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Mucoadhesive polymers in ophthalmic therapy

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ABSTRACTS

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V. Benjámin Gyarmati, **Gabriella Horvát**, Mária Budai-Szűcs, Szilvia Berkó, Barnabás Szilágyi, Erzsébet Csányi, András Szilágyi: Mucoadhesive thiolated poly(aspartic acid), *Polymer Network Groups Meeting and Gel Symposium, Tokyo, Japan, 10th to 14th November 2014.*

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ophthalmic use, *1st European Conference on Pharmaceutics – Drug Delivery, Reims, France, 13th to 14th April 2015.*

VIII. Benjámin Gyarmati, Barnabás Szilágyi, **Gabriella Horvát**, Mária Budai-Szűcs, Erzsébet Csányi, András Szilágyi: In situ gelling poly(aspartic acid)s for pharmaceutical applications, *16. Österreichische Chemietage 2015, Joint Meeting of the Italian and Austrian Chemical Societies, Innsbruck, Austria, 21st to 24th September 2015.*

IX. Barnabás Áron Szilágyi, Benjámin Gyarmati, **Gabriella Horvát**, Mária Budai-Szűcs, Erzsébet Csányi, András Szilágyi: Thiolated poly(aspartic acid): an in situ gelling mucoadhesive polymer, *16. Österreichische Chemietage 2015, Joint Meeting of the Italian and Austrian Chemical Societies, Innsbruck, Austria, 21st to 24th September 2015.*

X. **Horvát Gabriella**, Csányi Erzsébet, Budai-Szűcs Mária: Szemészeti terápia során alkalmazható első és második generációs mukoadhezív polimerek, *Gyógyszertechnológiai és Ipari Gyógyszerészeti Konferencia 2015, Siófok, Magyarország, 2015. október 15-17.*

TABLE OF CONTENTS

ABBREVIATIONS
1. INTRODUCTION 1
2. LITERATURE SURVEY 2
2.1. Possible drug delivery routes in the eye 2
2.2. Challenges in ocular drug delivery formulation 3
2.3. Regulatory considerations 5
2.4. Possible ways to increase the bioavailability of drugs in topical ophthalmic therapy 6
2.4.1. First generation mucoadhesive polymers 6
2.4.2. Second generation mucoadhesive polymers 7
2.5. Mucoadhesion 8
2.5.1. Mechanism of mucoadhesion 9
2.5.2. Mucoadhesion theories 9
2.6. Experimental aims 11
3. MATERIALS AND METHODS 12
3.1. Materials 12
3.1.1. Hyaluronic acid derivatives 13
3.1.2. Thiolated poly(aspartic acid) polymers 14
3.2. Methods 14
3.2.1. Preformulation measurements 14
3.2.2. Cytotoxicity 15
3.2.3. Rheology 15
3.2.3.1. Rheological data analysis 16
3.2.4. Swelling 16
3.2.4.1. Swelling data analysis 17
3.2.5. Tensile test 17
3.2.5.1. Tensile test data analysis 17
3.2.6. ‘Wash away’ measurement 18
3.2.7. Drug release 18
3.2.7.1. Drug release data analysis 18
3.2.8. Statistical analysis 18

4. RESULTS AND DISCUSSION 19
4.1. First generation mucoadhesive polymers 19
4.1.1. Rheology of the gels 19
4.1.2. Cytotoxicity 20
4.1.3. Mucoadhesion 21
4.1.3.1. Rheology 21
4.1.3.2. Tensile test 23
4.1.4. Drug release 25
4.1.5. Conclusion 26
4.2. Second generation mucoadhesive polymers 27
4.2.1. Preformulation measurements 27
4.2.2. Cytotoxicity 29
4.2.3. Gel formation 30
4.2.4. Swelling of ThioPASP hydrogels 31
4.2.5. Mucoadhesion 32
4.2.5.1. Rheology 32
4.2.5.2. Tensile tests 35
4.2.5.3. ‘Wash away’ measurements 37
4.2.6. Effects of blinking on the gel structure 38
4.2.7. Drug release measurements 39
4.2.8. Effect of the stabilizing agent on the ThioPASP properties 40
4.2.8.1. Mucoadhesion measurements 41
4.2.8.2. Drug release 42
4.2.9. Conclusion 43
5. SUMMARY 44
6. REFERENCES 46

ABBREVIATIONS

% S	percentage swelling
A	work of adhesion
ACC	acetylcysteine stabilized thiolated poly(aspartic acid)
API	active pharmaceutical ingredient
CLNaHA	crosslinked sodium hyaluronate
CDI	1-[3(dimethylamino)propyl]-3-ethylcarbodiimide methiodide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol stabilized thiolated poly(aspartic acid)
F	adhesive force
F_{swp}	swelling power
G'	storage modulus
G''	loss modulus
GSH	glutathione stabilized thiolated poly(aspartic acid)
HBSS	Hank's balanced salt solution
HEC	hydroxyethylcellulose
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
η^*	complex viscosity
NaHA	sodium hyaluronate
PASP	poly(aspartic acid)
PBS	phosphate buffered saline solution
RCE	corneal epithelial cells of rabbits
SD	sodium diclofenac
ThioPASP	thiolated poly(aspartic acid)
ZnHA	zinc hyaluronate

1. INTRODUCTION

With the aging of the population, the need for the treatment of ocular diseases and disorders has become more important than ever. Increasingly high incidences of age-related macular degeneration, glaucoma, diabetic retinopathy and ocular inflammatory diseases demand better, more effective and innovative treatments. If we are to maintain the quality of life for this aging population, the preservation of vision is critical.

Unfortunately, the ophthalmic formulations on the market suffer from poor bioavailability (< 2%) and it would be useful to design a new formulation which is able to prolong the residence time and reduce the administration frequency. Since topical ocular delivery treatments are considered to be the safest, least invasive and most self-administrable, their development is highly sought.

The formulation of ocular drug delivery systems poses many challenges, but also offers many opportunities to overcome the inadequacies of the current formulations. The corneal epithelium has a complex hydro- and lipophilic character that limits drug absorption, and the eye has many protective mechanisms, including blinking, tear turnover and reflex lacrimation. There is therefore a need for the frequent instillation of eye drops, which is accompanied by discomfort and a decrease in patient compliance, especially in the long term.

One way to overcome the natural anatomical barriers of the eyes is to take advantage of the mucosal layer and to formulate a drug delivery system with mucoadhesive properties. Polymer matrices which exhibit strong mucoadhesion are promising platforms in ocular drug delivery from the aspect of improved bioavailability.

In my Ph.D. work, first (hyaluronic acid (HA) derivatives) and second generation (thiolated polymers) mucoadhesive polymers were characterized as potential ocular drug delivery systems. I carried out gel characterization (rheology) and determinations of mucoadhesion and drug release. Thiolated polymers, as new potential excipients in ophthalmic therapy, were characterized in a wide range.

2. LITERATURE SURVEY

2.1. Possible drug delivery routes in the eye

The main routes of ocular drug delivery system administration are topical, systemic/oral, periocular and intravitreal (Fig. 1). The most important processes in the eye are: trans-corneal permeation from the lacrimal fluid into the anterior chamber (Fig. 1, 1); non-corneal drug permeation across the conjunctiva and sclera into the anterior uvea (Fig. 1, 2); drug distribution from the blood stream via the blood–aqueous barrier into the anterior chamber (Fig. 1, 3); elimination of the drug from the anterior chamber by the aqueous humour turnover to the trabecular meshwork and Sclemm's canal (Fig. 1, 4); drug elimination from the aqueous humour into the systemic uveoscleral circulation (Fig. 1, 5); drug distribution from the blood into the posterior eye across the blood–retina barrier (Fig. 1, 6); intravitreal drug administration (Fig. 1, 7); drug elimination from the vitreous via the posterior route across the blood−retina barrier (Fig. 1, 8); and drug elimination from the vitreous via the anterior route to the posterior chamber (Fig. 1, 9) (Amo and Urtti, 2008; Almeida et al., 2014).

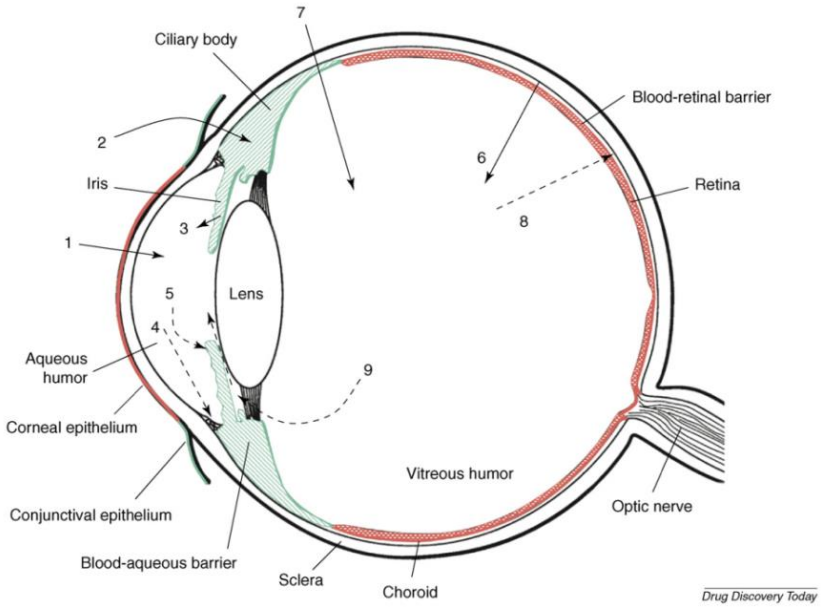

Fig. 1. Routes of drug absorption and elimination (Amo and Urtti, 2008)

In cases of topical application, eye drops, gels and ointments are used to target the anterior segment (cornea, conjunctiva, sclera, iris and ciliary body) of the eye. The most important benefits of this therapy are the non-invasiveness and administration by the patients themselves (Davis et al., 2004). Systemic delivery (oral) is non-invasive and very patient−compliant drug

administration, but unfortunately high dosage concentrations can be required, which can cause toxicity and side−effects (Gaudana et al., 2010). Periocular delivery (injections and implants in the eye) is more invasive and less patient−compliant, but more efficient, especially if the posterior segment of the eye is targeted (Ghate and Edelhauser, 2006). Intravitreal injections or implants are the most invasive forms of administration, which can involve several risks for the patient (haemorrhage or retinal detachment). For these reasons, the patient−compliance is very low, but higher concentrations of the active pharmaceutical ingredient (API) can be maintained in the retina or vitreous (Amo and Urtti, 2008; Lorentz and Sheardown, 2014).

2.2. Challenges in ocular drug delivery formulation

In ophthalmic therapy, there is an obvious need for more efficient formulations, but a number of factors must be taken into consideration, such as anatomical and biopharmaceutical aspects, patient-driven challenges and, not least, mandatory regulatory factors (Almeida et al., 2014; Lorentz and Sheardown, 2014).

The anatomy of the eye poses considerable difficulties for ocular drug delivery. The most important anatomical barriers of the eye are the barriers responsible for drug removal from the ocular surface (blinking and the tear film) and the lacrimal fluid−eye barriers (the cornea and the conjunctiva) (Urtti, 2006; Ruponen and Urtti, 2015). The volume of a dispensed eye drop is 5-6 times greater than the tear fluid volume on the ocular surface. During eye drop instillation, the fluid may flow out of the eye, followed by reflex blinking and a possibly increase in tear secretion, especially if the eye drop contains an irritant (Urtti and Salminen, 1993; Ghate and Edelhauser, 2006; Reimondez-Troitiño et al., 2015). Both the pH of the drug delivery system and the osmolality of the formulation must be similar to those of the natural tear film, as otherwise the formulation can cause increased tearing and irritation, resulting in poor therapeutic efficiency (Baeyens and Gurny, 1997). The corneal surface and conjunctiva are covered by a mucin coat, secreted by the goblet cells of the conjunctiva, with the functions of hydration, cleaning, lubrication and defence against pathogens. The corneal epithelium contains five cell layers, which are very well sealed, because the cells are joined by tight junctions and gap junctions, and they provide resistance against both hydrophilic and lipophilic active ingredients (Ghate and Edelhauser, 2006; Reimondez-Troitiño et al., 2015). Another possibility for drug removal is absorption of the drug into the systemic circulation (Urtti, 2006).

The biopharmaceutical-driven challenges involve the hydrophilicity or lipophilicity, and the size and the charge of the API. APIs with an amphiphilic character have the greatest chance of penetrating through the cornea and conjunctiva (Ahmed et al., 1987; Sasaki et al., 1995). The molecular mass of the drug and its delivery system plays important roles in the penetration (Sunkara and Kompella, 2003; Rabinovich et al., 2004). The components of the tears (buffers and proteins) must be taken into consideration during the formulation of a new ocular drug delivery system, because they can bind to the API and change its ionization state (Shell, 1982). All these physicochemical properties can affect the route and the rate of permeation in the cornea.

The needs of patients must be satisfied by novel formulations. The optimum drug delivery system for patients must be effective, should require few applications per day and should be easy to handle and dispense; it must not cause local or systemic adverse events and only minimal or no visual interference, no ocular discomfort or foreign body sensation and no blockage of puncti or canaliculi; it must be as non-invasive as possible; and it must be inexpensive (Lorentz and Sheardown, 2014). Studies have shown that the more instillations or injections required and the more invasive the procedure, the greater the degree of patient non-compliance (Fig. 2) (Ghate and Edelhauser, 2006).

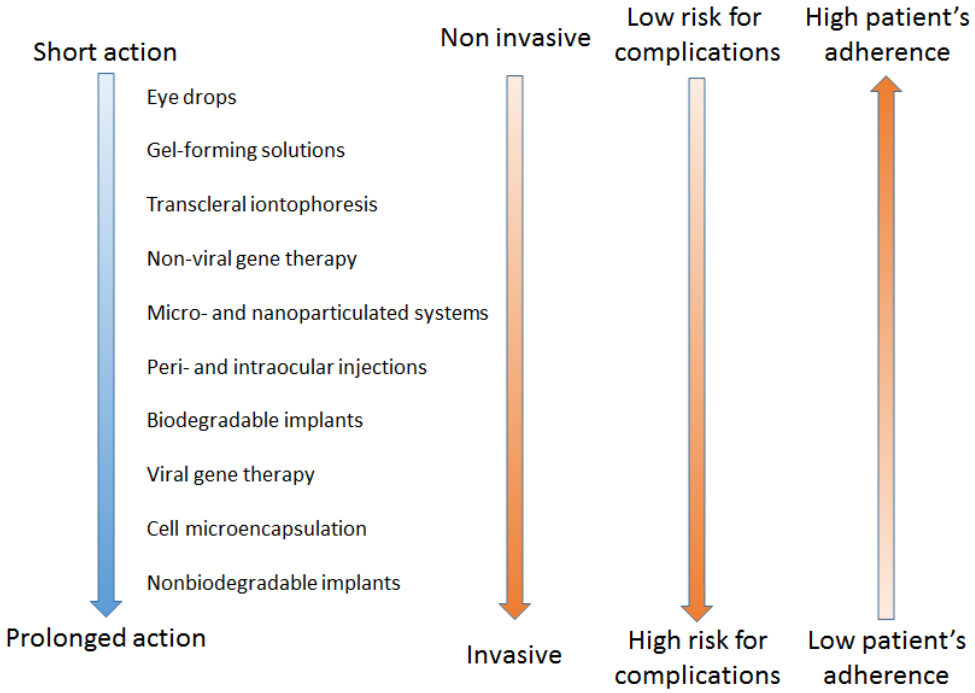

Fig. 2. Possible drug delivery systems in the eye and their invasiveness, risk of complications and patient compliance

2.3. Regulatory considerations

To launch an ophthalmic product, knowledge of the regulations is necessary. The regulation requirements of ophthalmic formulations have not been well defined, they differ considerably around the world and there is a need for mutual approvals. New drugs or delivery systems require human clinical trials in accordance with the Investigational New Drug Application in the United States or the Clinical Trial Notification in Europe (Ali and Lehmussaari, 2006).

The European Commission has issued protocols for local toxicity and eye irritation measurements, in which the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for the rabbit corneal epithelial (RCE) cell line is included. This test provides essential information on the biocompatibility of the measured API or excipients at a cellular level (DB-ALM, DataBase service on ALternative Methods to animal experimentation), which can be a basis of further *in vivo* experiments.

During drug delivery formulation, it must be taken into consideration that excipients are neither inert nor inactive substances and they can also cause adverse reactions (Baldrick, 2000; Pifferi and Restani, 2003).

As concerns the formulation requirements, *The International Pharmacopoeia (Ph. Int. Fifth Edition,* published by the WHO*)* includes a collection of recommended procedures for analysis and specifications for the determination of pharmaceutical substances, excipients and dosage forms. In connection with the definition of ophthalmic drops, the Fourth Supplement to the Fifth Edition of *The International Pharmacopoeia* specifies that the preparation of aqueous ophthalmic drops requires careful consideration of the need for isotonicity, a certain buffering capacity, the desired pH, the addition of antimicrobial agents and/or antioxidants, the use of viscosity-increasing agents, and the choice of appropriate packaging, which also correspond to the guidelines of the American Society of Hospital Pharmacists on Pharmacy-Prepared Ophthalmic Products (ASHP, 1993; The International Pharmacopoeia). Although tests of these factors are not listed in the regulatory directives, the manufacturers must fulfil them and these preformulation measurements are included in the Drug Master File or Applications (Ali and Lehmussaari, 2006).

2.4. Possible ways to increase the bioavailability of drugs in topical ophthalmic therapy

Numerous strategies have been developed to improve topical ocular bioavailability. The most common are eye ointments, prodrugs, penetration enhancers, liposomes, niosomes, nanoparticles, nanospheres, nanosuspensions, microemulsions and viscosity enhancers (mucoadhesive polymers, gels and *in situ* forming gels) (Lorentz and Sheardown, 2014; Reimondez-Troitiño et al., 2015).

The use of mucoadhesive drug delivery system prolongs the contact time between the preparation and the corneal/conjunctival epithelium (Ludwig, 2005; Patel et al., 2010, Ruponen and Urtti, 2015). The mucoadhesive polymers can be classified into two main categories: first and second generation mucoadhesive polymers (Smart, 2005; Andrews et al., 2009; Serra et al., 2009; Carvalho et al., 2010; Karolewicz, 2015).

2.4.1. First generation mucoadhesive polymers

The first-generation mucoadhesive polymers are natural or synthetic hydrophilic molecules, which can be anionic, cationic or non-ionic. These polymers are considered to be non-specific mucoadhesive systems, because the adhesion may occur at sites other than expected.

Anionic polymers are used in pharmaceutical formulations, thanks to their mucoadhesivity and low toxicity. These polymers are characterized by the presence of carboxyl and sulfate functional groups. They include poly(acrylic acid), sodium carboxymethylcellulose, polycarbophil, carbomer, alginates, hyaluronic acid, etc.

Cationic polymers are able to bind to mucus via ionic interactions, thanks to the negatively charged surface of the mucus layer in addition to hydrogen−bonding. The most widely studied cationic polymer is chitosan.

Non−ionic polymers are weaker mucoadhesives as compared with the anionic and cationic polymers. This group of polymers includes hydroxypropylmethylcellulose, hydroxyethylcellulose (HEC) and methylcellulose (Andrews et al., 2009; Serra et al., 2009; Carvalho et al., 2010; Karolewicz, 2015).

2.4.2. Second generation mucoadhesive polymers

The second-generation mucoadhesives are derivatives of the first-generation polymers (e.g. thiolated polymers) and include several new mucoadhesives (e.g. lectins and bacterial adhesives) (Andrews et al., 2009; Serra et al., 2009).

Lectins are naturally present proteins that play a fundamental role in biological recognition phenomena (cells and proteins). They are glycoproteins which are able to bind non-covalently to glycosylated components of the cellular membrane, but not of the mucus, and adhesion can therefore be called cytoadhesion. The disadvantages of these systems are their toxicity and immunogenicity and they can induce antibodies, which can render individuals susceptible to systemic anaphylaxis on subsequent exposure (Andrews et al., 2009; Han et al., 2015).

The function of bacterial adhesions is based on the phenomenon of the pathogenic bacteria adhering to the mucosal membranes in the gastrointestinal tract. K99−fimbriae (from *E. coli*) are covalently attached to polyacrylic acid networks, which increase the *in vitro* adhesion relative to the unmodified polymer, through the adhesion to the epithelial surface of the erythrocytes (Serra et al., 2009; Carvalho et al., 2010).

Thiolated polymers (thiomers) are mucoadhesive polymers with thiol group-containing side-chains (Bernkop-Schnürch, 2005). The most commonly used thiomers are synthetized from chitosan, alginate, polyacrylates and cellulose derivatives (Andrews et al., 2009). In contrast with the first-generation polymers, they are capable of forming covalent (disulfide) bonds with cysteine-rich subdomains of the mucus layer (Bernkop-Schnürch, 2005).

Other advantages of thiomers include permeation enhancement through the reversible opening of the tight junction, enzyme inhibition and efflux pump inhibition (Iqbal et al., 2011; Rahmat et al., 2012; Gradauer et al., 2013). As a result of these advantages, these polymers ensure the prolongation of the residence time and increase the bioavailability. They can be used in many medical fields (e.g. topical ocular therapy) in various dosage forms, such as liquid drops, gels or mini-tablets (Bernkop-Schnürch, 2005).

Earlier studies (Marschutz and Bernkop-Schnürch, 2002; Bernkop-Schnürch et al., 2003) revealed the lower stability of thiolated polymers in solution, thanks to thiol oxidation at $pH \geq 6$. During the oxidation process, inter- and intramolecular disulfide bonds are formed, limiting the permeation enhancement and mucoadhesivity of the solutions. At higher pH of the thiomer

solution, the thiol groups are oxidized more rapidly, thanks to the decrease in H^+ concentration leading to an increase of the negative thiolate anions, S^-, which are more capable of oxidation.

There are two ways for the stabilization of thiolated polymers in solution: 1) the use of reducing agents (antioxidants) or 2) thiol group protection by already−formed disulfide bonds (Marschutz and Bernkop-Schnürch, 2002; Bernkop-Schnürch et al., 2003; Dünnhaupt et al., 2012).

The addition of a reducing agent during or after synthesis ensures the stability of thiol groups in solution, providing free thiol groups for better mucoadhesion and permeation. In earlier studies 2-mercaptoethanol (Bernkop-Schnürch et al., 2003), dithiothreitol (DTT), sodium borohydride (Bernkop et al., 2004), hydroxylamine (Kafedjiiski et al., 2005), EDTA (Martien et al., 2011) and sodium cyanoborohydride (Rahmat et al., 2011) were used to avoid the oxidation of thiol groups.

In the case of thiol group protection, thiol groups are protected by already−formed disulfide bonds. In earlier studies, this type of protection was performed with pyridyl sulfhydryl (Dünnhaupt et al., 2012), 6-mercaptonicotinamide (Dünnhaupt et al., 2012, Laffleur et al., 2015), 2-mercaptonicotinamide (Wang et al., 2012; Hintzen et al., 2013) or 3-methyl-1-phenylpyrazole-5-thiol (Müller et al., 2013). Thanks to the addition of these protective agents, the thiolated polymers have improved stability and mucoadhesive, enzyme-inhibitory, permeation-enhancing and efflux-pump inhibiting properties (Dünnhaupt et al., 2012). The disadvantage of this method is the longer synthesis.

