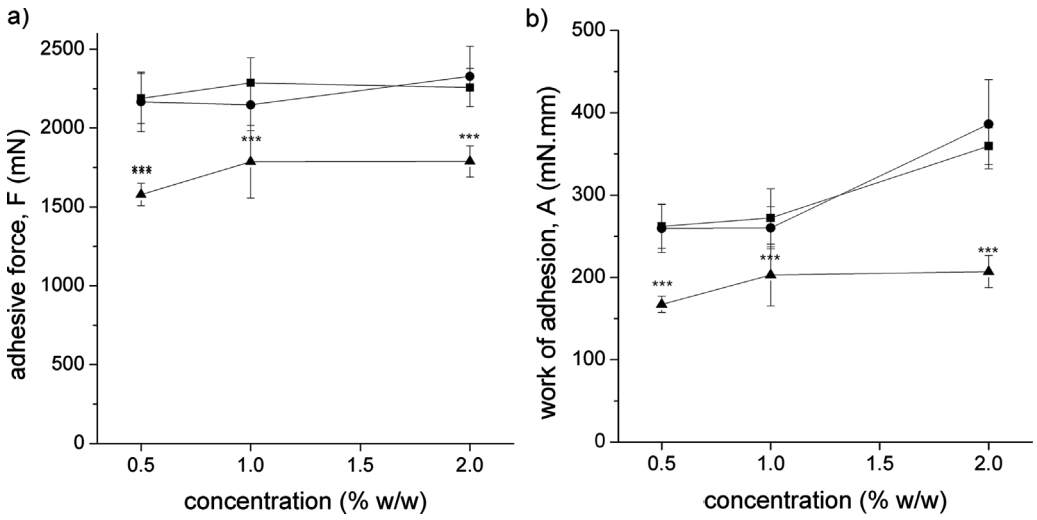

Fig. 5. Adhesive force (a) and work of adhesion (b) of (●) CLNaHA, (■) NaHA and (▲) ZnHA as a function of the concentration of the polymer in contact with mucin (***$p \leq 0.001$ highly significant compared with CLNaHA and NaHA).

The drug release mechanism can be characterized with the following equation:

$$\frac{M_t}{M_\infty} = kt^n \quad (2)$$

where M_t/M_∞ is the fraction of drug released, k is the kinetic constant and n is the release exponent describing the mechanism of the release (Chaturvedi et al., 2011; Kajjari et al., 2014; Peppas et al., 2000). The slopes were determined by power law fitting to the curve of the released drug amount (% w/w) *versus* time (h) of CLNaHA, NaHA and ZnHA in the first hour of the measurements.

The slopes in the first hour indicated non-Fickian drug release in the cases of CLNaHA ($n = 0.6081$, $R^2 = 0.9996$) and NaHA ($n = 0.5814$, $R^2 = 0.9997$), because the n values were between 0.5 and 1. In these anomalous processes of drug release, both, Fickian diffusion through the hydrated layers of the matrix and polymer chain relaxation/erosion are involved. The Fickian contribution to the overall release process decreases with increasing amount of drug released. Thus, the relaxation of the polymer chains becomes more pronounced, which is expected since water is taken up simultaneously with drug release, and this water leads to polymer chain relaxation (Baumgartner et al., 2006; Mundargi et al., 2008; Park and Munday, 2002; Peppas and Buri, 1985; Peppas et al., 2000; Ritger and Peppas, 1987). In the case of ZnHA ($n = 1.0013$, $R^2 = 0.9988$) zero-order kinetics was observed, which confirms the possibility of interactions between SD and ZnHA.

In conclusion, it can be established that all the derivatives undergo rapid release, and up to 6 h release more than 65% (w/w) of the SD. This release profile is beneficial in ocular therapy, because the therapeutic dosage can be reached at the beginning of the application, which is followed by a sustaining dosage.