2.5. Mucoadhesion

One of the most important phenomena in ocular formulations is the adhesion between the drug delivery system and the eye tissues. In bioadhesion, physical or chemical bonds are formed between the biological and synthetic surfaces. Mucoadhesive drug delivery vehicles exploit the adhesion between the polymer component and the biological tissue, a mucosal membrane, the mechanism being referred to as mucoadhesion (Chickering and Mathiowitz, 1999). In the case of the ocular mucus, the conjunctival goblet cells, the conjunctival epithelium and the corneal epithelium are responsible for the secretion of mucin. Mucins are large glycoproteins which are mainly composed of a protein core and carbohydrates and are well glycosylated. There are two

types of ocular mucins: membrane-associated and secreted mucins (Lorentz and Sheardown, 2014; Ruponen and Urtti, 2015).

The new delivery systems with mucoadhesive properties have various advantages: better bioavailability, a lower active ingredient concentration is sufficient and the administration frequency can be decreased, thanks to the enhanced residence time (Saettone et al., 1985; Andrews et al., 2009).

The mechanisms governing mucoadhesion are determined by the intrinsic properties of the formulation and by the environment in which it is applied. The polymer properties include its molecular mass, the presence of functional groups, the chain flexibility, the concentration, the degree of cross-linking and the degree of hydration. The environmental-related factors are the pH, the initial contact time, the swelling and the physiological variations (Leung and Robinson, 1990; Robinson and Mlynek, 1995; Leitner et al., 2003; Ludwig, 2005; Andrews et al., 2009; Carvalho et al., 2010).

2.5.1. Mechanism of mucoadhesion

Mucoadhesion can be described in three steps: 1) the formation of an intimate contact between the mucoadhesive preparation and the mucus, followed by the wetting of the mucoadhesive formulation; 2) the swelling of the macromolecules and the formation of an interpenetrating network with the mucus macromolecules; and 3) chemical bond formation (primary or secondary) between the entangled chains (Duchêne et al., 1988; Caramella et al., 2015).

Physical and chemical interactions can arise during the process of mucoadhesion. Physical interactions may occur during the interpenetration of the polymer chains into the mucin layers, and primary (covalent) and secondary chemical bonds (i.e. ionic bonds, hydrogen-bonds and van der Waals interactions) can evolve between the entangled chains (Dodou et al., 2005).

2.5.2. Mucoadhesion theories

Numerous theories have been put forward to explain the complex phenomenon of mucoadhesion, such as electronic, adsorption, wetting, diffusion and fracture theories. It is difficult to compare these theories, but they may well supplement each other and reflect the complex nature of mucoadhesion (Fig. 3).

The *electronic theory* is based on the different electronic structures of the polymer and mucin; it follows that a double layer of electrical charge is formed on the interface, the attractive forces within this electronic double layer determining the mucoadhesive strength.

The *adsorption theory* is based on the formation of van der Waals interactions, hydrogen-bonds, etc. Such forces have been considered the most important in the adhesive interaction phenomenon because, although they are individually weak, a great number of interactions can result in intense global adhesion.

The *wetting theory* relates to the ability of the mucoadhesive polymer to spread over a tissue. The general rule states that the lower the contact angle, the greater the affinity.

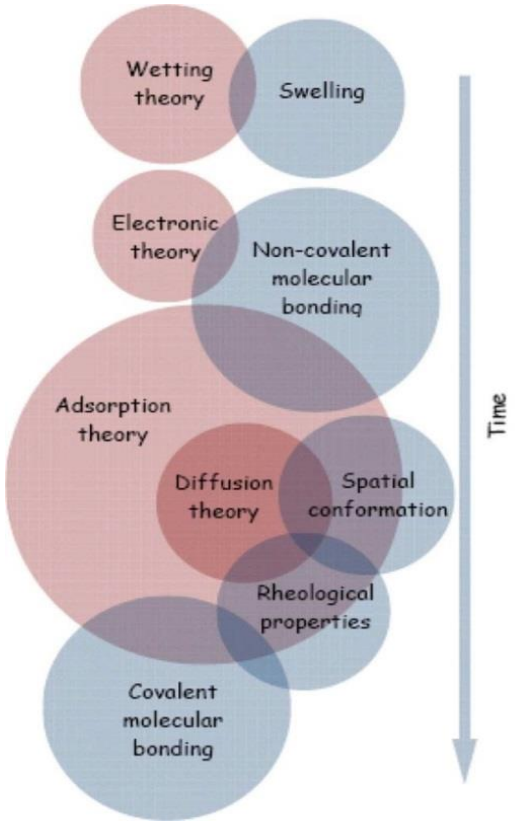

Fig. 3. Mucoadhesion theories (Dodou et al., 2005)

The most important step in the *diffusion theory* is the interpenetration of the polymer chains into the mucus. It is believed that the adhesion force increases with the degree of penetration of the polymer chains. In order for diffusion to occur, it is important that the components involved should have good mutual solubility, which means that the bioadhesive and the mucus should have similar chemical structures.

The *fracture theory* analyses the forces required to separate the two surfaces after adhesion.

The mechanical theory assumes that adhesion arises from the interlocking of a liquid adhesive into the irregularities on the rough surfaces, and provides an increased surface area available for interaction together with an enhanced viscoelastic and plastic dissipation of energy during joint failure, which are thought to be more important than a mechanical effect in the adhesion process (Chickering and Mathiowitz, 1999; Smart, 2005; Serra et al., 2009; Carvalho et al., 2010).

2.6. Experimental aims

In ophthalmic drug delivery systems, the polymers applied play an important role in the increase of the bioavailability. The use of mucoadhesive polymers can increase the residence time on the ocular surface or in the cul-de-sac. For this reason, it is very important to determine the mucoadhesive properties of the polymers. Since these polymers are planned to be used in ophthalmic therapy, the matrix also has to be characterized with regard to its potential for drug release.

In my Ph.D. work, I characterized hyaluronic acid derivatives as first generation and thiolated poly(aspartic acid) (ThioPASP) polymers as second generation mucoadhesive polymers, as potential vehicles for ocular drug delivery systems.

The aims of my experimental work can be summarized as follows (Fig. 4):

- Comparisons of a nanosized cross-linked sodium salt (CLNaHA), a linear sodium salt (NaHA) and a linear zinc salt of hyaluronic acid (ZnHA):
    - investigation of their biocompatibility,
    - rheological characterization of the matrix of the HA derivatives,
    - mucoadhesion determination:
        - *in vitro* (rheology and tensile test) measurements,
        - *ex vivo* (tensile test) measurements,
    - drug release profile determination.
- Characterization of ThioPASP as a potential new type of excipient in ophthalmic therapy:
    - preformulation measurements from the aspect of ophthalmic drug delivery system formulation,
    - investigation of biocompatibility,
    - polymer matrix characterization:
        - swelling capability,

- rheological properties,

- determination of mucoadhesion:
  - *in vitro* (rheology and tensile test) measurements,
  - *ex vivo* (tensile and 'wash away' test) measurements,
- drug release profile determination,
- determination of the effects of the stabilizing agents (dithiothreitol, glutathione and acetylcysteine stabilization) on the properties of the ThioPASP polymers:
  - determination of mucoadhesion (rheology and tensile test),
  - drug release profile determination.

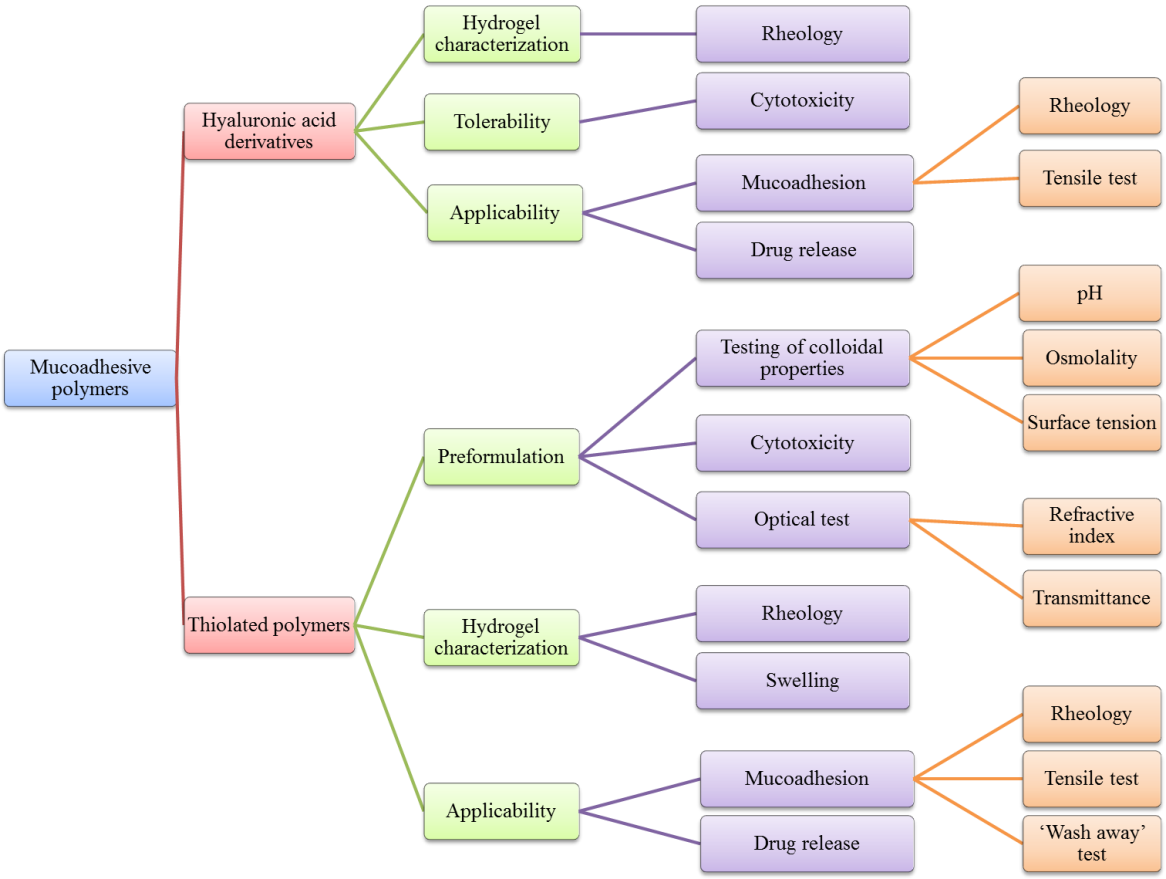

Fig. 4. Measurements performed with first and second generation mucoadhesive polymers

3. MATERIALS AND METHODS

3.1. Materials

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} $MgCl_2.6H_2O$ and 73.5 mg dm^{-3} $CaCl_2 \cdot H_2O$ in distilled water, the pH being adjusted with 1 M HCl.

2,2-(Ethylenedioxy)bis(ethylamine), 1-[3(dimethylamino)propyl]-3-ethylcarbodiimide methiodide, mucin (porcine gastric mucin type II), MTT, HBSS (Hank's Buffered Salt Solution), dimethyl sulfoxide, sodium diclofenac (SD) and sodium fluorescein were purchased from Sigma Aldrich (USA). Mucin dispersions were prepared with PBS or simulated lacrimal fluid and stirred for 8 h. HEC (Natrosol Pharm) was bought from Hercules.

3.1.1. Hyaluronic acid derivatives

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 (Debrecen, Hungary).

As topical use, HA is applied in the treatment of dry eye and Sjögren's syndrome. In higher concentrations, with a gel-like 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 (Price et al., 2007; Vasi et al., 2014).

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

Another HA modification involves ZnHA 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 scavenging, bactericidal, bacteriostatic and fungicidal effects, which are useful in ocular therapy, because the traditional preservative may then be omitted from the formulation (Nagy et al., 1998; Illés et al., 2002).

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.

3.1.2. Thiolated poly(aspartic acid) polymers

In our work, thiol-containing side-groups were bonded to poly(aspartic acid) (PASP). PASP polymers were synthetized by the Soft Matters Group at Budapest University of Technology and Economics (Fig. 5). PASP is a biocompatible and biodegradable polymer by virtue of its protein-like structure, and its degradation products are excreted by the physiological mechanisms of the body. It is not toxic and does not generate immunogenicity. In *in vitro* and *ex vivo* experiments, 1 M $NaBrO_3$ solution was used as a model oxidant (Gyenes et al., 2008; Gyarmati et al., 2013; Gyarmati et al., 2014).

The following reducing agents were used as antioxidants during the synthesis: dithiotreitol (Merck), glutathione (Merck) and N-acetylcysteine (Reanal Hungary).

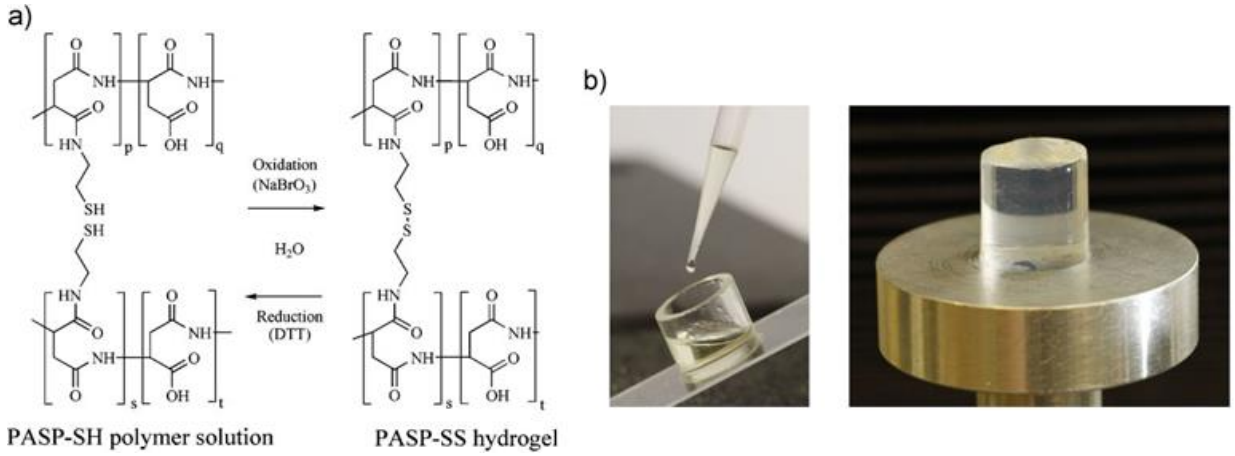

Fig. 5. a) Reaction of reversible thiol-disulfide exchange; b) oxidation−induced sol-gel transition (Gyarmati et al., 2013)

3.2. Methods

3.2.1. Preformulation measurements

Osmolality, surface tension, refractive index and transmittance were measured in aqueous solutions of ThioPASP at five concentrations (1, 3, 5, 7 and 10% w/w).

Osmolality measurements were carried out with an automatic osmometer (Knauer Semi-micro Osmometer, Germany) by measurement of the freezing point depression of the solution.

Surface tension measurements were performed with the OCA Contact Angle System (Dataphysics OCA 20, Dataphysics Inc., GmbH, Germany), using the pendant drop method. The Young-Laplace equation was used for the calculation of surface tension (OCA Manual).

Refractive index was measured with an Abbe refractometer.

The *pH* of ThioPASP solutions prepared with distilled water and PBS was determined with a pH-meter (Testo 206-pH2, UK).

Optical tests were performed by the measurement of transmittance with a UV-spectrophotometer (Unicam Heλios α Thermospectronic UV-spectrophotometer v4.55, UK) in the wavelength range 200-600 nm (Budai-Szűcs et al., 2015).

3.2.2. Cytotoxicity

For the cytotoxicity measurements, MTT tests were performed on the RCE cell line by a method described previously (Sandri et al., 2012; Mori et al., 2014; Horvát et al., 2015a,b). CLNaHA, NaHA and ZnHA formulations of 4% w/w were used in 20-fold dilution. ThioPASP solutions were measured in concentrations of 5, 7 and 10% w/w. All samples were brought into contact with cells for 3 h.

3.2.3. Rheology

The rheological properties were studied with a Physica MCR101 rheometer (Anton Paar, Austria). The tests were performed by a method described previously (Horvát et al., 2015a,b,c; Budai-Szűcs et al., 2015).

Measurements were carried out with CLNaHA, NaHA and ZnHA gels with and without mucin (the final mucin concentration in the mixtures was 5% w/w). Flow curves and viscoelastic character were determined. Measurements were made over the frequency range from 0.01 to 100 Hz, whereby the storage modulus (*G'*), loss modulus (*G''*) and complex viscosity (η^*) were determined.

ThioPASP was dissolved in PBS and gelation was initiated by the addition of oxidant (20% w/w). The precursor solutions of the hydrogels consisting of the ThioPASP and oxidant were mixed on the plate of the rheometer. Measurements were performed with and without mucin (the final mucin concentration in the mixtures was 5% w/w). The gelation and the viscoelastic character (frequency sweep tests) were made over the angular frequency range from 0.1 to 100 s^{-1}, whereby *G'*, *G''* and η^* were determined. In order to investigate the effect of blinking on the gel structure, accelerated blinking cycles were applied by using the automation function of the instrument. Tests were performed at 10% w/w ThioPASP.

3.2.3.1. Rheological data analysis

Rheological synergism between mucin and polymer mixtures can be proposed as an *in vitro* parameter through which to determine the mucoadhesive behaviour of polymers (Hassan and Gallo, 1990). The rheological method is based on the determination of the changes in rheological parameters after the mucoadhesive polymer is mixed with mucin. Hassan and Gallo demonstrated that a synergistic increase in viscosity could be observed when the mucoadhesive polymer and mucin were mixed together. This viscosity change, called the bioadhesive viscosity component (η_b), is caused by chemical and physical bonds formed in mucoadhesion. It can be calculated as follows:

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

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 solutions (Hassan and Gallo, 1990; Caramella et al., 1999; Marschütz and Bernkop-Schnürch, 2002).

More recently, the rheological synergism parameters have been measured by dynamic oscillatory rheometry. In this case, the absolute synergism parameters *(*$\Delta G'$ and $\Delta\eta^*$) can be calculated as follows (Madsen et al., 1998):

$$\Delta G' = G'_{(mix)} - (G'_{(polymer)} + G'_{(mucin)}) \quad (2)$$

$$\Delta\eta^* = \eta^*_{(mix)} - (\eta^*_{(polymer)} + \eta^*_{(mucin)}) \quad (3)$$

where *mix* is the polymer-mucin mixture.

If the calculated synergism parameters are negligible, it is reasonable to use the relative rheological synergism parameters ($\Delta G'_{rel}$ and $\Delta\eta^*_{rel}$), which express the relative increments in viscoelasticity with regard to the polymer ($G'_{(polymer)}$ and $\eta^*_{(polymer)}$) and mucin ($G'_{(mucin)}$ and $\eta^*_{(mucin)}$) solutions alone (Madsen et al., 1998; Horvát et al., 2015c):

$$\Delta G'_{rel} = \frac{\Delta G'}{G'_{(polymer)} + G'_{(mucin)}} \quad (4)$$

$$\Delta\eta^*_{rel} = \frac{\Delta\eta^*}{\eta^*_{(polimer)} + \eta^*_{(mucin)}} \quad (5)$$

3.2.4. Swelling

The water absorption capacity of the ThioPASP gels was determined gravimetrically by a method described previously (Horvát et al., 2015c). 20% w/w mixtures of ThioPASP with oxidant (1 M $NaBrO_3$, 20% w/w) were measured.

3.2.4.1. Swelling data analysis

The percentage swelling (*% S*) gives information on the water uptake capacity of the polymer, which can be calculated from the following equation:

$$\% S = \frac{M_t - M_0}{M_0} \times 100 \quad (6)$$

where M_0 is the mass of the dry gel (g) and M_t is the mass of the swollen gel (g).

Another important factor involved in the swelling process is the swelling power (F_{swp}), which gives information concerning the mechanism of the swelling:

$$F_{swp} = \frac{M_t - M_0}{M_0} = Kt^n \quad (7)$$

where *t* is time (min). The swelling constants (*K)* and the swelling exponents (*n*) can be determined by power law fitting to the curve of F_{swp} vs. *t* (min).

The mechanism of water uptake is indicated by the value of *n*. A value in the range 0.45-0.5 corresponds to Fickian diffusion, while a value of 0.5-1 means that the diffusion mode is non-Fickian (Karadağ et al., 2002).

3.2.5. Tensile test

Tensile tests were performed with a TA-XT Plus (Texture analyser (ENCO, Spinea,I)) instrument equipped with a 1 kg load cell and a cylinder probe with a diameter of 1 cm. Samples were placed in contact with a filter paper disc wetted with 50 µl of 8% w/w mucin dispersion (*in vitro*), simulated lacrimal fluid (blank) or excised porcine conjunctiva (*ex vivo*).

The measurements were performed by a method described previously (Horvát et al., 2015a,b,c).

3.2.5.1. Tensile test data analysis

In the tensile test, the normalized mucoadhesion parameters (*ΔAUC/AUC*) were calculated as followed (Salcedo et al., 2012):

$$\frac{\Delta \mathrm{AUC}}{\mathrm{AUC}} = \frac{\mathrm{AUC_m\text{-}AUC_b}}{\mathrm{AUC_b}} \quad (8)$$

where AUC_m is the work of adhesion in presence of mucin and AUC_b is the work of adhesion of blank measurements (with simulated lacrimal fluid).

3.2.6. 'Wash away' measurement

To perform the 'wash away' measurements, an earlier-developed modified Franz diffusion cell was used (Bonferoni et al., 1999; Rossi et al., 1999). The measurements were performed by a method described previously (Horvát et al., 2015c). *Ex vivo* tests were made on excised porcine conjunctiva placed on the acceptor chamber and simulated lacrimal fluid was streamed through the donor chamber. 250 mg of polymer gel (5, 7 or 10% w/w) was used, with sodium fluorescein (0.008% w/w) as the measured marker. HEC gels under the same experimental conditions were used as reference.

3.2.7. Drug release

The drug release profile of SD was determined with a vertical Franz diffusion cell system (Hanson Microette Plus TM). 1% w/w formulations of CLNaHA, NaHA or ZnHA and 7 and 10% w/w ThioPASP gel concentrations were prepared. All samples contained 0.1% w/w SD. The measurements were performed by a method described previously (Horvát et al., 2015a,b,c).

3.2.7.1. Drug release data analysis

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

$$\frac{M_t}{M_\infty} = kt^n \quad (9)$$

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. These values can be determined from the equation of the power law fitted to the curve of the amount of drug released (% w/w) against time (min).

An *n* value in the range 0.45-0.5 corresponds to Fickian diffusion, while a value of 0.5-1 means that the diffusion mode is non-Fickian (Peppas et al., 2000).

3.2.8. Statistical analysis

The results were evaluated and analysed 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.

4. RESULTS AND DISCUSSION

4.1. First generation mucoadhesive polymers

4.1.1. Rheology of the gels

The viscoelastic characters of CLNaHA, NaHA and ZnHA were determined by frequency sweep testing in the frequency range 0.1 to 100 Hz. Figure 6 depicts the frequency sweep test results on the measured samples at 1% w/w polymer concentration.

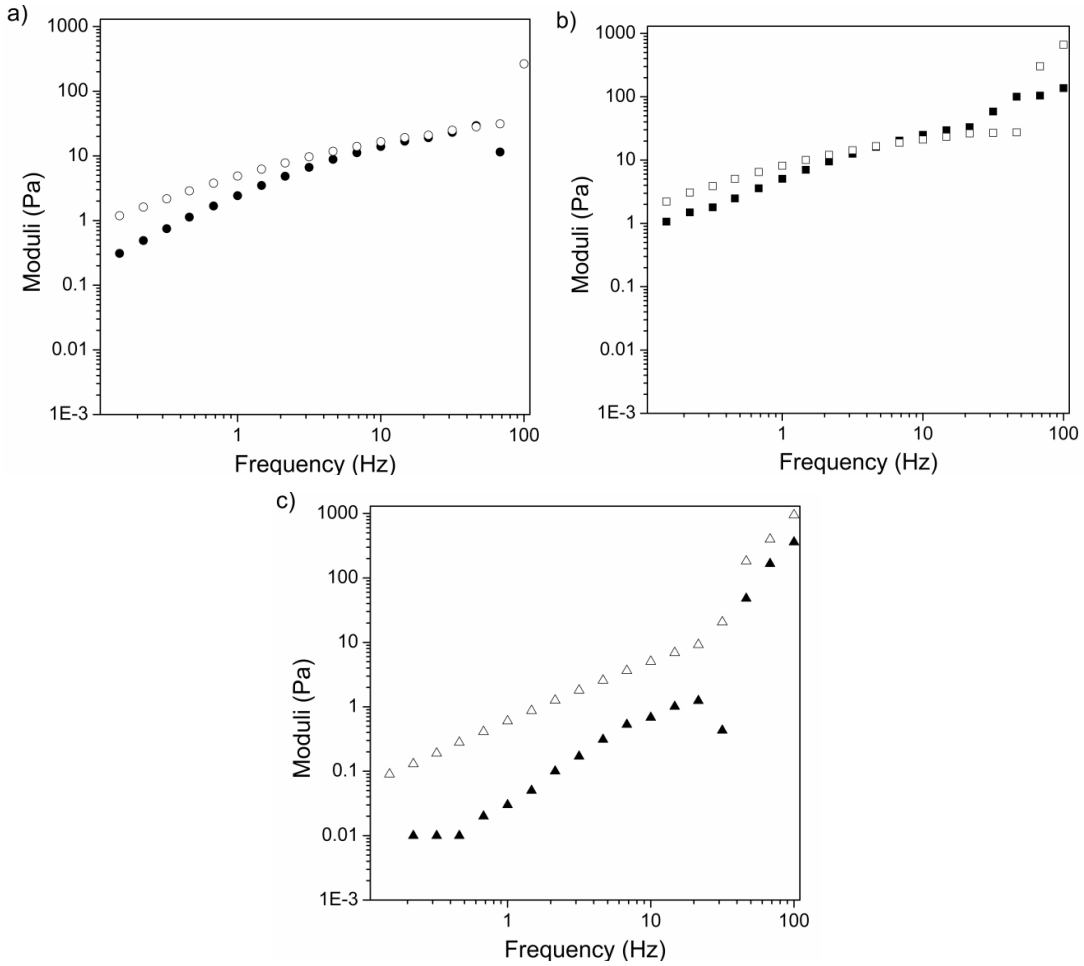

Fig. 6. *G'* (solid symbols) and *G"* (open symbols) values of (◑) CLNaHA, (◨) NaHA and (◭) ZnHA as a function of frequency

The highest moduli values were observed for NaHA, which corresponds to its long linear structure. CLNaHA exhibited lower values, 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 the 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 shows less elastic behaviour. In contrast with CLNaHA and NaHA, ZnHA behaves as a viscous fluid; *G"* predominates over *G',* and no cross-over point can be detected.

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.

4.1.2. Cytotoxicity

Figure 7 illustrates the results of the biocompatibility determination of CLNaHA, NaHA and ZnHA on RCE cells by the MTT test. As control, HBSS was used.

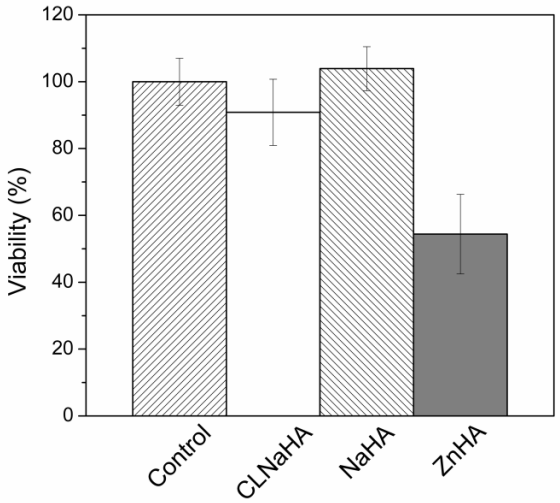

Fig.7. Biocompatibility of CLNaHA, NaHA and ZnHA formulations

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

Under *in vivo* conditions, zinc 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; Ugarte and Osborne, 2001; Bozym et al., 2010; Mehr, 2011; Ugarte et al., 2013).

Our results demonstrate 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.

4.1.3. Mucoadhesion

4.1.3.1. Rheology

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. 8.

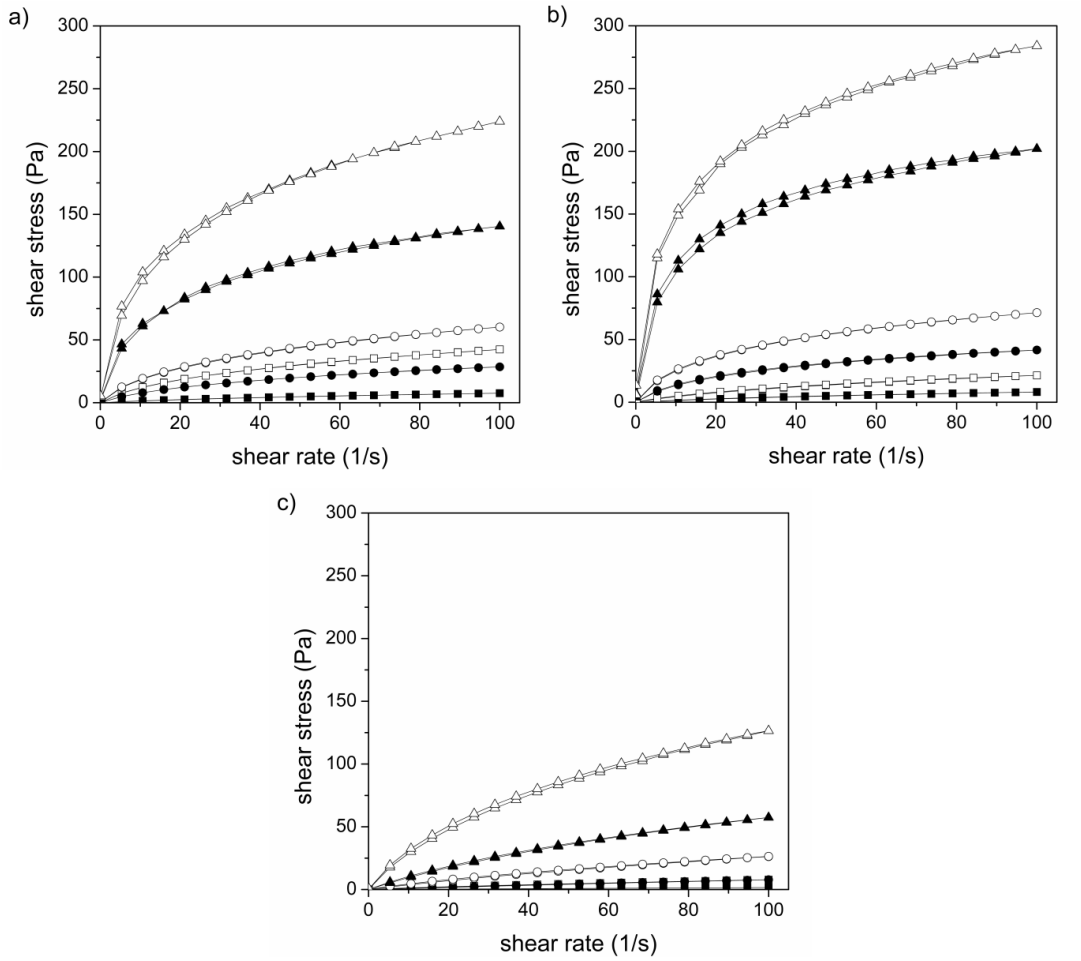

Fig. 8. Flow curves of CLNaHA (a), NaHA (b) and ZnHA (c) at (◧) 0.5% w/w, (◑) 1% w/w and (◭) 2% w/w, with mucin (open symbols) or without mucin (solid symbols)

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 (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 mucin. Mucin has a gel-strengthening effect, because more network links are created by entanglements and secondary bond (hydrogen-bond) formation. The calculated absolute synergism parameters (Eq. 3; section 3.2.3.1) of viscosity at a shear rate of 100 s^{-1} are illustrated in Fig. 9.

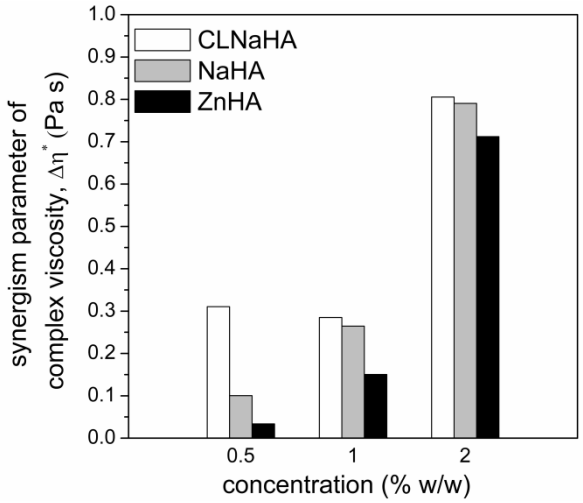

Fig. 9. Calculated absolute synergism parameter values of viscosity at a shear rate of 100 s^{-1}

The calculated values revealed that the mucoadhesive behaviour increased with 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 not a 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 surface.

4.1.3.2. Tensile test

The tensile test involves measurement of the force of detachment and the total work of adhesion needed to separate the surfaces, which results from the area under the force−distance curve (Woertz et al., 2013). Earlier studies 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. 10).

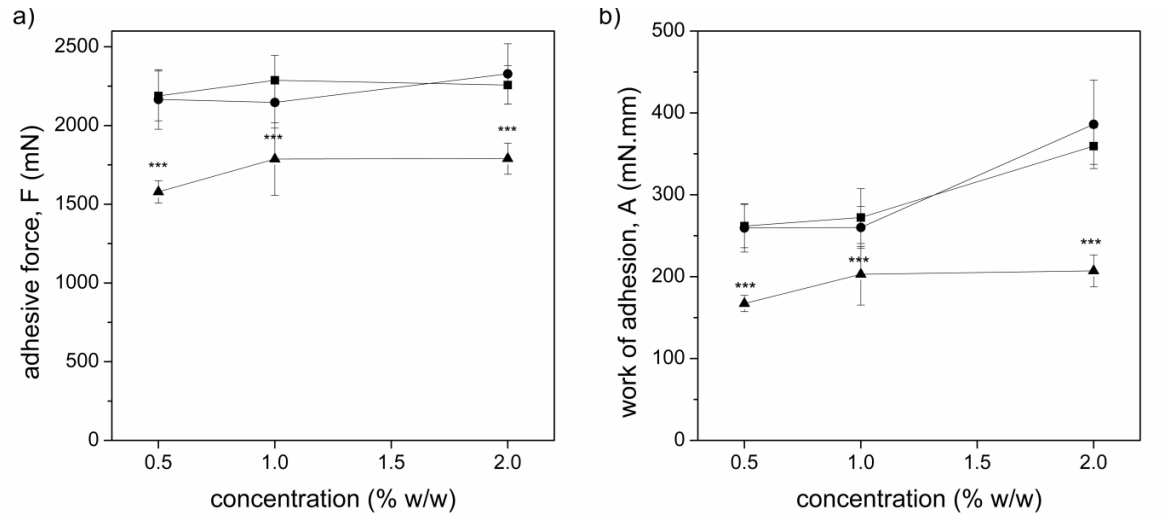

Fig. 10. Adhesive force (a) and work of adhesion (b) of (●) CLNaHA, (■) NaHA and (▲) ZnHA as a function of the concentration of the polymer in contact with mucin (***: $p \leq 0.001$ highly significant compared with CLNaHA and NaHA)

The values of *F* for all three derivatives did not increase with increase of the 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.

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. 11). These measurements related to conditions closer to the real mucoadhesive circumstances of the eye.

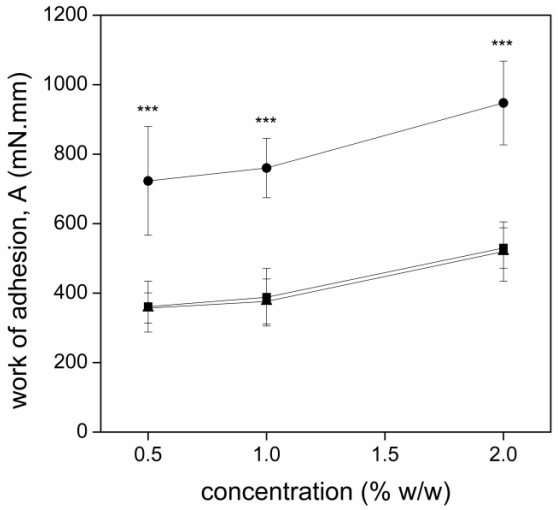

Fig. 11. Work of adhesion of (●) CLNaHA, (■) NaHA and (▲) ZnHA as a function of the concentration of the polymer in contact with excised porcine conjunctiva (***: $p \leq 0.001$, highly significant compared with CLNaHA)

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 more

facile 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 an increase in mucoadhesion and no significant difference was observed between them.

4.1.4. 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. Figure 12 shows the amount of drug released (% w/w) during the examination time (h). The slopes were determined (Eq. 9; section 3.2.7.1) by power law fitting to the curve of the released drug amount (% w/w) versus time (h) of CLNaHA, NaHA and ZnHA.

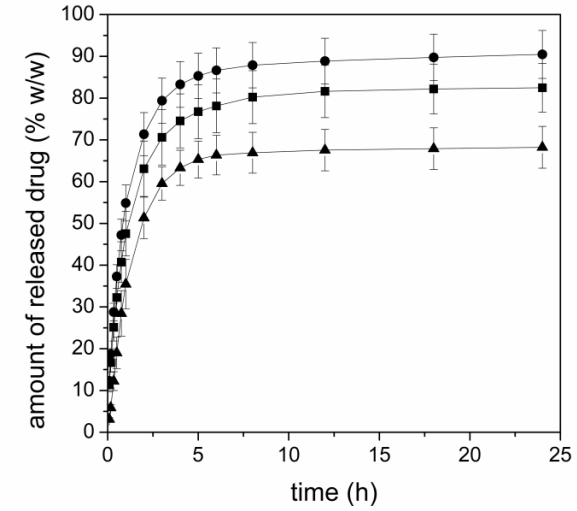

Fig. 12. Release of SD from (●) CLNaHA, (■) NaHA and (▲) ZnHA

In the first hour of measurements, a rapid diffusion of SD was observed from all three formulations, but their release profiles then diverged. 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.

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 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 (Peppas and Buri, 1985; Ritger and Peppas, 1987; Peppas et al., 2000; Park and Munday, 2002; Baumgartner et al., 2006). In the case of ZnHA (n = 1.0013, R^2 = 0.9988), zero-order kinetics was observed in the first few hours of diffusion, which confirms the possibility of interactions between SD and ZnHA.

In conclusion, it can be established that all the derivatives undergo rapid release, and release more than 65% w/w of the SD up to 6h. 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.

4.1.5. Conclusion

The investigated CLNaHA and NaHA are biocompatibile, while ZnHA displayed lower biocompatibility. CLNaHA showed the highest capability for mucoadhesion, due to its nanoparticulate structure, which can easily interpenetrate and form secondary bonds with mucin. The structure of ZnHA hampers interpenetration, entanglement and bond formation, which results in lower adhesive force and work of adhesion values. From all three derivatives, rapid SD release was observed in the initial period, which is especially beneficial in ocular therapy.

Although ZnHA has weaker mucoadhesive, drug release properties and lower biocompatibility *in vitro*, its application in ophthalmic formulations is favourable due to its scavenging, bactericidal, bacteriostatic and fungicidal effects, which allows omission of the preservative from the formulation. 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.

4.2. Second generation mucoadhesive polymers

4.2.1. Preformulation measurements

An ideal ocular dosage form is able to integrate easily into the environment of the eye or into its tissues; in the case of a surface−administered formulation (e.g. eye drops), this can mean the tear film. For this reason, the physiological properties of the tear film must be taken into consideration (Table 1).

In the event of an ocular drug delivery formulation, the needs of the patients' must be respected. Side-effects influencing vision can reduce their willingness to take their medication. Thus, ocular drug delivery systems must not cause a feeling of sand in the eyes, dry eye or blurry vision (Taylor et al., 2002; Lafuma et al., 2011).

Table 1. Physiological properties of the tear film

	Values	Literature
pH	7.4	Ludwig 2005
Osmolality	310-350 mOsm kg^{-1}	Ludwig 2005
Surface tension	44 mN m^{-1}	Ludwig 2005
Refractive index	male: 1.3368; female: 1.3371	Patel et al., 2000

During ocular drug delivery formulation, various excipients are used which can change the physical and chemical properties of the ocular surface and the stability of the tear film (Yañez-Soto et al., 2014).

In a hyperosmotic tear environment, water flows out of the cells to balance the osmolality of the intracellular fluids and the surrounding tears, resulting in dehydration of the cells in the ocular surface and damaging the cell membranes. Hypoosmolality is well tolerated by patients, but if it is very low it can cause irritation of the eye.

Eye drops of pH 6-9 do not cause discomfort, but outside this range an increased production of tear fluid can be observed due to the irritation (Ziemssen and Zierhut, 2008; Januleviciene et al., 2012).

I determined the osmolality, surface tension and refractive index of polymer solutions at five concentrations (Table 2).

Table 2. Measured values of osmolality, surface tension and refractive index of polymer solutions

Concentration (% w/w)	Osmolality (mOsm/l)	Surface tension (mN/m)	Refractive index
	mean ± SD	mean ± SD	
1	4.3 ± 0.5	75.3 ± 0.3	1.3330
3	8.0 ± 0.0	75.4 ± 0.3	1.3330
5	11.0 ± 1.6	75.3 ± 0.2	1.3332
7	17.0 ± 2.2	75.4 ± 0.1	1.3339
10	19.3 ± 0.5	75.4 ± 0.2	1.3342

The results revealed that the polymer has a very low osmotic activity. Increase of the polymer concentration resulted in an increase in osmolality, but this was not of great significance. These values are beneficial: after the osmolality of the eye drops has been set with an isotonizing agent, the ThioPASP will not result in a hyperosmotic solution.

The measured surface tension and refractive index values differ slightly from those of water (71.99 ± 0.05 mN m^{-1} and 1.3330, respectively) (Pallas and Harrison, 1990). Increase of the polymer concentration did not influence the surface tension, but the refractive index increased to a small extent. Thus, ThioPASP solutions do not lower the surface tension of the tears, leading to irritation, and do not cause visual interference.

The pH of the ThioPASP solution prepared with distilled water or with PBS was 5.4 and 7.4, respectively. For eye drop formulation, therefore, ThioPASP solution should be prepared in buffer so as to meet the pH requirements necessary to avoid irritation.

The transmittance spectrum of ThioPASP was measured in order to characterize the possible effects of the solution on the vision (Fig. 13).

Such spectral transmittance curves reveal that ThioPASP solutions are transparent in visible light, which means that they will not cause any visual disturbance. Increase of the polymer concentration resulted in a shift of the curves towards longer wavelengths, but even at the highest concentration the polymer solution does not have many effects on vision.

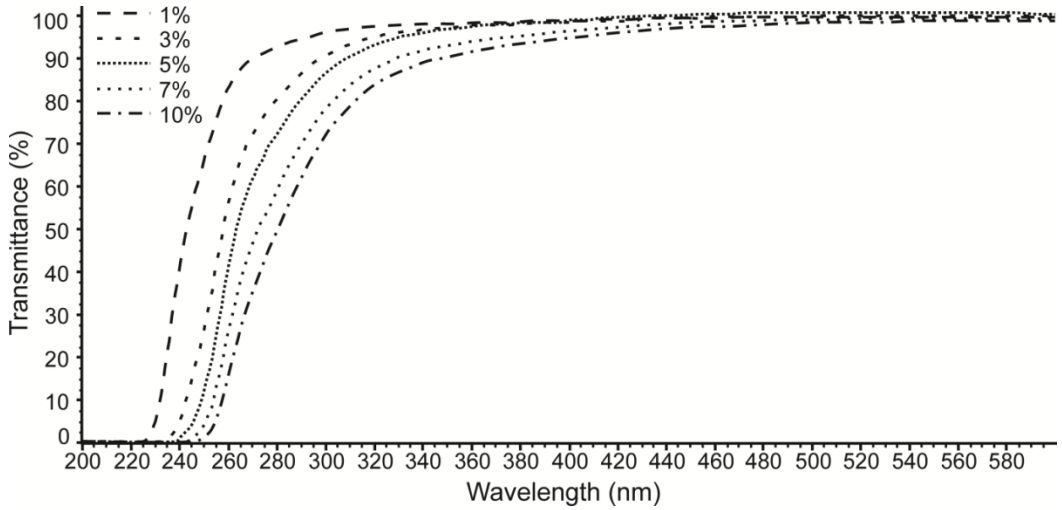

Fig. 13. Spectral transmittance curves of ThioPASP solution at five concentrations

The osmolality, surface tension, refractive index, transmittance and pH measurement results indicate that ThioPASP may be a very promising eye drop formulation. Thanks to its inert properties, ThioPASP solution does not affect the tear stability, and the ophthalmic requirements can be achieved through the addition of necessary excipients such as the isotonizing and surface tension−modifying agents.

4.2.2. Cytotoxicity

Cytotoxicity measurements were performed with the MTT assay on the RCE cell line. Only the viable cells are able to reduce the dye MTT to formazan. Figure 14 shows the viability of cells after contact with ThioPASP solution samples relative to control cells.

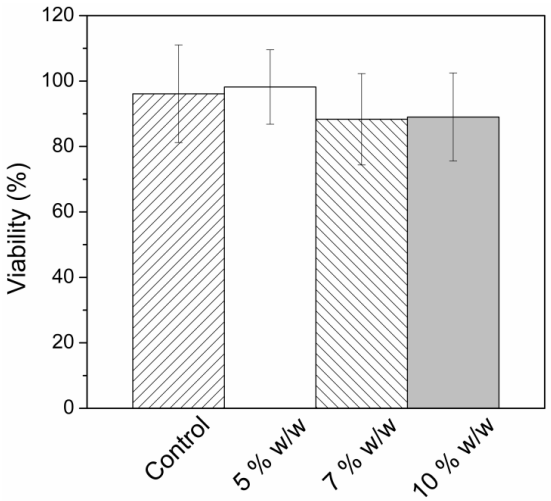

Fig. 14. Cell viability after contact with ThioPASP solutions

The results demonstrate that ThioPASP solution is biocompatible, because the cell viability was >90% after a contact time of 3 h in all cases. This is an extremely important finding, especially because RCE cells are very sensitive, so that it can be predicted that ThioPASP solution will highly probably not have a toxic effect on the eye.

4.2.3. Gel formation

The gelation process and the gel structure were characterized by means of rheology. The effects of the polymer concentration (7 or 10% w/w) were studied (Fig. 15).