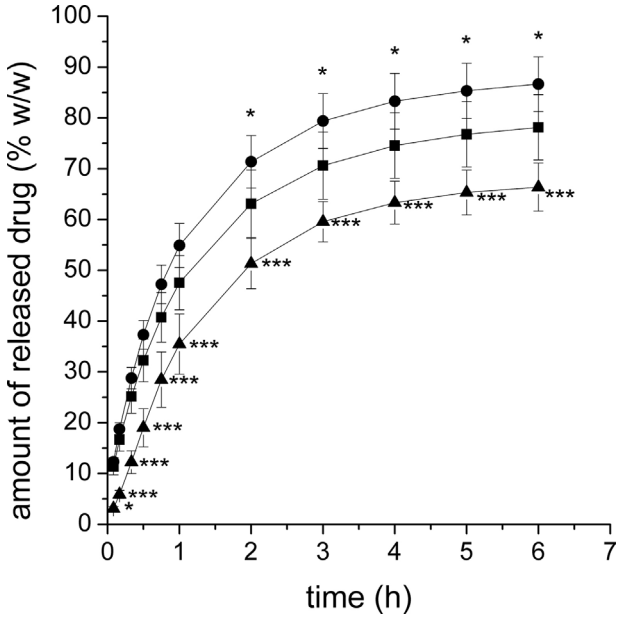

Fig. 7. Release of SD from (●) CLNaHA, (■) NaHA and (▲) ZnHA (*$p \leq 0.05$, significant compared with NaHA; and ***$p \leq 0.001$, highly significant compared with CLNaHA and NaHA).

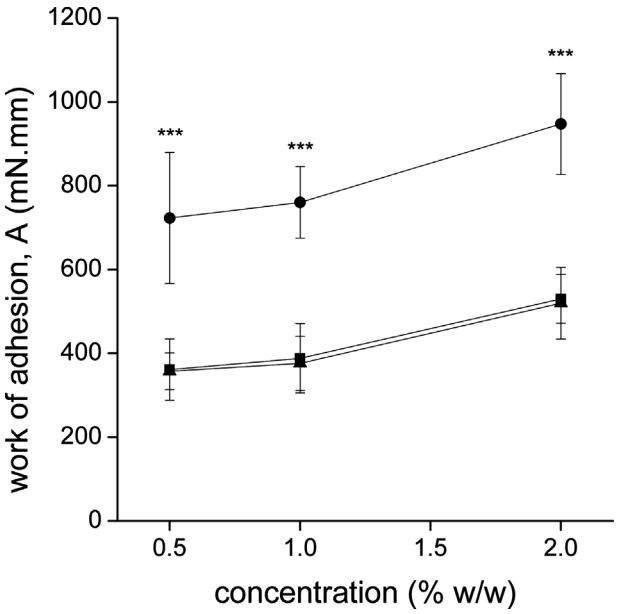

Fig. 6. Work of adhesion of (●) CLNaHA, (■) NaHA and (▲) ZnHA as a function of the concentration of the polymer in contact with excised porcine conjunctiva (***$p \leq 0.001$, highly significant compared with CLNaHA).

4. Conclusion

HA derivatives are widely used and researched in many therapeutic fields, due to their valuable properties such as a high capacity for lubrication, water binding and water retention, and they also play an important role in wound healing. In our work, nanosized CLNaHA, NaHA and ZnHA were investigated. They have different structures, which influence their behaviour. Hence, their structure characterization was first performed by means of rheology, which proved the viscoelastic behaviour of CLNaHA and NaHA, and the viscous fluid behaviour of ZnHA. According to the result of cytotoxicity measurement, CLNaHA and NaHA were biocompatibile, while ZnHA displayed lower biocompatibility. Rheological and tensile test *in vitro* measurements showed their capability for mucoadhesion. *Ex vivo* experiments, involving tensile test, were also performed, these circumstances being closer to those in the eye, to predict the mucoadhesive behaviour on the eye surface or in the *cul-de-sac* more precisely. In this case, higher adhesion work was measured, which predicts an increased level of interpenetration and chemical bond formation on the eye surface or in the *cul-de-sac*.

In all cases, CLNaHA showed the highest capability for mucoadhesion, due to its nanoparticulate structure, which can easily interpenetrate and form secondary bonds with the mucin. The structure of ZnHA hampers interpenetration, entanglement and bond formation, which results in lower adhesive force and work of adhesion values.

As potential drug delivery systems, SD release was also determined. From all three derivatives, rapid release was observed in the initial period, which is especially beneficial in ocular therapy. The advantageous rheological and mucoadhesive properties of the derivatives ensure resistivity during blinking, which prolongs the residence time on the eye. Although ZnHA has weaker mucoadhesive, drug release properties and lower biocompatibility *in vitro*, its application in ophthalmic formulations is favourable due to its scavenger, bactericidal, bacteriostatic and fungicidal effects, which allows omission of the preservative from the formulation. Consequently, all three investigated derivatives can serve as potential ocular mucoadhesive drug delivery systems with an appropriate drug release profile whereby the administration frequency can be decreased and the patient compliance might be increased. However, the nanosized CLNaHA with its increased mucoadhesion, even at lower concentrations, is preferable for use in ophthalmic preparations so as to increase the residence time of the active agent.

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