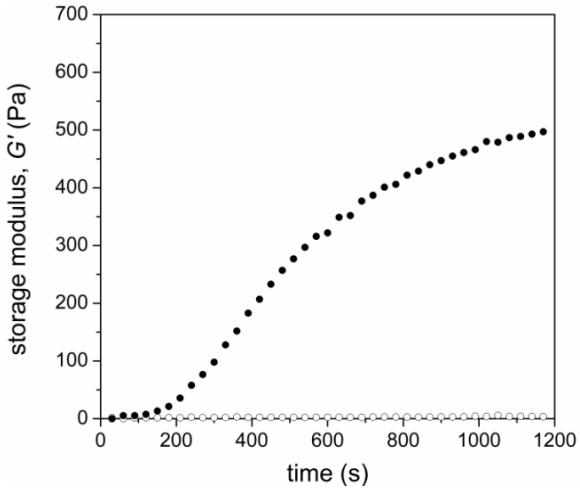

Fig. 15. Effects of polymer concentrations on the storage modulus (*G'*) of ThioPASP as a function of the gelation time; the polymer concentrations are (○) 7% w/w and (●) 10% w/w

Gelation did not proceed at polymer concentrations lower than 10% w/w. At high polymer concentration (10% w/w) after the addition of oxidant, cross-links were formed, resulting in the gelation of ThioPASP.

The frequency sweep test was started after full gelation. Table 3 presents *G'* and *G''* values of the formulations at an angular frequency of 1 s^{-1}.

Table 3. Storage (*G'*) and loss moduli (*G''*) of ThioPASP systems at different polymer concentrations (ω = 1 s^{-1})

ThioPASP conc. (% w/w)	*G'*	*G''*
3	0.18	0.02
5	0.11	0.02
7	16.28	1.35
10	533.0	12.6

At polymer concentrations lower than 7% w/w, changes of the polymer concentration did not affect the gelation, *G'* did not vary significantly and the precursor solutions remained in the liquid state even after the addition of oxidant (*G'* was similar in order of magnitude to *G''*). At polymer concentration of 7% w/w, a gel structure formed (*G'* was more than an order of magnitude higher than *G''*). At high polymer concentrations (7 and 10% w/w), the values of *G'* increased with increase of the polymer concentration. The gel obtained at 10% w/w ThioPASP displayed the strongest gel structure, indicating that the elevation of the polymer concentration enhanced the cross-linking density by increasing the concentration of disulfide linkages.

4.2.4. Swelling of ThioPASP hydrogels

The swelling of the hydrogels was characterized by a gravimetric method. Formulations of 20% w/w ThioPASP gels were measured. During the 6 h measurements, the swollen polymer discs maintained their coherent structure and shape, because of the formation of disulfide linkages between the polymer chains.

Figure 16 depicts the percentage swelling (*% S*), calculated from Eq. 6 section 3.2.4.1.

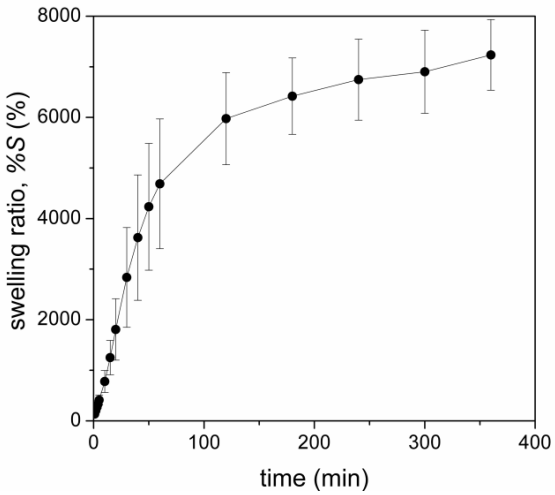

Fig. 16. Swelling kinetics of ThioPASP hydrogels

The polymer swelled faster initially and the water uptake then slowed as equilibrium was approached. The swelling ability of the hydrogel was large because of the lower cross-linking density resulting from the weaker elastic interactions inside the polymer network. This led to a marked water uptake of the formulation. ThioPASP was able to swell to 6000-7000% of the volume of its dry mass. The swelling exponent (*n*) was calculated via Eq. 7 (section 3.2.4.1) and curve fitting. In our case, non-Fickian diffusion was observed, because the *n* value was 0.874 (Karadağ et al., 2002).

The results of water uptake measurements indicated that the ThioPASP polymers have a very good water uptake capacity, which plays an important role in mucoadhesion and also in drug release.

4.2.5. Mucoadhesion

4.2.5.1. Rheology

Rheological measurements were performed with different concentrations of ThioPASP polymer and mucin. It was presumed that, if an *in situ* gelling system is used, the mucin can influence the gelation time of the formulation. For this reason, the gelation time was first determined in the presence of mucin, using the same method as described before. The results are shown in Fig. 17.

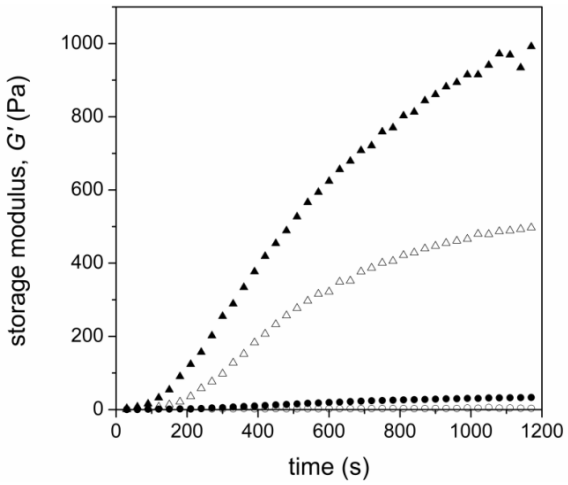

Fig. 17. Evolution of storage modulus (*G'*) as a function of time at (◑) 7% w/w and (◭) 10% w/w polymer concentrations with (solid symbols) or without (open symbols) mucin

As in the previous measurements without mucin, gelation was observed only at 7 and 10% w/w ThioPASP. Mucin did not cause an appreciable difference in the rheological parameters at ThioPASP concentrations lower than 7% w/w. The gelation time was also defined as the time at which a maximum was observed in the curve of the differential with respect to time (Table 4) (Ma et al., 2008).

Table 4. Gelation time (t_g) at 7 and 10% w/w ThioPASP concentrations

ThioPASP conc. (% w/w)	t_g without mucin (s)	t_g with mucin (s)
3	n. g.	n. g.
5	n. g.	n. g.
7	n. g.	450
10	330	300

n. g. – no gelation was observed

In the cases of 7 or 10% w/w polymer, the gelation time was shorter. The addition of mucin aided the gelation and in each case the gelation time was shorter in the presence of mucin. The rate of gelation and the final value of *G'* were higher in the presence of mucin.

Frequency sweep tests were performed after gelation to investigate the interaction between the mucin and the ThioPASP gels. Figure 18 presents the variation in *G'* with angular frequency for the formulations with and without mucin.

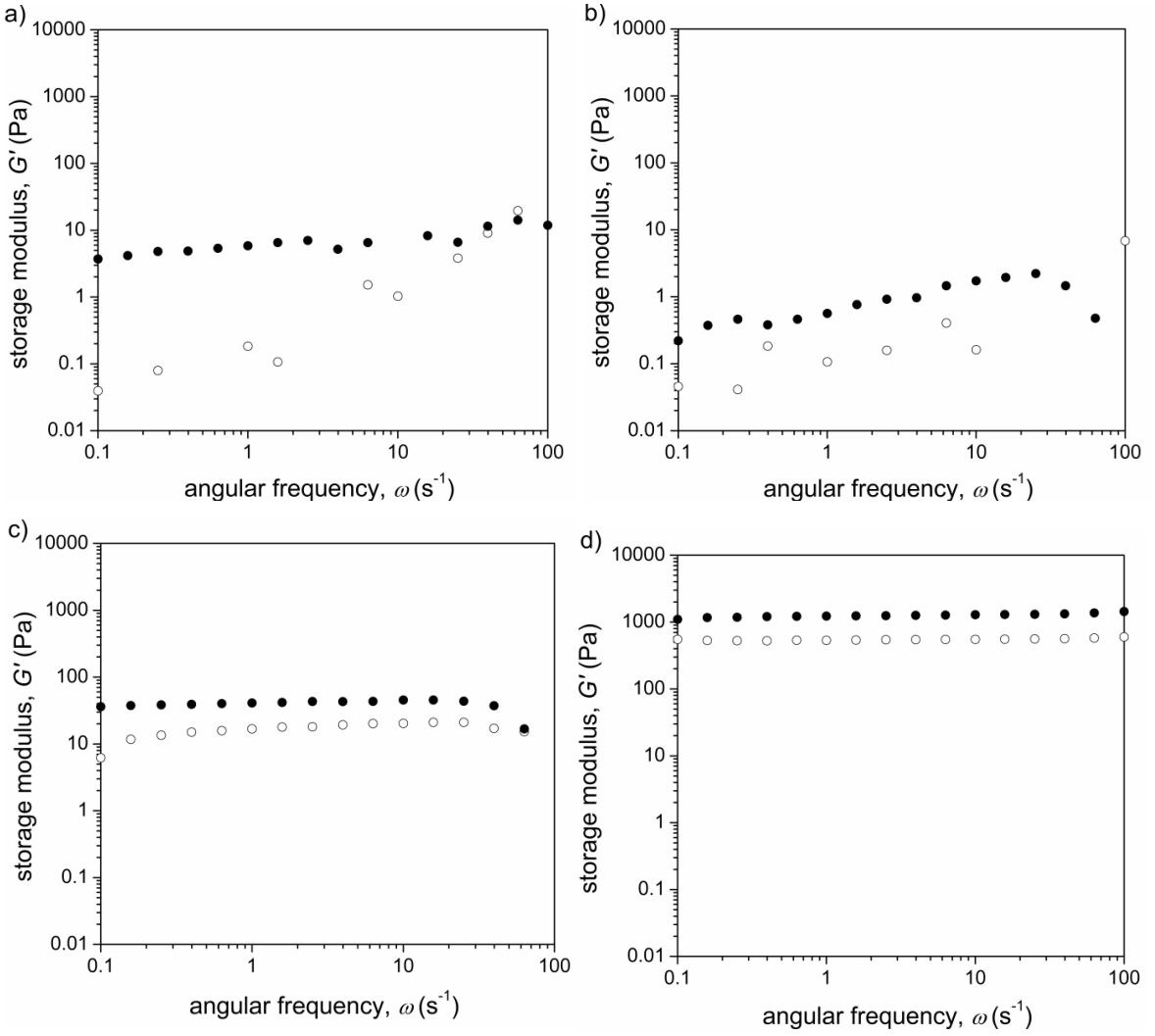

Fig. 18. Frequency sweep tests at (a) 3, (b) 5, (c) 7 and (d) 10% w/w polymer concentrations with (solid symbols) or without mucin (open symbols)

In all cases, mucin augmented the elastic modulus of the samples, indicating that interactions occurred between the polymer and the mucin. The shapes of the curves (the slopes of the *G'* vs. angular frequency curves, which show the frequency dependency of the systems) of the samples with 10% w/w polymer with or without mucin were similar to each other. At this concentration, the polymer gels exhibited a densely cross-linked gel structure even without mucin. Mucin did not change the rheological profile of the systems. The changes in the rheological behaviour of the samples containing lower polymer concentration (3 and 5% w/w) suggested the formation of a chemically cross-linked structure between the polymer and mucin chains in addition to physical entanglements. In a physically entangled structure, the moduli depend strongly on the

frequency: at low frequency, the *G'* values are decreased considerably (Ross-Murphy and McEvoy, 1986; Madsen et al., 1998). In our case, at lower ThioPASP concentrations, the added mucin decreased the slope of the curves, which indicated the occurrence of the cross-linking of the polymer with the mucin.

Table 5 shows the relative synergism parameters (Eqs. 4 and 5; section 3.2.3.1) η^* and *G'* at an angular frequency of 1 s^{-1}.

Table 5. Relative synergism parameters of viscosity and storage modulus between the polymer−mucin mixtures

ThioPASP conc. (% w/w)	$\Delta G'_{rel}$	$\Delta\eta^*_{rel}$
3	31.85	36.11
5	5.28	7.78
7	2.44	2.44
10	2.31	2.31

The stiffness of the gels was larger in the presence of mucin in each case. At higher polymer concentrations, the relative differences ($\Delta G'_{rel}$ and $\Delta\eta^*_{rel}$) were lower than at lower polymer concentrations. The mucoadhesive character was displayed most significantly at lower polymer concentrations (3 and 5% w/w). This result is in accordance with earlier studies in which it was concluded that there is an optimum polymer concentration for mucoadhesion (Madsen et al., 1998). In our work, this was probably because a loosely, chemically cross-linked structure was present, and the chains were flexible enough to be able to form more bonds with the mucin, resulting in a gel-strengthening effect in the mixture.

4.2.5.2. Tensile tests

Tensile test measurements were also made with 3, 5, 7 and 10% w/w polymer *in vitro* with mucin dispersion (Fig. 19).

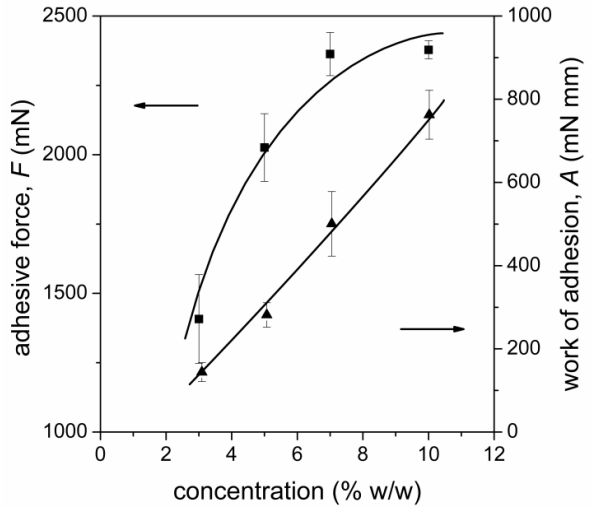

Fig. 19. (■) Adhesive force (*F*) and (▲) work of adhesion (*A*) as functions of polymer concentration

Figure 19 reveals that *A* increased continuously as the concentration was elevated, while the adhesive force (*F*, mN) reached a maximum at 7% w/w polymer. As indicated earlier (Park and Munday, 2002), in our work the chemical bonds probably have a larger effect at lower polymer concentration, and it is likely that covalent bonds and secondary bonds were formed with the mucin glycoproteins. Thus, *F* increased continuously with increasing polymer concentration. At high polymer concentration, the potential for chemical bonds reached a maximum because the free thiol groups were saturated at the interface. Accordingly, a plateau was observed in the *F* vs. concentration curve. *A* did not reach a maximum, but increased continuously, because interpenetration prevails in the process of mucoadhesion rather than chemical bonding at higher polymer concentrations. In our case, the ThioPASP gel at higher concentration has more thiol groups and more cross-links, resulting in a gel structure, which induces increased swelling, allowing deeper and improved interpenetration.

These tensile test results can be correlated to the rheological results, where the changes in the shape of the frequency sweep curves could be observed up to 7% w/w polymer concentration, which indicated the formation of chemical cross-links (Hägerström and Edsman, 2003).

Tensile test measurements were also performed *ex vivo* on excised porcine conjunctiva (Fig. 20.).

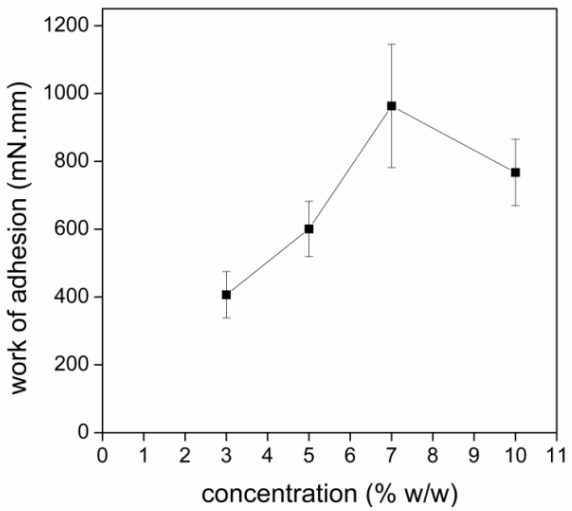

Fig. 20. Work of adhesion (*A*) as a function of polymer concentration in *ex vivo* measurements

In *ex vivo* tensile test measurements, *A* increased continuously as the polymer concentration was elevated up to 7% w/w. At high concentration (10% w/w), the ThioPASP polymer is not able to interpenetrate into the mucous layer of the porcine conjunctiva, probably because a highly cross−linked structure, which is less flexible is formed at this concentration.

We can conclude that ThioPASP concentration has a high effect on mucoadhesion. At high polymer concentration, the interpenetration into the mucous layer is limited. These results correspond with the calculated rheological relative synergism parameters, where it was also found, that the mucoadhesiveness of ThioPASP polymers decreases at 10% w/w polymer concentration.

4.2.5.3. 'Wash away' measurements

'Wash away' *ex vivo* measurements mimic the lacrimation of the eye, under conditions relatively close to real mucoadhesive circumstances of the eye. The amount of sodium fluorescein washed away from the porcine conjunctiva can indicate the amount of the dosage form remaining on the surface. In our work, HEC gels were used as reference.

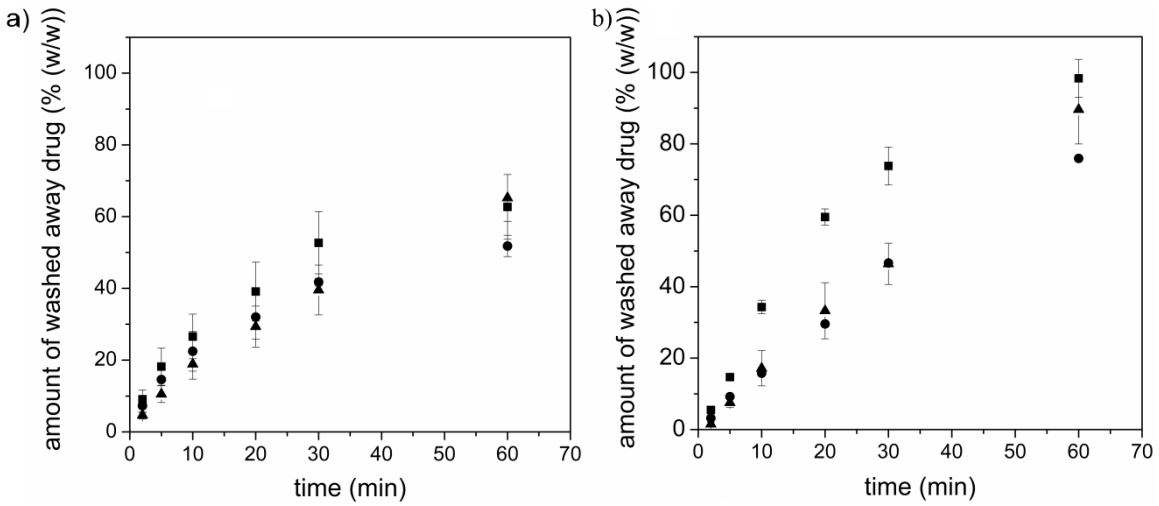

Fig. 21. (a) ThioPASP and (b) HEC containing the model drug. Polymer concentrations: (□) 5% w/w, (○) 7% w/w and (△) 10% w/w

It can be observed in Fig. 21a that increase of the ThioPASP concentration was accompanied by a slight decrease in the amount of model drug washed away. These differences were not pronounced after 1 h. In the case of the reference systems (HEC gels, Fig. 21b), the observations were similar; the gel with the lowest HEC concentration underwent the fastest washing−out. Comparison of the ThioPASP systems with the HEC gels indicated that the ThioPASP formulations have a longer residence time, because 40% w/w of the model drug remained on the conjunctiva, in contrast with 10-30% w/w for the reference systems.

4.2.6. Effects of blinking on the gel structure

During the formulation of a mucoadhesive ocular drug delivery system, eye movements and blinking must be taken into consideration, because the gel on the surface of the eye is exposed to a continuous shear force, which may thin or dislodge the formulation (Robinson and Mlynek, 1995). As a result, the gel structure may be disrupted under this shear. The strength of the gel was investigated in cycling strain tests, simulating the real circumstances in the eye. One blinking cycle can correspond to 1 min of blinking (Fig. 22).

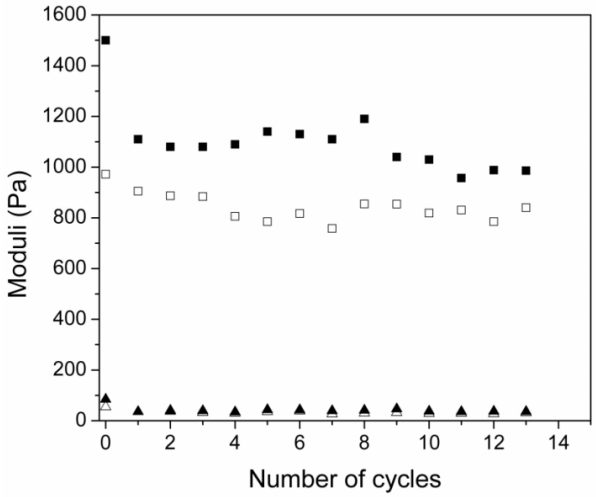

Fig. 22. Modification of gel structure at 10% w/w ThioPASP during blinking cycles: storage modulus (◨) and loss modulus (◭) with (filled symbols) or without (empty symbols) mucin

Figure 22 depicts the changes in the structure during the blinking cycles. It can be observed that the moduli decreased only in the first two cycles and later became practically constant. The form applied to the eye surface remained in a gel state during blinking, as indicated by the constant phase moduli. The large *G'* value and the difference between the moduli indicated the presence of the gel structure, which preserved its strength after several test cycles. There was no difference between the shapes of the curves in the cases of mucin-containing and mucin-free samples. The only difference was in the value of the storage modulus; the mucin-containing sample gave higher values, the mucoadhesivity being maintained under shear.

4.2.7. Drug release measurements

Drug release measurements were performed with a vertical Franz diffusion cell system with gels containing 10% w/w polymer and 0.1% w/w SD. Figure 23 shows the amount of drug released (% w/w) during time.

In the first hour, the diffusion of the SD was fast, and this was followed by a sustained release. The drug release results correspond with the swelling measurement results. The formulation has a higher water uptake, suggesting a lower cross-linking density, and the SD is therefore able to diffuse through this structure more easily.

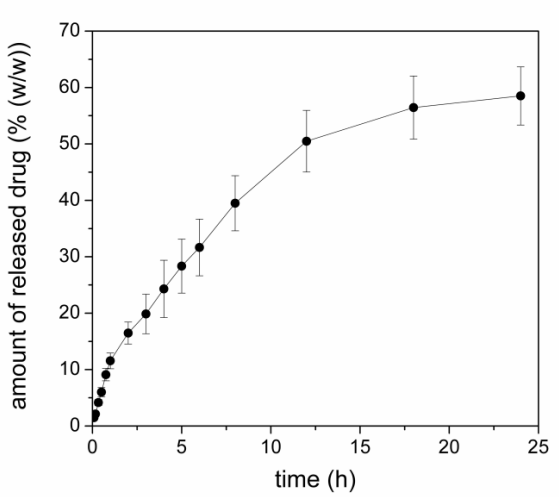

Fig. 23. Release of the model drug, SD, from ThioPASP gel

The swelling-controlled drug release mechanism can be characterized by Eq. 9 (section 3.2.7.1) where the *n* value can be determined from the equation of the power law fitted to the curve of the amount of drug released (% w/w) against time (min).

In our case, we have a non-Fickian release mechanism, because the value of *n* is 0.6561, which corresponds with our swelling results. During the drug release, our 10% w/w polymer gels underwent continuous swelling on the membrane, which led to the simultaneous absorption of water and desorption of the drug via a swelling-controlled diffusion mechanism. The combination of diffusion, swelling and relaxation is responsible for the non-Fickian release mechanism (Lee, 1985; Peppas and Buri, 1985; Ritger and Peppas, 1987; Peppas et al., 2000; Park and Munday, 2002; Baumgartner et al., 2006).

The advantage of these formulations is the rapid drug release in the first hour, followed by a prolonged release. This is important in therapy, because a higher dose is needed immediately after the application, in order to reach the therapeutic dosage, after which a sustaining dosage is required. From the aspect of ophthalmic preparations, this can increase patient compliance.

4.2.8. Effect of the stabilizing agent on the ThioPASP properties

I determined the effects of the stabilizing agent on the polymer structure and properties such as their mucoadhesion and drug release. Dithiothreitol (*DTT*), glutathione (*GSH*) and acetylcysteine (*ACC*) stabilized ThioPASP polymers were characterized.

4.2.8.1. Mucoadhesion measurements

To determine the effects of the stabilizing agents on the mucoadhesivity of the systems, rheological and tensile test measurements can be used with the calculation of synergism parameters in the case of rheology and normalized mucoadhesion parameters from the tensile test.

Figure 24 shows the calculated absolute (Eq. 2*;* section 3.2.3.1) and relative (Eq. 4*;* section 3.2.3.1) synergism parameters of *G'* at an angular frequency of 1 s^{-1}, the calculated normalized mucoadhesion parameters (Eq. 8*;* section 3.2.5.1) in the case of the tensile tests.

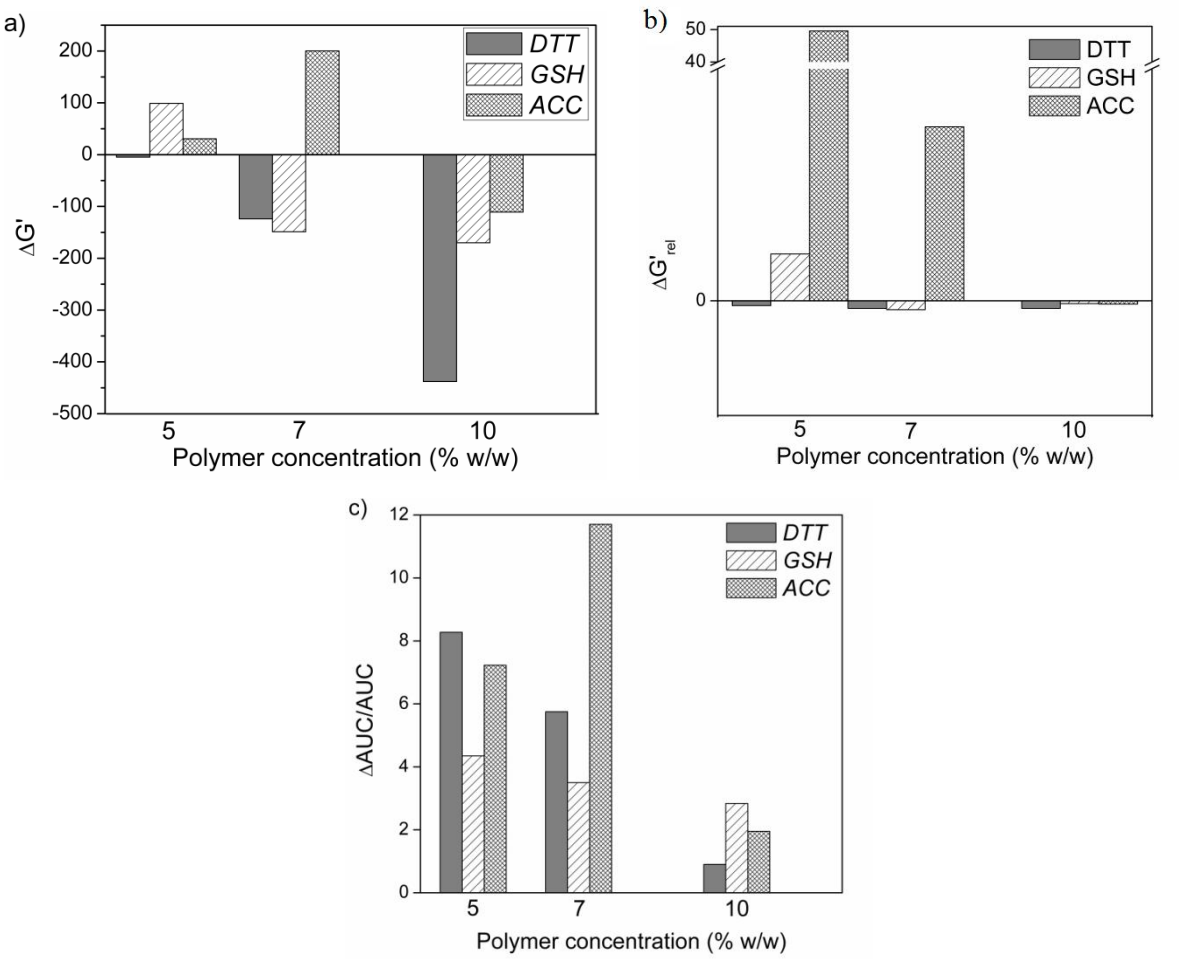

Fig. 24. The calculated a) absolute and b) relative synergism parameters of *G'* in rheological measurements and the calculated c) normalized mucoadhesion parameters in tensile tests

Dithiothreitol stabilized formulations (*DTT*) did not show mucoadhesion in the bulk rheological method; moreover, increase of the polymer concentration reduced the gel structure, resulting in increasingly more negative *ΔG'* and very low $\Delta G'_{rel}$ values. However in the case of

tensile tests as a surface method, mucoadhesion was observed, which decreased as the concentration was elevated. These calculations predict that dithiotreitol as stabilizing agent prefers polymer−polymer interactions, but even in this case it can provide a limited mucoadhesion on the interface.

The *GSH* as a glutathione stabilized sample at low polymer concentration was mucoadhesive in the bulk method, but with increase of the polymer concentration its mucoadhesivity decreased. As compared with the dithiothreitol stabilized samples, the tensile tests indicated a weak mucoadhesive property, which can be explained by its weak cohesivity (the gels fell apart during the experiments) and resistance against the tensile strength.

Up to 7% w/w polymer concentration, the acetylcysteine stabilized formulation (*ACC*) showed marked mucoadhesivity relative to the other two formulation types. At 7% w/w, a gel structure was formed which can provide optimum mucoadhesion, as proved by both bulk and tensile test methods (high values of $\Delta G'$, $\Delta G'_{rel}$ and $\Delta AUC/AUC$).

4.2.8.2. Drug release

The SD release from the *DTT*, *GSH* and *ACC* gels (at the optimum, 7% w/w polymer concentration) was determined with the vertical Franz diffusion cell system during 24 h (Fig. 25).

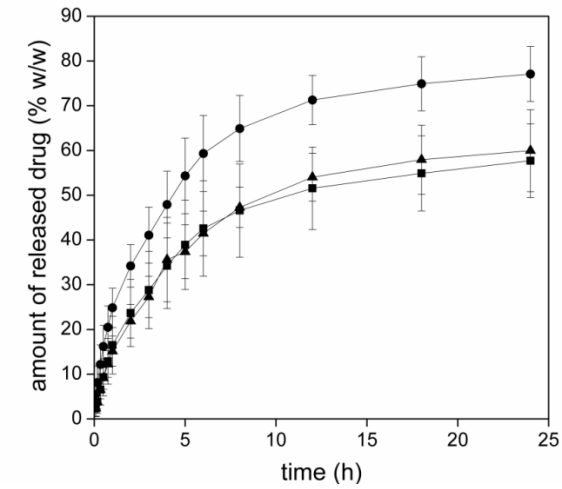

Fig. 25. Release of SD from ThioPASP gels: (■) *DTT*; (●) *GSH* and (▲) *ACC*

The results revealed that the *GSH* gels released the highest amount of SD (~80% w/w) during 24 h, while there was no significant difference between the lower amounts of SD released by *DTT* and *ACC*.

Table 6 shows the *n* values determined from the equation of the power law fitted to the curve of the amount of drug released (% w/w) against time (min) according to Eq. 9 (section 3.2.7.1).

Table 6. Release exponents (*n*) of *DTT*, *GSH* and *ACC*

	n	R^2
DTT	0.6781	0.9914
GSH	0.5542	0.997
ACC	0.6674	0.9948

The fitting results demonstrate that all samples display non-Fickian diffusion, because the *n* values are between 0.5 and 1. The non-Fickian release mechanism is a swelling−controlled mechanism, with simultaneous water uptake and API desorption (Peppas and Buri, 1985; Ritger and Peppas, 1987; Peppas et al., 2000; Park and Munday, 2002; Baumgartner et al., 2006).

It can be concluded that *GSH* has the fastest release thanks to the disrupted structure, which cannot be predicted and planned in advance. Even if *DTT* and *ACC* release lower amounts of SD, their release profile can be designed thanks to their stable structure. The *in vitro* results indicate that the formulations can provide 24 h continuous release.

4.2.9. Conclusion

The ThioPASP polymer is a new type of mucoadhesive polymer that is planned to be used in ophthalmic therapy. Since such polymers had not been used previously, preformulation measurements were first performed in order to verify the suitability of these polymers for ophthalmic formulations. The results demonstrated that ThioPASP solutions are biocompatible and will not cause blurred vision.

In situ gellable ThioPASP hydrogels were fabricated with the aim of obtaining delivery vehicles with increased adhesion to the eye surface, and the next important step was therefore to characterize these hydrogels in terms of gelation time, viscoelastic behaviour, mucoadhesion, resistance against blinking and lacrimation and drug release. Mucin exhibited a strong effect on cross-link formation, and the ThioPASP gels displayed strong mucoadhesion, especially at lower polymer concentrations (3 and 5% w/w). The formation of disulfide linkages with mucin glycoproteins contributed to the strong mucoadhesion, in addition to chain entanglement during the interpenetration of the polymer into the mucin. The addition of a small amount of oxidant

improved the mucoadhesion, because of the formation of a gel structure with a considerable number of free thiol groups.

The ThioPASP gels demonstrated high resistance against lacrimation of the eye, which confirmed the strong mucoadhesion. These polymers are also resistant against blinking. SD could be encapsulated into the ThioPASP polymers during formation of the gel structure and underwent rapid release in the first hour during *in vitro* measurements, followed by sustained release of the drug for a further 23 h.

The next important step was the stabilization of ThioPASP polymers, where three types of reducing agents were used (*DTT*, *GSH* and *ACC*). The aim was to find an optimum stabilizing agent which can ensure an appropriate gel structure for mucoadhesion and drug release. The results revealed that ThioPASP stabilized with acetylcysteine has an optimum cross−linked structure with free thiol groups ensuring polymer−mucin interactions, resulting in the best mucoadhesive properties.

5. SUMMARY

The aim of my research work was to characterize hyaluronic acid (HA) derivatives as first generation and thiolated poly(aspartic acid) (ThioPASP) polymers as second generation mucoadhesive polymers as potential ocular drug delivery system vehicles. The generally poor bioavailability of ophthalmic formulations can be improved by new formulations with a prolonged residence time.

Comparative studies of HA derivatives from the aspects of mucoadhesion and drug release have not been reported previously. Likewise, a cross−linked sodium salt of HA (CLNaHA) has not been used before as a potential ocular drug delivery system vehicle.

ThioPASP polymers as a new type of mucoadhesive polymers were studied first in a wide range from the aspects of ophthalmic preformulation (osmolality, surface tension, refractive index, transmittance and cytotoxicity) and formulation (hydrogel characterization, mucoadhesion and drug release).

The results of the measurements with the HA derivatives and the ThioPASP polymers led to the following conclusions:

- Both the HA derivatives and the ThioPASP polymers are biocompatible, as proved by the MTT test on RCE cell line.

- Their good mucoadhesive property was verified *in vitro* (rheological and tensile tests) and *ex vivo* (tensile tests). In the *ex vivo* tensile tests, higher values of adhesive work were measured, predicting a better *in vivo* mucoadhesion. Thanks to this property, the residence time on the eye can be prolonged.
- Both HA derivatives and ThioPASP polymers exhibit a fast initial release of sodium diclofenac, followed by a sustained release up to 24 h. This is beneficial in ophthalmic therapy because the therapeutic effect can be achieved at the beginning of the application, which is followed by a sustaining dosage.
- The use of HA derivatives gives an opportunity to find the optimum salt and structure for the required therapy.
- Although ZnHA displayed lower biocompatibility and mucoadhesion, its bactericidal and fungicidal properties can give an opportunity to decrease or eliminate the amount of preservatives from the formulation (such preservatives can cause cellular damage during long−term ophthalmic therapy).
- Of the HA derivatives tested, CLNaHA had the optimum structure for mucoadhesion and drug release. These results justify the use of CLNaHA in ophthalmic therapy in the future.
- *In situ* gelling can be achieved through the use of ThioPASP polymers. This property is very beneficial in ocular therapy because such polymers can be used as eye drops and will gellify *in situ*.
- ThioPASP polymers are inert excipients, which simplifies the formulation of ocular drug delivery system.
- ThioPASP polymers are also resistant against lacrimation and blinking, as proved by the "wash away" and rheological tests. These properties also play an important role in prolongation of the residence time on the ocular surface.
- The ThioPASP polymer stabilized with acetylcysteine exhibited the best mucoadhesive and drug release properties.

All of the results of the measurements performed excellently illustrated the potential of the application of the HA derivatives and the ThioPASP polymer as mucoadhesive ocular drug delivery system vehicles, with the beneficial property of reducing the necessary frequency of use.

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ANNEX

I.

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Thiolated poly(aspartic acid) as potential *in situ* gelling, ocular mucoadhesive drug delivery system

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ABSTRACT

The ophthalmic formulations on the market suffer from poor bioavailability, and it would therefore be useful to design a new formulation which is able to prolong the residence time and reduce the administration frequency. Polymer matrices which exhibit strong mucoadhesion are promising platforms in ocular drug delivery from the aspect of improved bioavailability. In the present study, an *in situ* gelling, mucoadhesive drug delivery system was fabricated from thiolated poly(aspartic acid) (ThioPASP). The thiol groups of ThioPASP are able to form disulphide linkages with the mucin glycoproteins and prolong the residence time on the eye. The effects of the thiol groups on the structure, swelling behaviour and mucoadhesive character of the gel and on the drug release profile were determined. The gel structure was characterized by means of rheology. The ThioPASP gel was demonstrated by rheology, tensile test and 'wash away' measurements to display strong mucoadhesion. The drug release from the ThioPASP gel was studied on a vertical Franz diffusion cell: a burst release of sodium diclofenac occurred in the first hour, followed by sustained release of the encapsulated drug for up to 24 h. The results proved the importance of the presence of the thiol groups and suggested that a ThioPASP formulation can be useful as an *in situ* gelling, ocular dosage form.

1. Introduction

The formulation of ocular drug delivery systems poses many challenges, but also offers many opportunities to overcome the inadequacies of the current formulations. In consequence of the limited area and time for absorption in the eye, the bioavailability of ocular drugs is very poor (less than 5%). The corneal epithelium has a complex hydro- and lipophilic character that limits drug absorption and the eye has many protective mechanisms, including blinking, the tear turnover and reflex lachrymation. The conjunctiva is the major site of systemic absorption which also plays an important role in drug elimination from the ocular surface thanks to its rich blood flow, large surface area and more permeable membrane compared to the corneal membrane. There is therefore a need for the frequent instillation of eye drops, which is accompanied by discomfort and a decrease in patient compliance, especially in long-term therapy (Urtti et al., 1990; Urtti and Salminen, 1993; Ludwig, 2005; Junginger et al., 2002; Pepić et al., 2013).

One of the most important phenomena in ocular formulations is the adhesion between the drug delivery system and the eye tissues. In the case of bioadhesion, physical or chemical bonds are formed between the biological and synthetic surfaces. Mucoadhesive drug delivery vehicles exploit the adhesion between the polymer component and the biological tissue, a mucosal membrane, the mechanism being referred to as mucoadhesion (Chickering and Mathiowitz, 1999). In case of ocular mucus, the conjunctival goblet cells, the conjunctival epithelium, and the corneal epithelium are responsible for the secretion of mucin. Mucins are large glycoproteins which are mainly composed of a protein core, carbohydrates and are well glycosylated. There are two types of ocular mucins: membrane-associated and secreted mucins (Lorentz and Sheardown, 2014).

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Mucoadhesion can be described in three steps: (1) the formation of an intimate contact between the mucoadhesive preparation and the mucus, followed by the wetting of the mucoadhesive formulation; (2) the swelling of the macromolecules and the formation of an interpenetrating network with the mucus macromolecules; and (3) chemical bond formation (primary or secondary) between the entangled chains (Duchêne et al., 1988). Thus physical and chemical interactions can arise during the process of mucoadhesion.

Numerous theories have been put forward to explain the complex phenomenon of mucoadhesion, such as electronic, adsorption, wetting, diffusion and fracture theories. It is difficult to compare these theories, but they may well supplement each other and reflect the complex nature. The electronic theory is based on the different electronic structures of the polymer and mucin; it follows that a double layer of electrical charge is formed on the interface. The adsorption theory is based on the formation of van der Waals interactions, hydrogen-bonds, etc. The wetting theory relates to the ability of the mucoadhesive polymer to spread over a tissue. The most important step in the diffusion theory is the interpenetration of the polymer chains in the mucus. The fracture theory analyses the forces required to separate the two surfaces after adhesion (Chickering and Mathiowitz, 1999; Serra et al., 2009). Numerous experimental methods have been described for the measurement of mucoadhesion. Most are based on two approaches: (1) surface analysis, such as contact angle determination and spectroscopic techniques, and (2) determination of the bioadhesive bond strength in tensile or rheological tests (Peppas and Buri, 1985).

The new delivery systems with mucoadhesive properties have various advantages: better bioavailability, a lower active ingredient concentration is sufficient and the administration frequency can be decreased, thanks to the enhanced residence time (Andrews et al., 2009; Saettone et al., 1985). The strength of mucoadhesion is determined by the polymer: its molecular mass, the presence of functional groups, the chain flexibility, the concentration, the degree of cross-linking and the degree of hydration (Andrews et al., 2009; Leitner et al., 2003; Ludwig, 2005; Leung and Robinson, 1990; Robinson and Mlynek, 1995).

The mucoadhesive polymers can be classified into two main categories. The first-generation mucoadhesive polymers can be anionic (e.g. poly(acrylic acid), sodium carboxymethylcellulose), cationic (e.g. chitosan) or non-ionic. The second-generation mucoadhesives are derivatives of the first-generation polymers (e.g. thiolated polymers) and include several new mucoadhesives (e.g. lectins, bacterial adhesions) (Andrews et al., 2009; Serra et al., 2009).

Thiolated polymers (thiomers) are mucoadhesive polymers with thiol group-containing side-chains (Bernkop-Schnürch, 2005). The most commonly used thiomers are synthetized from chitosan, alginate, polyacrylates and cellulose derivatives (Andrews et al., 2009). In contrast with the first-generation polymers, they are capable of forming covalent (disulphide) bonds with cysteine-rich subdomains of the mucus layer (Bernkop-Schnürch, 2005). Other advantages of thiomers include permeation enhancement through the reversible opening of the tight junction, enzyme inhibition and efflux pump inhibition (Gradauer et al., 2013; Iqbal et al., 2011; Rahmat et al., 2012). As a result of these advantages these polymers ensure the prolongation of the residence time and increase the bioavailability, and they can be used in many medical fields (e.g. topical ocular therapy) in various dosage forms, such as liquid drops, gels or mini-tablets.

In our work, thiol-containing side-groups were bonded to poly(aspartic acid) (PASP). PASP is a biocompatible and biodegradable polymer by virtue of its protein-like structure, and its degradation products are excreted by the physiological mechanisms of the body. It is not toxic and does not generate immunogenicity. PASP is currently used as a material component in dialysis membranes, artificial skin, drug delivery systems and orthopaedic implants in biomedical applications (Gyarmati et al., 2013; Gyenes et al., 2008; Roweton et al., 1997; Zrínyi et al., 2013).

The goal of our work was to develop a mucoadhesive formulation from thiolated PASP (ThioPASP) polymers which could be used as an *in situ* gelling, ophthalmic drug delivery system by spreading it on the surface of the eye or instilling it into the cul-de-sac. An important step was to find the ideal oxidized state of the polymers. The gel structure formed was characterized by rheology. The functions of the thiol groups (their effects on the gel structure and on the mechanism and strength of mucoadhesion) were determined. Rheology, tensile tests and 'wash away' measurements were performed to characterize the mucoadhesion. Since the ThioPASP polymers were planned to be used in ocular therapy, measurements were performed to determine the release profile of active ingredients from the gels.

2. Materials and methods

2.1. Materials

Imidazole (puriss p.a.) and Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) were purchased from Sigma–Aldrich. S-Aspartic acid (99%), dibutylamine (DBA, 99%), methanol (MeOH, 99.9%), cysteamine hydrochloride (97%), potassium chloride (KCl, 99.5%), sodium bromate ($NaBrO_3$, 99%), dithiothreitol (DTT, for biochem.), glutathione (for biochem.), sodium borohydride (for synthesis), mesitylene (for synthesis) and sulfolane were purchased from Merck. Phosphoric acid (H_3PO_4, cc. 85%), hydrochloric acid (HCl, 35%) and dimethylformamide (DMF, pure) were bought from Lach Ner. N-acetyl-L-cysteine (ACR: 160280250), sodium hydroxide (NaOH, a. r.) and sodium chloride (NaCl, a. r.) were obtained from Reanal Hungary. Milli-Q reagent-grade water ($\kappa > 18.2\ M\Omega\ cm$, Millipore) was used for the preparation of aqueous solutions. All reagents and solvents were used without further purification. The modified and cross-linked polymers were synthetized and all the measurements were carried out at 25 °C, unless otherwise indicated.

In *in vitro* and *ex vivo* experiments, 1 M $NaBrO_3$ solution was used as a model oxidant. Buffer solutions of pH = 8 were prepared from imidazole ($c = 0.1$ M); the pH was adjusted with 1 M HCl. The pH of the buffer solutions was measured with a pH/ion analyser (Radelkis OP-271/1, Hungary). The ionic strength of the solutions was adjusted to 0.15 M with KCl. 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. Lachrymal 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, the pH being adjusted with 1 M HCl.

Mucin (porcine gastric mucin type II), sodium diclofenac (SD) and sodium fluorescein were purchased from Sigma Aldrich. No other modifications were carried out on porcine gastric mucin type II. Mucin dispersions were prepared with PBS or simulated lachrymal fluid and stirred for 8 h. Hydroxyethylcellulose (HEC) (Natrosol Pharm) was bought from Hercules.

2.2. Synthesis

The pre-cursor polymer of ThioPASP, polysuccinimide (PSI), was synthetized by the procedure reported earlier (Gyarmati et al., 2013). The chemical structure of the PSI was confirmed by ^1H

NMR (300 MHz, DMSO-d_6, δ): 5.10 (d, 1H, CH); 3.20 and 2.75 (s, s, 2H, CH_2). The average molecular weight of the PSI, estimated by measuring the molecular weight of its hydrolysed derivative, PASP, with HPLC size exclusion chromatography (SEC), proved to be M_w = 56.1 kDa, PDI = 1.07.

ThioPASP was synthetized by the modification of PSI with cysteamine hydrochloride in DMF in equimolar amount with DBA as deprotonating agent (Fig. 1). The procedure was as follows: 0.485 g of PSI (containing 5 mmol of succinimide repeating units) and 0.114 g (1 mmol) of cysteamine hydrochloride were dissolved in 9.272 g of DMF under a nitrogen atmosphere. DBA (170 µl, 0.129 g, 1 mmol) was added dropwise to the solution. Solutions of thiolated PSI were poured between silicon frames (1.0 mm thickness) on a glass plate and were oxidized by the air into disulphide cross-linked PSI gels. After 2 days of oxidation, the PSI gels were immersed into imidazole buffer of pH = 8 (I = 0.15 M). Water-swollen, transparent PASP gels were obtained after 3 days of hydrolysis.

The PASP gels were dissolved by the addition of solid DTT to the swelling solution. The molar ratio of DTT to thiol groups was 1:1. Dissolution was complete after 15 min. The reduced ThioPASPs were dialysed (cut-off M_w = 12–14 kDa) against water. Glutathione was used as antioxidant in a concentration of 1% w/w relative to the weight of the polymer. The solid polymers that resulted after lyophilization were stored at 8 °C until further use.

The total thiol content of the polymers was determined by Ellman's assay. 100 µl of polymer solution (c = 100 mM) was diluted with 200 µl of a freshly-prepared solution of $NaBH_4$ (10% w/w, pH = 8) and the reduction was performed for 30 min. The excess of reducing agent was decomposed by the addition of 500 µl of 1 M HCl to the solution, which was neutralized after 30 min with 500 µl of 1 M NaOH. Ellman's assay was performed by adding 20 µl of Ellman's reagent (1 M) after dilution of the mixture to 2000 µl with buffer solution of pH = 8. Absorption spectra were recorded with an Analytic Jena Specord 200 spectrophotometer (Germany). N-acetylcysteine was used as standard for the calibration curve. The molar ratio of thiols to repeating units was calculated to be 7.0% n/n.

2.3. Rheology

ThioPASP was dissolved in PBS and the gelation was initiated by adding various amounts of oxidant. The precursor solutions of the hydrogels consisting of the ThioPASP and oxidant were mixed on the plate of the rheometer. For the mucoadhesive investigation, the polymer solutions were mixed with a mucin dispersion in

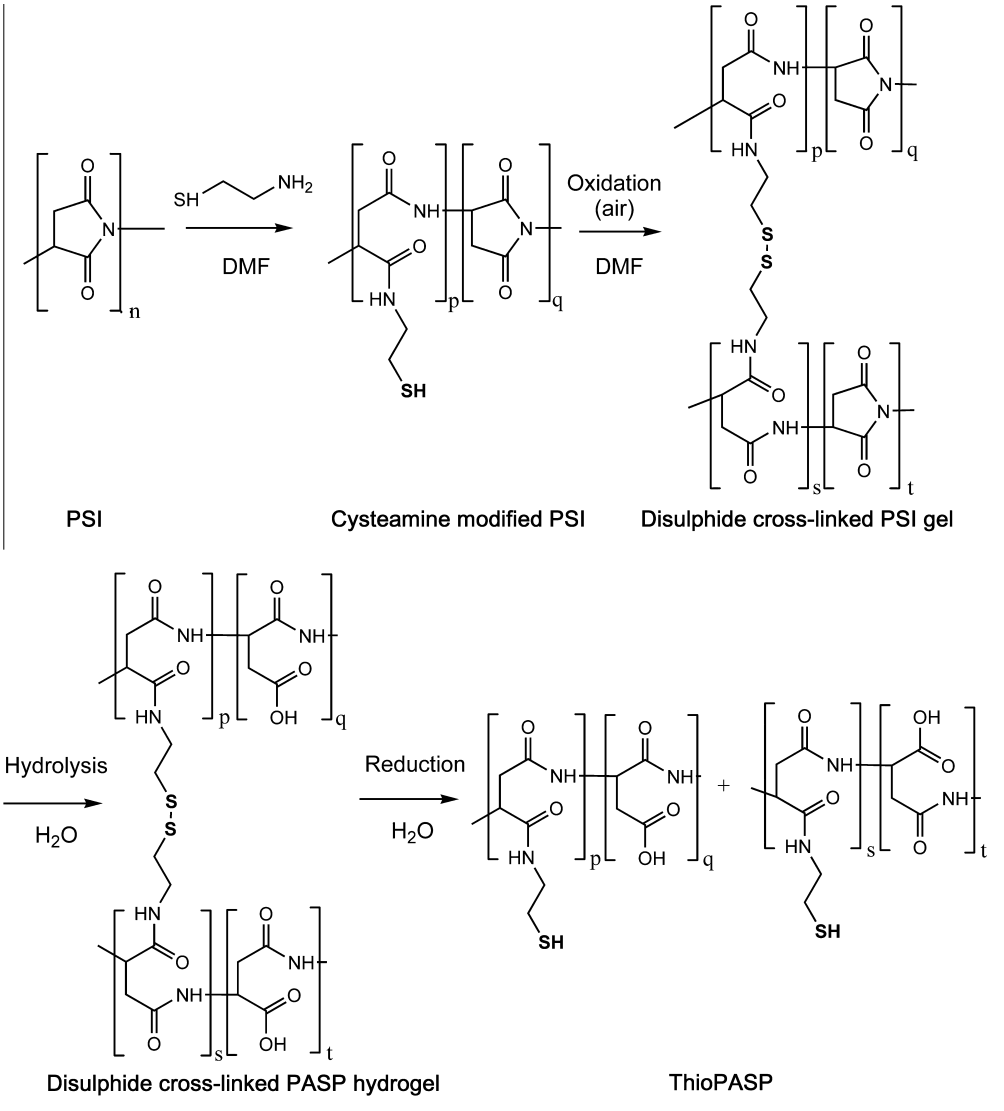

Fig. 1. Synthesis of thiolated poly(aspartic acid) (ThioPASP) from polysuccinimide (PSI).

PBS before the oxidation (final mucin concentration in the mixtures 5% w/w). The rheological properties were studied with a Physica MCR101 rheometer (Anton Paar, Austria). The measuring device was of cone and plate type (diameter 25 mm, the gap height in the middle of the cone 0.046 mm, and cone angle 1°). The gelation of the ThioPASP polymer was followed at a constant frequency of 1.0 Hz at a constant strain of 1% at 25 °C. Each measurement was carried out on a freshly-made sample and was started immediately after the mixing of the compositions. Viscoelastic character was determined by frequency sweep tests after the gelation, with a strain of 1% at 25 °C. Storage modulus (G'), loss modulus (G'') and complex viscosity (η^*) were determined over the angular frequency range from 0.1 to 100 1 s^{-1}. The strain value (1%) used in the measurements was in the range of the linear viscoelasticity of the gels.

2.4. Swelling of ThioPASP hydrogels

The water absorption capacity of the ThioPASP gels was determined gravimetrically. 20% w/w mixtures of ThioPASP with different oxidant concentrations (1 M $NaBrO_3$, 20% or 40% w/w) were made in a syringe with a cut ending and held at 4 °C for 20 min. The gel was then pushed out of the cylinder, cut into four equal parts (for the four parallel measurements) and dried in vacuum at 30 °C to constant weight. The weights of the discs were measured and they were placed into distilled water at room temperature. The discs were taken out of the water after defined time periods, the surplus water was removed by blotting and the weights of the gels were measured gravimetrically.

2.5. Tensile tests

The measurements were carried out in two different ways; either the oxidant was added to the polymer solution, or the oxidant was added to the mucin. Tensile tests were performed with a TA-XT Plus (Texture analyser (ENCO, Spinea, I)) instrument equipped with a 1 kg load cell and a cylinder probe with a diameter of 1 cm. Samples were placed in contact with a filter paper disc wetted with 50 µl of an 8% w/w mucin dispersion or with excised porcine conjunctiva. The mucin dispersion was made with simulated lachrymal fluid (pH = 7.4). 10 parallel measurements were carried out (Szűcs et al., 2008).

20 mg of the sample was attached to the cylinder probe and placed in contact with the biological substrate. A 2500 mN preload was used for 3 min. The cylinder probe was moved upwards to separate the sample from the substrate at a prefixed speed of 2.5 mm min^{-1}. Measurements were made both with excised porcine conjunctiva and with blank (simulated lachrymal fluid). The porcine conjunctiva was obtained from a slaughterhouse, freshly detached from the connective tissue and stored at −20 °C until the measurement. After the complete thaw, the conjunctiva was placed on the previously wetted (with simulated lachrymal fluid) filter paper and fixed in the lower probe (Sandri et al., 2006). The work of adhesion (A, mN mm) was calculated as the area (AUC) under the "force versus distance" curve (Sandri et al., 2012).

2.6. 'Wash away' measurements

To perform the 'wash away' measurements, an earlier-developed modified Franz diffusion cell was used (Bonferoni et al., 1999; Rossi et al., 1999). Simulated lachrymal fluid was streamed through the donor chamber by a HPLC pump, the effluent fluid being collected in a beaker. The measurements were made on excised porcine conjunctiva, placed on the acceptor chamber filled with distilled water and closed with Parafilm. Filter paper was impregnated with simulated lachryimal fluid, and positioned between the Parafilm and the conjunctiva to keep it hydrated. The donor chamber was connected to the acceptor chamber (used to regulate the temperature of the conjunctiva). 250 mg of polymer gel was used, with sodium fluorescein (0.008% w/w) as the measured marker. The formulation was prepared in the donor chamber on the conjunctiva, by adding 5%, 7% or 10% w/w polymer solution containing sodium fluorescein and 20% w/w oxidant solution. After 10 min, the donor chamber was filled with lachrymal fluid and a stream at 1 ml min^{-1} was set up to mimic the lachrymation of the eye. Measurements were carried out in duplicate and were made at 35 °C, corresponding to the temperature of the eye surface. Lachrymal fluid washings were collected in beakers after 2, 5, 10, 20, 30 and 60 min, and measured with a spectrofluorimeter at 494 nm excitation wavelength and 521 nm emission wavelength (LS50B, Perkin Elmer) (Sandri et al., 2006). HEC gels under the same experimental conditions were used as reference.

2.7. Drug release measurements

The drug release profile of SD was determined with a vertical Franz diffusion cell system (Hanson Microette Plus TM). 300 µl of formulation containing 10% w/w polymer solution with 0.1% w/w SD and 20% or 40% w/w oxidant solution was placed on a Porafilm membrane filter (pore size of 0.45 µm) impregnated with pH 7.4 buffer solution. The two solutions were mixed on the membrane filter as a donor phase. PBS (pH = 7.4) was used as acceptor phase and thermostated at 35 °C. Measurements were performed for 24 h and five parallel measurements were carried out. 0.8 ml samples were taken from the acceptor phase by the autosampler and replaced with fresh PBS. The SD released was quantified by UV spectrophotometry at 275 nm (Csizmazia et al., 2011).

3. Results and discussion

3.1. Gel formation

The gelation process and the gel structure were characterized by means of rheology. The effects of the polymer concentration

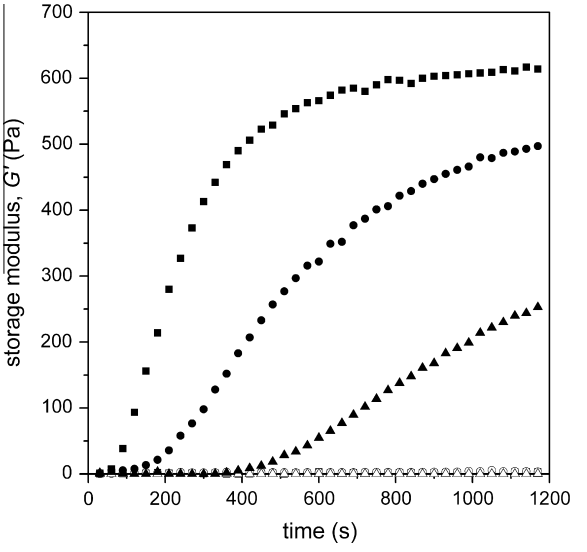

Fig. 2. Effects of polymer and oxidant concentrations on the storage modulus (G') of ThioPASP as a function of the gelation time; the polymer and oxidant concentrations: (△) 7% w/w and 10% w/w; (○) 7% w/w and 20% w/w; (□) 7% w/w and 40% w/w; (▲) 10% w/w and 10% w/w; (●) 10% w/w and 20% w/w; (■) 10% w/w and 40% w/w, respectively.

(3%, 5%, 7% or 10% w/w) and the weight ratio of oxidant (10%, 20% or 40% w/w) were studied (Fig. 2).

Gelation did not proceed at polymer concentrations lower than 10% w/w, regardless of the concentration of oxidant. The fastest gelation and the largest G' were obtained with 40% w/w oxidant and 10% w/w ThioPASP. Increase of the oxidant concentration resulted in a larger number of cross-links via disulphide formation and a stiffer gel structure. The oxidant concentration also influenced the gelation time: an increase of the weight ratio of the oxidant accelerated the gelation process. The gelation time can usually be determined from the cross-point of the moduli G' and the G'' (Fatimi et al., 2009; Winter and Chambon, 1986). In our case, this cross-point could not be observed, because G' predominated over G' throughout the measurement. This might be explained by the presence of a loosely cross-linked physical network. Prior to the chemical cross-linking, second-order interactions formed between the polymer chains, particularly-hydrogen bonds between the free thiol groups. Consequently, the gelation time was defined as the time at which a maximum could be observed in the curve of the differential with respect to time (Fig. 3) (Ma et al., 2008).

The frequency sweep test was started after full gelation. Table 1 presents G' and G' of the formulations at an angular frequency of 1 l s^{-1}.

At polymer concentrations lower than 7% w/w, the changes of the polymer or oxidant concentration did not affect the gelation, G' did not change significantly and the precursor solutions remained in the liquid state even after the addition of oxidant (G' was similar in order of magnitude to G''). A gel structure formed (G' was more than an order of magnitude higher than G'') at 7% w/w polymer concentration in the case of higher amounts of oxidant (20% and 40% w/w). At high polymer concentrations (7% and 10% w/w), the values of G' increased with increase of the polymer concentration and the oxidant amount. The gel obtained at 10% w/w ThioPASP displayed the strongest gel structure, indicating that the elevation of both the polymer and the oxidant concentration enhanced the cross-linking density by increasing the concentration of disulphide linkages.

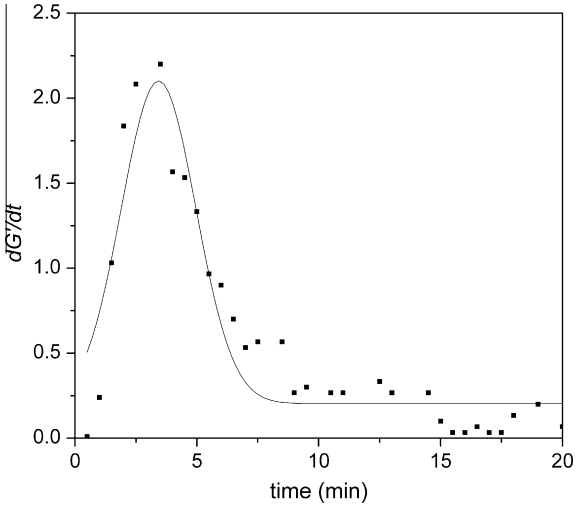

Fig. 3. The differential curve of the storage modulus (G') of the 10% w/w ThioPASP gel with 40% w/w oxidant with respect to time (t).

Table 1
Storage (G') and loss moduli (G'') at different oxidant and polymer concentrations (ω = 1 s^{-1}).

ThioPASP conc. (% w/w)	Oxidant conc. (% w/w)					
	10		20		40	
	G'	G''	G'	G''	G'	G''
3	~0	0.06	0.18	0.02	0.07	0.02
5	~0	0.23	0.11	0.02	0.08	0.28
7	0.78	0.37	16.28	1.35	10.20	0.80
10	433.0	11.0	533.0	12.6	637.0	9.1

3.2. Swelling of ThioPASP hydrogels

The swelling of the hydrogels was characterized by a gravimetric method. Two formulations of 20% w/w ThioPASP gels were measured, with different oxidant concentrations (20% and 40% w/w). During the 6 h measurements, the swollen polymer discs maintained their coherent structure and shape, because of the formation of disulphide linkages between the polymer chains.

Fig. 4 depicts the percentage swelling (%S), calculated from the following equation (Karadağ et al., 2002):

$$\%S = \frac{M_t - M_0}{M_0} \times 100 \quad (1)$$

where M_0 is the mass of the dry gel (g) and M_t is the mass of the swollen gel (g). This value gives information about the water uptake capacity of the polymer.

The polymers swelled faster initially and the water uptake then slowed as equilibrium was approached. In the first period of the swelling, there was no significant difference between the two samples, but later the curves diverged. The samples with lower oxidant concentration contained a lower concentration of disulphide linkages. The swelling ability of these hydrogels was larger because of the lower cross-linking density resulting from the weaker elastic interactions inside the polymer network. This led to a marked water uptake of the formulation containing less oxidant. The polymers were able to swell to 6000–7000% of the volume of their dry mass.

Another important factor involved in the swelling process is the swelling power (F_{swp}), which gives information about the mechanism of the swelling (Karadağ et al., 2002):

$$F_{swp} = \frac{M_t - M_0}{M_0} = Kt^n \quad (2)$$

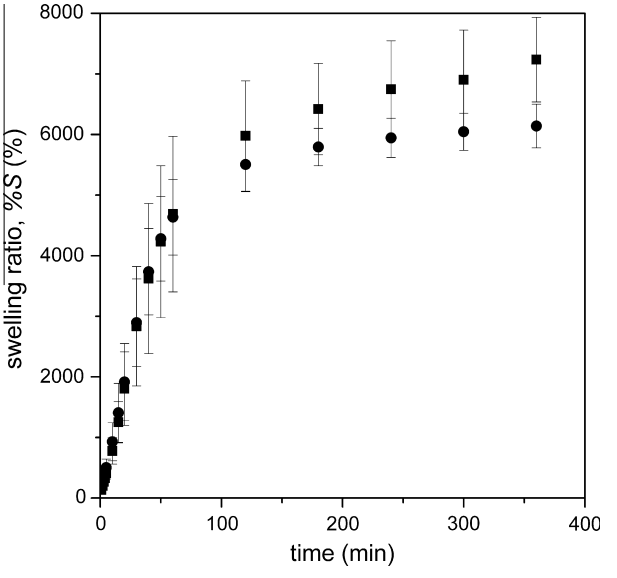

Fig. 4. Swelling kinetics of ThioPASP hydrogels at ((■) 20% w/w and (●) 40% w/w) oxidant concentrations.

Table 2
Swelling constant and swelling exponents at different oxidant concentrations, calculated from the power law.

Oxidant conc. (% w/w)	K	n
20	1.1198	0.874
40	1.5007	0.809

where M_0 is the mass of the dry gel (g), M_t is the mass of the swollen gel (g) and t is time (min). Table 2 reports the swelling constants (K) and the swelling exponents (n) of the two oxidant concentrations, determined by power law fitting to the curve of F_{swp} vs. t (min).

The mechanism of water uptake is indicated by the value of n. A value in the range 0.45–0.5 corresponds to Fickian diffusion, while a value of 0.5–1 means that the diffusion mode is non-Fickian (Karadağ et al., 2002). In our case, non-Fickian diffusion was observed: the n values were 0.874 and 0.809.

The results of water uptake measurements indicated that the oxidant concentration did not affect the initial stages of the process, but in the second period a larger water uptake was observed when 20% w/w of oxidant was used.

3.3. Mucoadhesion

3.3.1. Rheology

Rheological synergism between mucin and polymer mixtures can be proposed as an *in vitro* parameter through which to determine the mucoadhesive behaviour of polymers (Hassan and Gallo, 1990). The rheological method is based on the determination of the changes in rheological parameters after the mucoadhesive polymer is mixed with mucin. Hassan and Gallo demonstrated that a synergistic increase in viscosity could be observed when the mucoadhesive polymer and mucin were mixed together. This viscosity change, called the bioadhesive viscosity component (η_b), is caused by chemical and physical bonds formed in mucoadhesion. It can be calculated as follows:

$$\eta_b = \eta_t - \eta_m - \eta_p \tag{3}$$

where η_t is the viscosity of the mucin-polymer solution system, and η_m and η_b are the viscosity components of the mucin and polymer solution (Caramella et al., 1999; Hassan and Gallo, 1990; Marschütz and Bernkop-Schnürch, 2002).

More recently, the rheological synergism parameters have been measured by dynamic oscillatory rheometry. In this case, the synergism parameters can be calculated as follows (Madsen et al., 1998):

$$\Delta G' = G'_{(mix)} - \left(G'_{(polymer)} + G'_{(mucin)}\right) \tag{4}$$

$$\Delta \eta' = \eta^*_{(mix)} - \left(\eta^*_{(polymer)} + \eta^*_{(mucin)}\right) \tag{5}$$

where G' is the storage modulus and η^* is the complex viscosity of the systems.

In our work it was difficult to compare the rheological profiles of the polymer solution and the hydrogel because the G' values of the ThioPASP and mucin solutions were negligible. It therefore appeared reasonable to use the relative rheological synergism parameters, which express the relative increments in viscoelasticity with regard to the polymer and mucin solutions alone (Madsen et al., 1998):

$$\Delta G'_{rel} = \Delta G' \Big/ \left(G'_{(polymer)} + G'_{(mucin)}\right) \tag{6}$$

$$\Delta \eta^*_{rel} = \Delta \eta^* \Big/ \left(\eta^*_{(polymer)} + \eta^*_{(mucin)}\right) \tag{7}$$

Rheological measurements were performed with different concentrations of ThioPASP polymer and mucin. It was presumed that, if an *in situ* gelling system is used, the mucin can influence the gelation time of the formulation. For this reason, the gelation time was first determined in the presence of mucin, using the same method as described before. The results are shown in Fig. 5 and Table 3.

As in the previous measurements without mucin, gelation was observed only at 7% and 10% w/w ThioPASP. Mucin did not cause an appreciable difference in the rheological parameters at ThioPASP concentrations lower than 7% w/w. In the case of 7% or 10% w/w polymer, the gelation time was shorter at the lower ThioPASP concentration, and the gelation time decreased with increasing concentration of the oxidant. The addition of mucin aided the gelation and in each case the gelation time was shorter in the presence of mucin. The rate of gelation and the final value of G' were higher in the presence of mucin.

Frequency sweep tests were performed after gelation to investigate the interaction between the mucin and the ThioPASP gels. Fig. 6 presents the variation in G' with angular frequency for the formulations with and without mucin. In all cases, mucin augmented the elastic modulus of the samples, indicating that interactions occurred between the polymer and the mucin. The shapes of the curves (the slopes of the G' vs. angular frequency curves, which

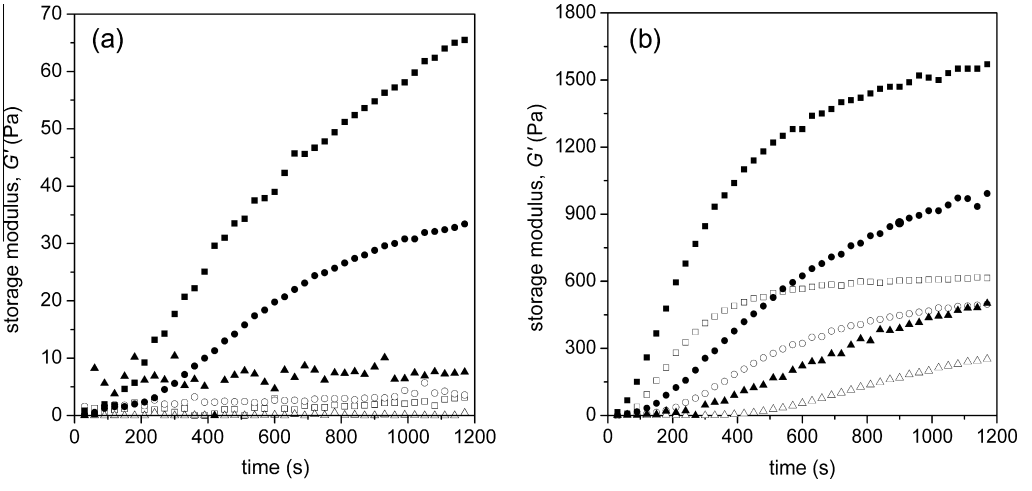

Fig. 5. Evolution of storage modulus (G') as a function of time at (a) 7% and (b) 10% w/w polymer concentrations; the oxidant concentration (◭) 10% w/w, (◑) 20% w/w, (◨) 40% w/w was also varied and mucin was (solid symbols) or was not (open symbols) added.

Table 3
Gelation time (t_g) at different compositions.

ThioPASP conc. (% w/w)	Oxidant conc. (% w/w)	t_g without mucin (s)	t_g with mucin (s)
7	10	n. g.	n. g.
	20	n. g.	450
	40	n. g.	420
10	10	780	540
	20	330	300
	40	150	120

n. g. – No gelation was observed.

show the frequency dependency of the systems) of the samples with 10% w/w polymer with or without mucin were similar to each other. At this concentration, the polymer gels exhibited a densely cross-linked gel structure even without mucin. Mucin did not change the rheological profile of the systems. The changes in the rheological behaviour of the samples containing 7% w/w or less polymer suggested the formation of a chemically cross-linked structure between the polymer and mucin chains in addition to physically entanglement. In a physically entangled structure, the moduli depend strongly on the frequency: at low frequency, the G' values are decreased considerably (Madsen et al., 1998; Ross-Murphy and McEvoy, 1986). In our case, at lower ThioPASP concentrations (and especially 7% w/w), the added mucin decreased the slope of the curves, which indicated the occurrence of the cross-linking of the polymer with the mucin.

Table 4 shows the relative synergism parameters of η^* and G' at an angular frequency of 1 1 s^{-1}.

The stiffness of the gels was larger in the presence of mucin in each case. At higher polymer concentrations, the relative differences ($\Delta G'$ and $\Delta\eta^*$) were lower at each oxidant content than at lower polymer concentrations. The mucoadhesive character was displayed most significantly at lower polymer concentrations (3% and 5% w/w) and especially at 20% w/w oxidant amount. This result is in accordance with earlier studies in which it was concluded that there is an optimum polymer concentration for mucoadhesion (Madsen et al., 1998). In our work, this was probably because a lower amount of oxidant resulted in a loosely, chemically cross-linked structure, and the chains were flexible enough to be able to form more bonds with the mucin, resulting in a gel-strengthening effect in the mixture.

3.3.2. Tensile tests

Adhesive strength is defined as the external force required for the separation of the two interfaces in the tensile test measurements.

The first important step was to determine the ideal oxidant amount for mucoadhesion. 10%, 20% and 40% w/w of the oxidant was used In one case the oxidant solution was added to the polymer solution, and in the second case the oxidant was added to

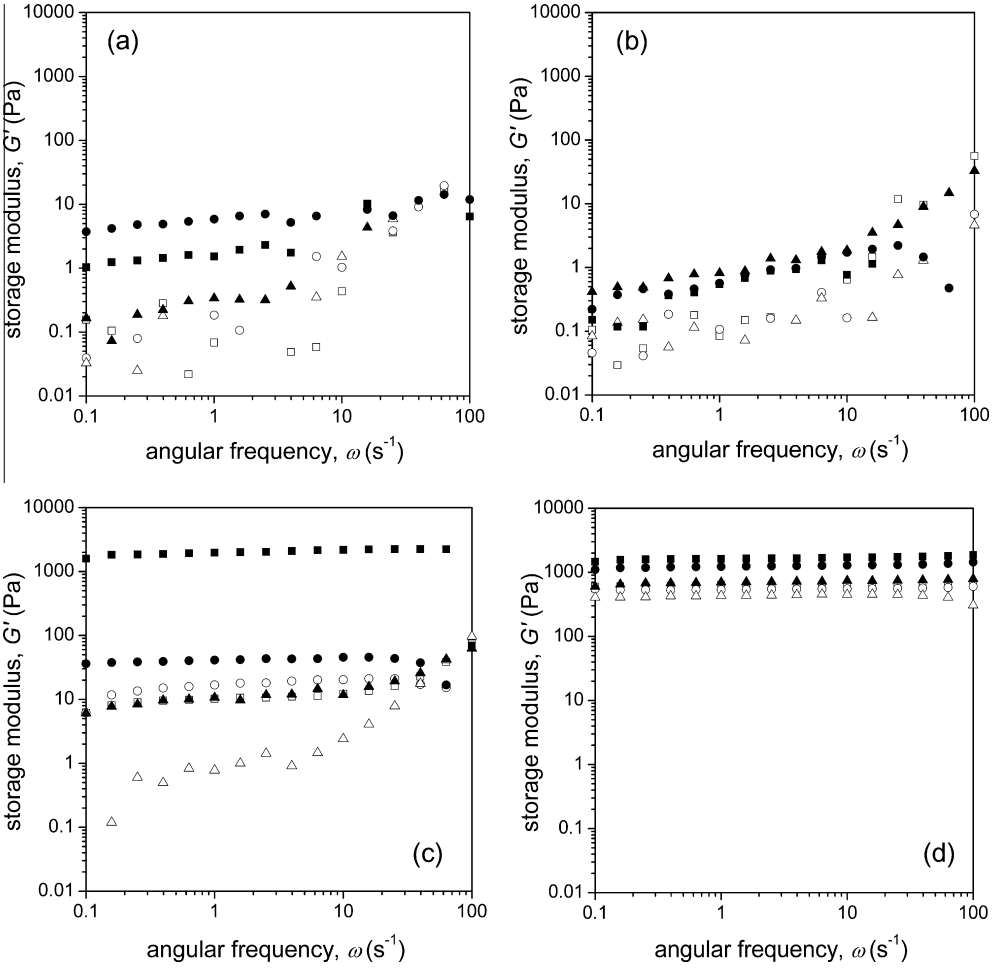

Fig. 6. Frequency sweep tests at (a) 3%, (b) 5%, (c) 7% and (d) 10% w/w polymer concentrations and at (◭) 10% w/w, (◑) 20% w/w and (◨) 40% w/w oxidant concentrations with (solid symbols) or without mucin (open symbols).

Table 4
Relative synergism parameters of viscosity and storage modulus between the polymer-mucin mixtures.

ThioPASP conc. (% w/w)	Oxidant conc. (% w/w)	$\Delta G'$	$\Delta\eta^*$
3	10	1.88	8.92
	20	31.85	36.11
	40	22.43	27.03
5	10	6.87	1.65
	20	5.28	7.78
	40	6.44	2.84
7	10	13.67	12.69
	20	2.44	2.44
	40	7.46	7.47
10	10	1.61	1.61
	20	2.31	2.31
	40	2.56	2.56

the mucin dispersion. The polymer can form cross-links with the thiol groups of the PASP, which therefore has fewer free thiol groups capable of forming disulphide bonds with the glycoproteins of the mucin. However, the oxidant is also needed to form bonds between the polymer and the mucin. These investigations could help to clarify which procedure is better for ideal mucoadhesion. Fig. 7 shows the work of adhesion (A, mN mm) of the samples with 10% w/w and 5% w/w polymer concentrations.

The results reveal that A was similar at the two concentrations. The same formulation prepared in the different ways displayed different mucoadhesive behaviour. It was found that the samples in which the oxidant was added to the polymer before the contact with the mucin exhibited greater mucoadhesive strength. An exception was the formulation with 10% w/w polymer and 40% w/w oxidant. In this case the samples oxidized in advance demonstrated lower A values. At this concentration, this is probably due to the high cross-link ratio, the low number of free thiol groups for mucoadhesion and the rigid structure of the network.

The formulation with 20% w/w oxidant added to the polymer solution had the optimal mucoadhesive properties (best A value) at both polymer concentrations. This amount of oxidant is probably needed for the optimum gel structure for interpenetration in the mucin, but still containing sufficient free thiol groups able to form disulphide bonds with the mucin glycoproteins. With regard to the results, 20% w/w was chosen as best oxidant concentration. Other measurements were made with 20% w/w oxidant added to the polymer solution with blank (lachrymal fluid) and conjunctiva.

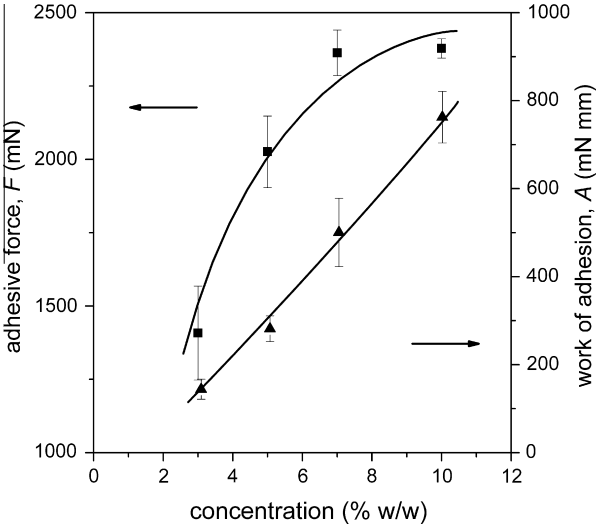

Fig. 8. (■) Adhesive force (F) and (▲) work of adhesion (A) as functions of polymer concentration.

Measurements were also made with 3% and 7% w/w polymer (Fig. 8).

Fig. 8 shows that A increased continuously as the concentration was elevated, while the adhesive force (F, mN) reached a maximum at 7% w/w polymer. It was earlier established (Park and Munday, 2002) that the F depends on the formation of chemical bonds between the functional groups of the polymer investigated and the mucin, whereas A is dependent on the formation of chemical bonds and also on mechanisms such as physical entanglement or interpenetration. In our work, the chemical bonds probably have a larger effect at lower polymer concentration, and it is likely that covalent bonds and secondary bonds were formed with the mucin glycoproteins. Thus, F increased continuously with increasing polymer concentration. At high polymer concentration, the potential for chemical bonds reached a maximum because the free thiol groups were saturated at the interface. Accordingly, a plateau was observed in the F vs. concentration curve. A did not reach a maximum, but increased continuously, because interpenetration prevails in the process of mucoadhesion rather than chemical bonding at higher polymer concentrations. In our case, the ThioPASP gel at higher concentration has more thiol groups and more

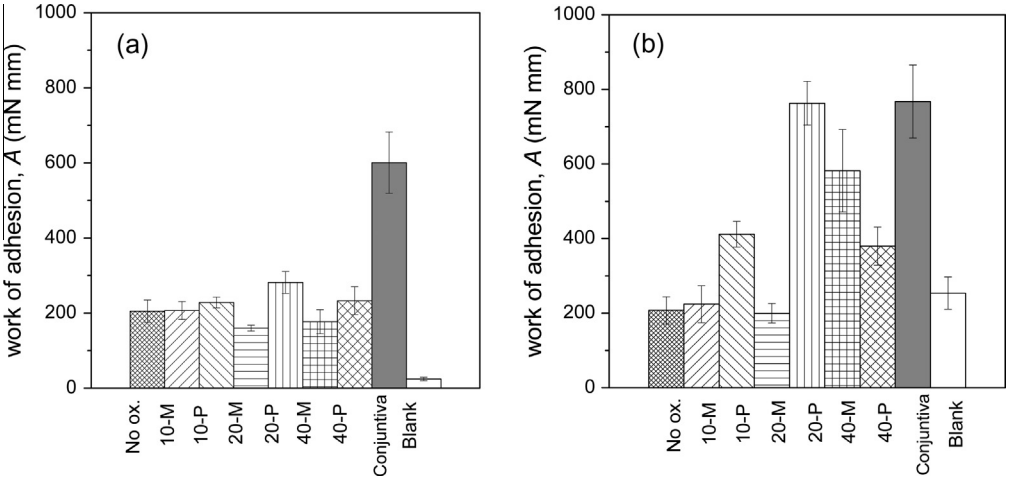

Fig. 7. Work of adhesion (A) at (a) 10% w/w and (b) 5% w/w polymer concentrations; samples are labelled as $x - P$ or $x - M$, where x is the oxidant concentration in % w/w, and P and M stand for ThioPASP and mucin, respectively.

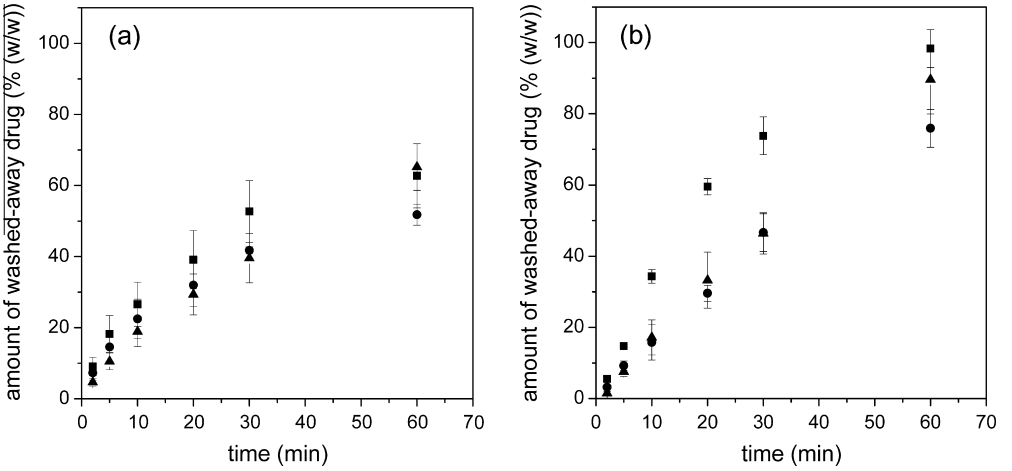

Fig. 9. (a) ThioPASP and (b) HEC containing the model drug. Polymer concentrations: (□) 5% w/w, (○) 7% w/w and (△) 10% w/w.

cross-links resulting in a gel structure, which induces increased swelling, allowing deeper and improved interpenetration.

These tensile test results can be correlated to the rheological results, where the changes in the shape of the frequency sweep curves could be observed up to 7% w/w polymer concentration, which indicated the formation of chemical cross-links (Hägerström and Edsman, 2003).

3.3.3. 'Wash away' measurements

'Wash away' *ex vivo* measurements mimic the lachrymation of the eye, under conditions relatively close to real mucoadhesive circumstances of the eye. The amount of sodium fluorescein washed away from the porcine conjunctiva can indicate the amount of the dosage form remaining on the surface. In our work, HEC gels were used as reference.

It can be observed in Fig. 9a that increase of the ThioPASP concentration was accompanied by a slight decrease in the amount of model drug washed away. These differences were not pronounced after 1 h. In the case of the reference systems (HEC gels, Fig. 9b), the observations were similar; the gel with the lowest HEC concentration underwent the fastest washing out. Comparison of the ThioPASP systems with the HEC gels indicated that the ThioPASP formulations have a longer residence time, because 40% w/w of the model drug remained on the conjunctiva, in contrast with 10–30% w/w for the reference systems.

3.4. Drug release measurements

Drug release measurements were performed with a vertical Franz diffusion cell system with gels containing 10% w/w polymer, SD and 20% or 40% w/w oxidant. Fig. 10 shows the amount of drug released (% w/w) during time.

In the first hour, the diffusion of the SD was fast and there was no significant difference between the two formulations. After 5 h, the release profiles became dissimilar in the cases of different concentrations of oxidant, and the formulation with 20% w/w oxidant released a larger amount of the SD. These results correspond with the results of the swelling measurements. The formulation with 20% w/w oxidant has a higher water uptake, suggesting a lower cross-linking density, and the SD is therefore able to diffuse through this structure more easily.

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

$$\frac{M_t}{M_\infty} = kt^n \tag{8}$$

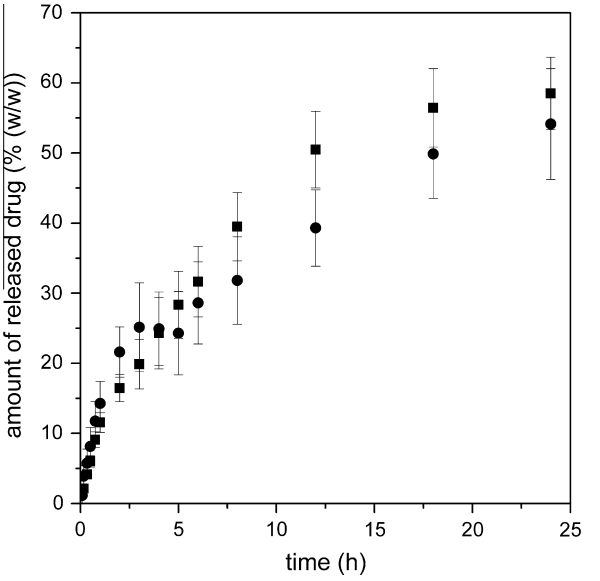

Fig. 10. Release of model drug, SD from ThioPASP gel at (■) 20% w/w and (●) 40% w/w oxidant concentrations.

Table 5
Release exponent (n) and kinetic constant (k) at two oxidant concentrations.

Oxidant conc. (% w/w)	n	k
20	0.6561	8.8906
40	0.5815	10.322

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 (Peppas et al., 2000). These values were determined from the equation of the power law fitted to the curve of the amount of drug released (% w/w) against time (min) (Table 5).

In our case, we have a non-Fickian release mechanism, because the value of n is between 0.5 and 1, and these results also correspond with our swelling results (Table 2). Non-Fickian (anomalous) transport occurs due to a coupling of Fickian diffusion and a polymer relaxation. In the anomalous processes of drug release, Fickian diffusion through the hydrated layers of the matrix and polymer chain relaxation/erosion are both involved. The contribution of these two mechanisms to the overall release are considered to be

additive. The Fickian contribution to the overall release process is decreasing with the increasing amount of the released drug. 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 enables polymer chain relaxation (Baumgartner et al., 2006; Park and Munday, 2002; Peppas et al., 2000; Peppas and Buri, 1985; Ritger and Peppas, 1987). During the drug release, our polymer gels of 10% w/w placed on the membrane was continuously swelling, which led to a simultaneous absorption of the water and desorption of drug via a swelling-controlled diffusion mechanism. The combination of diffusion, swelling, and relaxation is responsible for the non-Fickian release mechanism (Lee, 1985).

The advantage of these formulations is the rapid drug release in the first hour, followed by a prolonged release. This is important in therapy, because a higher dose is needed immediately after the application, to reach the therapeutic dosage, after which a sustaining dosage is required. From the aspect of ophthalmic preparations, this can increase patient compliance.

4. Conclusion

The generally poor bioavailability of ophthalmic formulations can be improved by new formulations with a prolonged residence time. *In situ* gellable ThioPASP hydrogels were fabricated with the aim of obtaining delivery vehicles with increased adhesion to the eye surface and these hydrogels were characterized in terms of gelation time, viscoelastic behaviour, mucoadhesion and drug release. Mucin exhibited a strong effect on cross-link formation, and the ThioPASP gels displayed strong mucoadhesion, especially at lower polymer concentrations (3%, 5% w/w). The addition of a small amount of oxidant improved the mucoadhesion, as indicated by the *A* values during the tensile tests, because of the formation of a gel structure with a considerable number of free thiol groups. The ThioPASP gels demonstrated high resistance against lachrymation of the eye, which also confirmed the strong mucoadhesion. SD could be encapsulated into the ThioPASP polymers during formation of the gel structure and underwent rapid release in the first hour during *in vitro* measurements, which was followed by a sustained release of the drug for a further 23 h. This drug release profile and the strong mucoadhesion excellently illustrate the potential of the application of the ThioPASP polymer as an *in situ* gelling, ocular drug delivery formulation for a once-daily dose administration.

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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

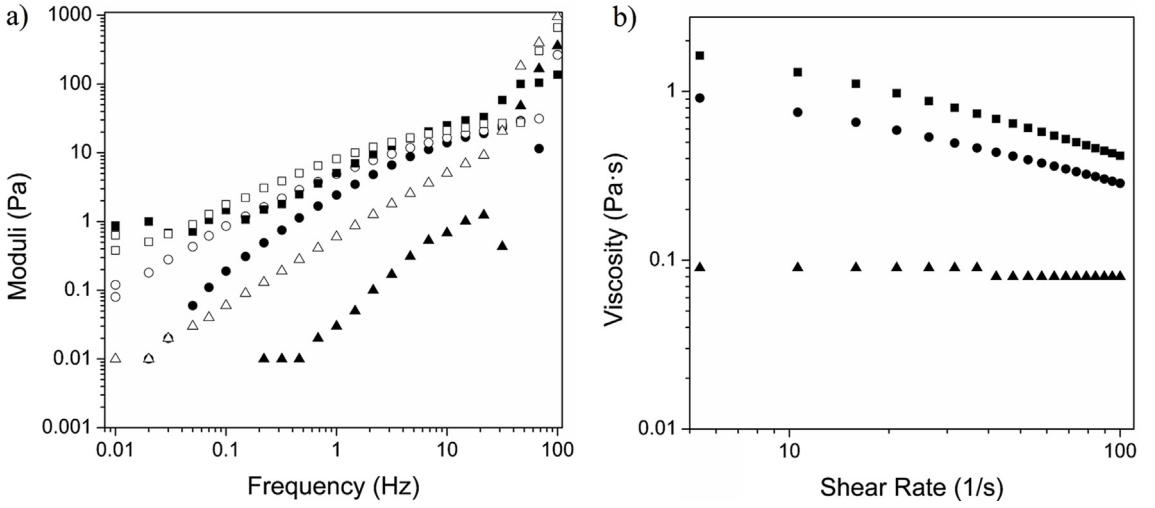

Fig. 1. (a) G' (solid symbols) and G'' (open symbols) values as a function of frequency of (◑) CLNaHA, (◨) NaHA and (◭) ZnHA; and (b) viscosity curves of (●) CLNaHA, (■) NaHA and (◭) ZnHA.

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

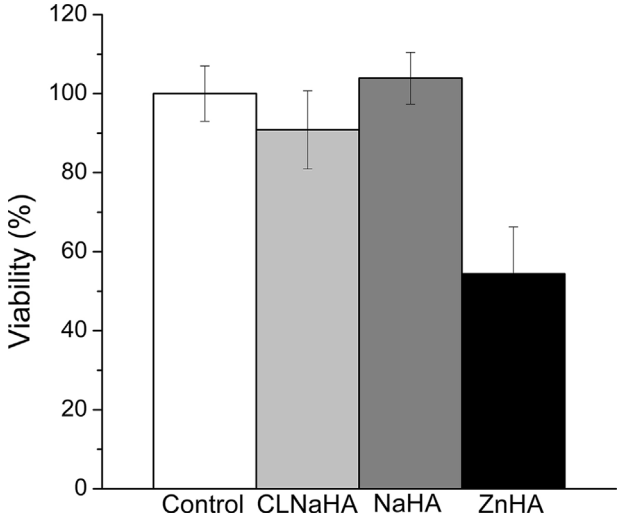

Fig. 2. Biocompatibility of CLNaHA, NaHA and ZnHA.

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,

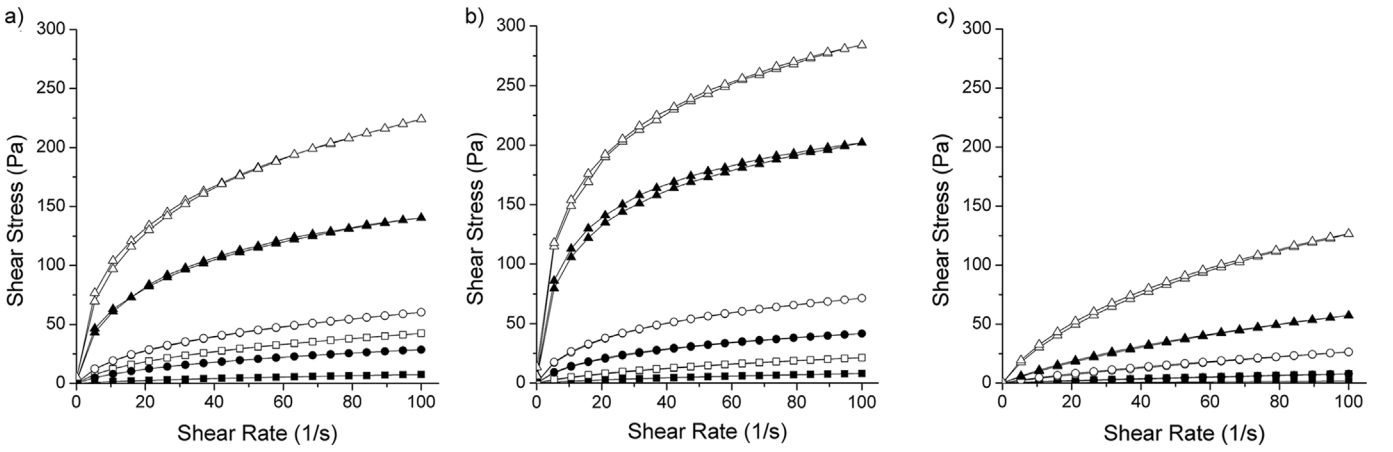

Fig. 3. Flow curves of CLNaHA (a), NaHA (b) and ZnHA (c) at: (◨) 0.5% (w/w), (◑) 1% (w/w) and (◭) 2% (w/w), with mucin (open symbols) or without mucin (solid symbols).

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.

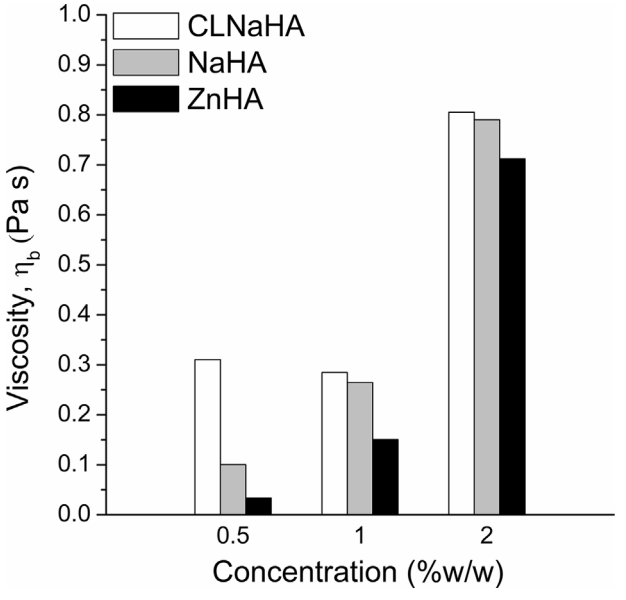

Fig. 4. Calculated synergism parameter values of viscosity at a shear rate of 100 s^{-1}.

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.

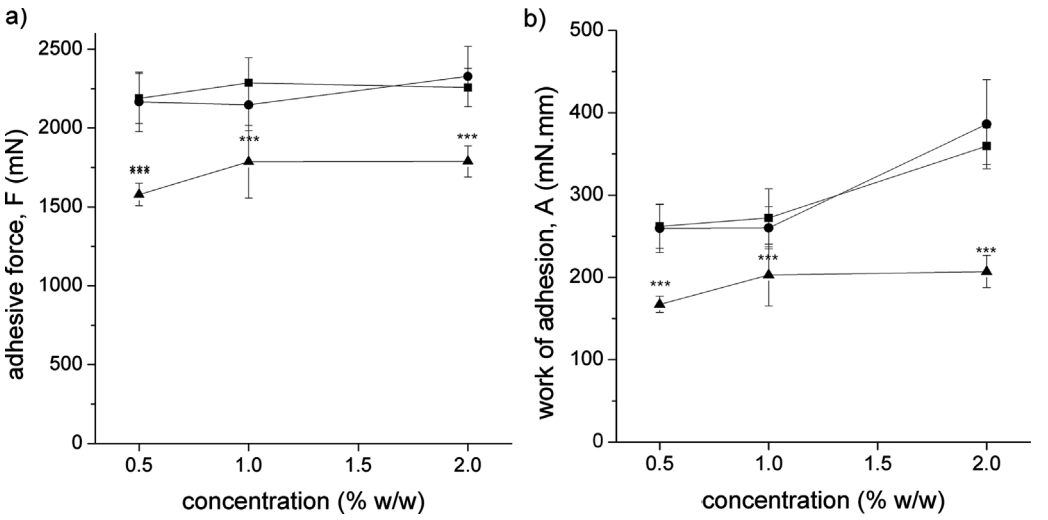

Fig. 5. Adhesive force (a) and work of adhesion (b) of (●) CLNaHA, (■) NaHA and (▲) ZnHA as a function of the concentration of the polymer in contact with mucin (***$p \leq 0.001$ highly significant compared with CLNaHA and NaHA).

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.

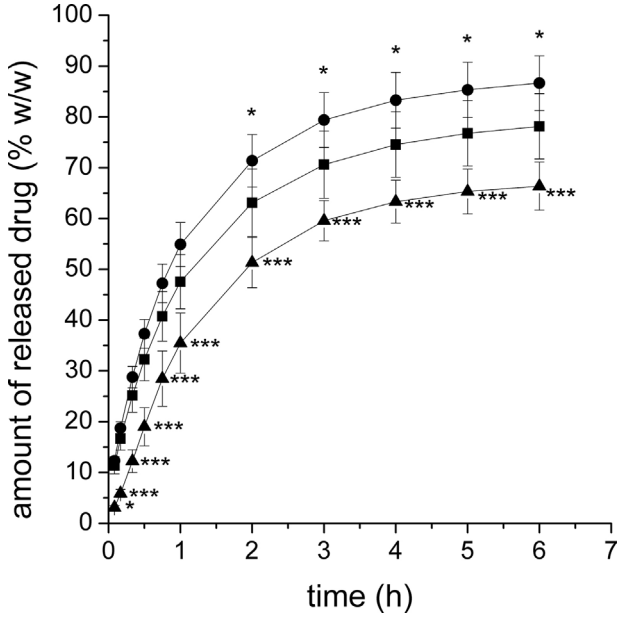

Fig. 7. Release of SD from (●) CLNaHA, (■) NaHA and (▲) ZnHA (*$p \leq 0.05$, significant compared with NaHA; and ***$p \leq 0.001$, highly significant compared with CLNaHA and NaHA).

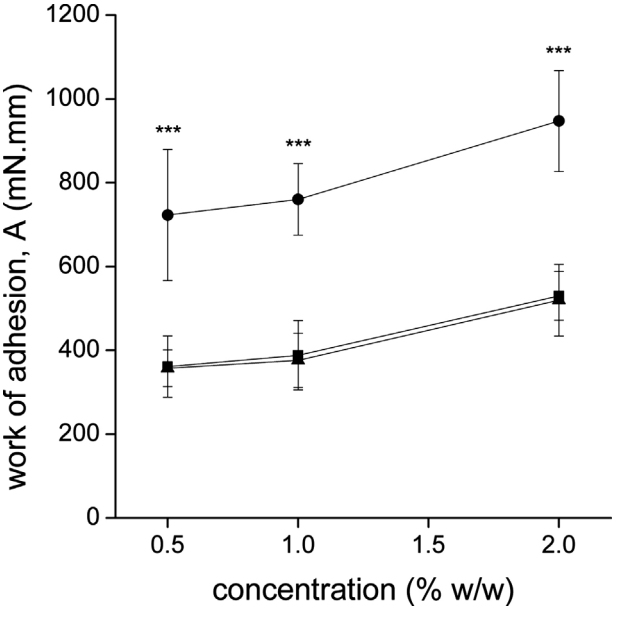

Fig. 6. Work of adhesion of (●) CLNaHA, (■) NaHA and (▲) ZnHA as a function of the concentration of the polymer in contact with excised porcine conjunctiva (***$p \leq 0.001$, highly significant compared with CLNaHA).

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.

Acknowledgements

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RESEARCH ARTICLE

In vitro testing of thiolated poly(aspartic acid) from ophthalmic formulation aspects

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ABSTRACT

Ocular drug delivery formulations must meet anatomical, biopharmaceutical, patient-driven and regulatory requirements. Mucoadhesive polymers can serve as a better alternative to currently available ophthalmic formulations by providing improved bioavailability. If all requirements are addressed, a polymeric formulation resembling the tear film of the eye might be the best solution. The optimum formulation must not have high osmotic activity, should provide appropriate surface tension, pH and refractive index, must be non-toxic and should be transparent and mucoadhesive. We would like to highlight the importance of *in vitro* polymer testing from a pharmaceutical aspect. We, therefore, carried out physical–chemical investigations to verify the suitability of certain systems for ophthalmic formulations. In this work, *in situ* gelling, mucoadhesive thiolated poly(aspartic acid)s were tested from ophthalmic formulation aspects. The results of preformulation measurements indicate that these polymers can be used as potential carriers in ophthalmic drug delivery.

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Introduction

The eye is a very complex organ with several barriers. The long-term treatment of certain eye diseases may cause adverse effects. Unfortunately, recently marketed ophthalmic formulations have very poor bioavailability[1]. Accordingly, there is an obvious need for more efficient formulations but a number of factors must be taken into consideration, such as anatomical and biopharmaceutical (API) aspects, patient-driven challenges and, not least, mandatory regulatory factors[2].

The anatomy of the eye poses considerable difficulties for ocular drug delivery. The most important anatomical barriers of the eye are the barriers responsible for drug removal from the ocular surface (blinking and the tear film) and the lacrimal fluid-eye barriers (the cornea and the conjunctiva)[3]. The volume of a dispensed eye drop is 5–6 times greater than the tear fluid volume on the ocular surface. During eye drop instillation, the fluid may flow out of the eye, followed by reflex blinking and a possible increase in tear secretion, especially if the eye drop contains an irritant[4,5]. Both the pH of the drug delivery system and the osmolality of the formulation must be similar to those of the natural tear film as otherwise the formulation can cause increased tearing and irritation, resulting in poor therapeutic efficiency[6]. The corneal surface and conjunctiva are covered by a mucin coat, secreted by the goblet cells of the conjunctiva, with the functions of hydration, cleaning, lubrication and defense against pathogens. The corneal epithelium contains five cell layers, which are very well sealed, because the cells are joined by tight junctions and gap junctions, and they provide resistance against both hydrophilic and lipophilic active ingredients[4]. Another possibility for drug removal is the absorption of the drug into the systemic circulation. All these processes result in low ocular bioavailability (< 5%)[3].

A strategy to increase the bioavailability of the API is to prolong the residence time on the ocular surface by the application of mucoadhesive formulations. The most recent ocular mucoadhesive drug delivery system developments can be divided into two main groups: the micro- or nano-sized drug deliveries[7–12] and the *in-situ* forming gels or films[13–16].

The biopharmaceutical-driven challenges involve the hydrophilicity or lipophilicity, the size and the charge of the API. Active agents with an amphiphilic character have the greatest chance of penetrating through the cornea and conjunctiva[17,18]. The molecular mass of the drug and its delivery system play an important role in the penetration[19–21]. The components of the tears (buffers and proteins) must be taken into consideration during formulation of a new ocular drug delivery system because they can bind to the active ingredient and change its ionization state[22]. All these physicochemical properties can affect the route and the rate of permeation in the cornea.

The needs of patients must be satisfied by novel formulations. The optimum drug delivery system must be effective, should require few applications per day and should be easy to handle and dispense; it must not cause local or systemic adverse events and only minimal or no visual interference, no ocular discomfort or foreign body sensation and no blockage of puncta or canaliculi; it must be as non-invasive as possible; and it must be inexpensive[2].

The regulation requirements of ophthalmic formulations have not been as well defined as concerns drug delivery development as in the case of solid oral dosage forms. The most important factors of the guidelines issued by the American Society of Hospital Pharmacists on pharmacy-prepared ophthalmic products are the tonicity, the pH, the buffering capacity, the inherent toxicity of the drug and the form, the need for a preservative, the solubility,

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the viscosity and the stability in an appropriate vehicle, and the packaging and storage of the finished product[23]. Although the test of these factors is not listed in the regulatory directives, the manufacturers must fulfill them and these preformulation measurements are included in the Drug Master File[24]. The European Commission has issued protocols for local toxicity and eye irritation measurements, in which the MTT assay for the rabbit corneal epithelial (RCE) cell line is included. This test provides essential information on the biocompatibility of the measured active ingredient or excipients at a cellular level[25].

In our work, we decided to perform physical–chemical investigations in order to verify the suitability of a system for ophthalmic formulations. We set out to prove that an *in situ* gelling mucoadhesive drug formulation, thiolated poly(aspartic acid) (ThioPASP), meets all of the important requirements of each of the aspects discussed above. Such formulations must spread on the corneal surface or in the cul-de-sac and provide a short gelation time, their pH must be ~7.4, they must be isotonic and non-toxic and they must not cause visual interference (appropriate refractive index and transmittance)[26].

PASP has been proven to be biocompatible and biodegradable, thanks to its protein-like structure. It is not toxic and does not generate immunogenicity, but the biocompatibility of ThioPASP as a novel derivative must be investigated carefully. ThioPASP is redox-sensitive and *in situ* gelling[16,27,28]. We determined the pH, osmolality, surface tension, refractive index, transmittance and toxicity of polymer solutions and the mucoadhesion and rheological parameters of the gels obtained from the polymer solutions.

Materials and methods

Materials

Previously synthetized ThioPASP (Mw 12–14 kDa, thiolated to an extent of 10% n/n) was characterized from ophthalmic biopharmaceutical aspects[16–28]. Polymer solutions were prepared with distilled water or phosphate buffer solution (pH 7.4). 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} $Na_2HPO_4.2H_2O$ and 0.12 g dm^{-3} KH_2PO_4 in distilled water, the pH being adjusted with 0.1 M HCl. Mucin (porcine gastric mucin type II) for mucoadhesion measurements, MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and dimethyl sulfoxide (DMSO) for cytotoxicity determination were purchased from Sigma-Aldrich (St. Louis, MO). 1 M $NaBrO_3$ solution was used as a model oxidant to facilitate the gelation of ThioPASP.

Methods

Osmolality, surface tension, refractive index and pH determination

Osmolality, surface tension and refractive index were measured in aqueous solutions of ThioPASP at five concentrations (1, 3, 5, 7 and 10% w/w).

Osmolality measurements were carried out with an automatic osmometer (Knauer Semi-micro Osmometer, Berlin, Germany) in three parallels. The osmolality of a solution was determined by measurement of the freezing point depression of the solution. One hundred and fifty microliters of the solution in the test tube was placed into the instrument. In the first segment of the measurement, the sample was overcooled, to a temperature lower than its freezing point. In the second segment, mixing was applied, which promoted crystallization of the sample. During the crystallization, the temperature automatically rose to the freezing point of the sample and remained at that temperature for a while. The osmolality (in mOsmol/l) of the sample was calculated from the freezing point depression.

Surface tension measurements were performed in 10 parallels with the OCA Contact Angle System (Dataphysics OCA 20, Dataphysics Inc., GmbH, Germany), using the pendant drop method. The Young–Laplace equation was used for the calculation of surface tension[29].

Refractive index was measured with an Abbe-type refractometer.

The *pH* of the ThioPASP solutions prepared with distilled water and PBS was determined with a pH-meter (Testo 206-pH2, UK).

Optical test

Optical tests were performed by the measurement of transmittance with a UV-spectrophotometer (Unicam Helios α Thermospectronic UV-spectrophotometer v4.55, UK) in the wavelength range 200–600 nm. Transmittance was determined in aqueous solutions of ThioPASP at five concentrations (1, 3, 5, 7 and 10% w/w).

Cytotoxicity measurement

Cytotoxicity measurements were performed by means of the MTT assay. In living cells, cellular reducing agents and dehydrogenase reduce the dye MTT (yellow) to its insoluble form, formazan (purple). RCE cells were seeded in 96-well plates with an area of 0.34 cm^2 at a density of 10^5 cells cm^{-2} and incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h. ThioPASP solutions were measured in concentrations of 5, 7 and 10% w/w. The samples were kept in contact with the cells for 3 h and then removed. The cells were subsequently treated with 50 µl MTT at 0.25 g ml^{-1} and 150 µl Hank's buffered salt solution (HBSS)(pH 7.4) and incubated for 3 h at 37 °C under 5% CO_2. At the end of this incubation time, the MTT was aspirated off and the cells were washed with HBSS, and 100 µl/well of DMSO was added in order to break the cell membrane to allow solubilization and the release of formazan crystals formed by the enzymatic conversion (mitochondrial dehydrogenase) of MTT. The absorbance of formazan solutions was assayed at 570 nm with an ELISA plate reader (Microplate Absorbance ReaderiMARKTM, Bio-Rad Laboratories Srl, Segrate, Italy), with the reference wavelength set at 690 nm after vigorous shaking for 60 s. Cell viability was calculated as the percentage 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[30–32].

Rheology

Rheological measurements were carried out with a Physica MCR101 rheometer (Anton Paar, Graz, Austria). The measuring device was of the cone and plate type (diameter 25 mm, gap height at the middle of the cone 0.046 mm and cone angle 1°). ThioPASP was dissolved in PBS and gelation was initiated by the addition of oxidizing agent (20% w/w) on the plate of the rheometer (final ThioPASP concentrations 5, 7 and 10% w/w). For the mucoadhesive investigation, the polymer solutions were mixed with mucin (final mucin concentration in the mixtures 5% w/w). The gelation of the ThioPASP polymer was followed at a constant angular frequency of 1.0 s^{-1} and at a constant strain of 1% at 25 °C. Measurements were carried out on freshly-made samples and were started immediately after the mixing of the compositions. The viscoelastic character was determined by frequency sweep tests after the total gelation, with a strain of 1% at 25 °C. Storage modulus (G') and loss modulus (G'')

were determined over the angular frequency range from 0.1 to 100 $1s^{-1}$. The strain value (1%) used in the measurements was in the range of the linear viscoelasticity of the gels[16]. In order to investigate the effect of blinking on the gel structure, accelerated blinking cycles were applied by using the automation function of the instrument. Tests were performed at 10% w/w ThioPASP. One blinking cycle comprises three sections: the strain section (with a strain of 2000%, a frequency of 0.50 Hz and a duration of 30 s) corresponding to blinking; followed by the rest section for 1 min and finally the measurement of G' and G'' at low strain value (a constant strain of 1% and a frequency of 1 Hz). The cycles were repeated until constant moduli values were attained; in our case, 13 loops were used.

Results and discussion

Osmolality, surface tension, refractive index and pH determination

In the event of ocular drug delivery formulation, it is necessary to take the physiological properties of the tear film into consideration, such as pH (7.4), osmolality (310–350 mOsmol kg^{-1}), surface tension (44 mN m^{-1}) and refractive index (male: 1.3368; female: 1.3371)[1,33].

During ocular drug delivery formulation, several excipients are used which can change the physical and chemical properties of the ocular surface and the stability of the tear film[34]. Surfactants are able to change the surface tension of the tear and the permeability of the epithelial membranes, resulting in increases in the solubility of the drugs and the drug uptake.

In a hyperosmotic tear environment, water flows out of the cells to balance the osmolality of the intracellular fluids and the surrounding tears. In this case, the cells in the ocular surface become dehydrated, damaging the cell membranes. Hypoosmolality is well tolerated by patients, but if it is very low it can cause irritation of the eye.

Eye drops of pH 6–9 do not cause discomfort, but outside this range an increased production of tear fluid due to irritation can be observed[35,36].

We determined the osmolality, surface tension and refractive index at five concentrations. The results are shown in Table 1.

The results revealed that the polymer has a very low osmotic activity. The increase of the polymer concentration resulted in an increase in osmolality, but this was not of great significance. These values are beneficial: after the osmolality of the eye drops has been set with an isotonizing agent, the ThioPASP will not result in a hyperosmotic solution (thanks to its low osmotic activity) that can cause cell damage.

The measured surface tension and refractive index values differ slightly from those of water (71.99 ± 0.05 mN m^{-1} and 1.3330, respectively)[37]. The increase of the polymer concentration did not influence the surface tension, but the refractive index increased to a small extent. Thus, ThioPASP solutions do not lower the surface tension of the tears, leading to irritation, and do not cause visual interference.

Table 1. Measured values of osmolality, surface tension and refractive index.

Concentration (% w/w)	Osmolality (mOsm/l) Mean ± SD	Surface tension (mN/m) Mean ± SD	Refractive index
1	4.3 ± 0.5	75.3 ± 0.3	1.3330
3	8.0 ± 0.0	75.4 ± 0.3	1.3330
5	11.0 ± 1.6	75.3 ± 0.2	1.3332
7	17.0 ± 2.2	75.4 ± 0.1	1.3339
10	19.3 ± 0.5	75.4 ± 0.2	1.3342

The pH of the ThioPASP solution prepared with distilled water or with PBS was 5.4 and 7.4, respectively. For eye drop formulation, therefore ThioPASP solution should be prepared in the buffer so as to meet the pH requirements necessary to avoid irritation.

The osmolality, surface tension, refractive index and pH measurement results indicate that ThioPASP may be a very promising eye drop formulation. Thanks to its inert properties, ThioPASP solution does not affect the tear stability and the ophthalmic requirements can be achieved through the addition of necessary excipients such as the isotonizing and surface tension modifying agents.

Optical test

Side effects influencing a patient's vision can reduce their willingness to take their medication. Thus, ocular drug delivery systems must not cause a feeling of sand in the eyes, dry eye or blurry vision[38,39]. The transmittance spectrum of ThioPASP was, therefore, measured in order to characterize the possible effects of the solution on the vision (Figure 1).

The spectral transmittance curve reveals that ThioPASP solutions are transparent in visible light, which means that they will not cause any visual disturbance. The increase of the polymer concentration resulted in a shift of the curves toward longer wavelength, but even at the highest concentration the polymer solution does not have any effect on vision.

Cytotoxicity

Ocular toxicity depends on the concentration of the material used, the frequency of application and the rate of removal[40]. Possible irritation of the eye can be determined very sensitively by means of the MTT assay[41].

Cytotoxicity measurements were performed with the MTT assay on the RCE cell line. Only the viable cells are able to reduce the dye MTT to formazan. Figure 2 shows the viability of cells after contact with ThioPASP solution samples relative to control cells.

The results demonstrate that ThioPASP solution is biocompatible because the cell viability was >90% after a contact time of 3 h in all cases. This is an extremely important finding, especially because RCE cells are very sensitive so that it can be predicted that ThioPASP solution will highly probably not have a toxic effect on the eye.

Rheology

In order to investigate the structure of the gels formed, rheological methods were used. As the systems gel *in situ*, the gelation time and the gel structure were first analyzed by means of oscillation tests. The systems were studied both in the absence and in the presence of mucin; the latter situation imitated the possible *in vivo* interaction of the polymer and the mucus layer of the eye.

Table 2 lists the gelation times with and without mucin. The gelation time can be taken as the time at which a maximum is observed in the curve of the differential with respect to time[16].

It is clear that, whereas no gelation occurred at 5% w/w polymer concentration, at higher concentrations the gelation was faster and a stronger gel structure was formed (higher G' values) in the presence of mucin (Table 2). The gelation time decreased on the increase of the polymer concentration. These results predict faster gelation during *in vivo* application in the eye, which is very beneficial, because the gelation time can be decreased and a stronger gel structure may evolve.

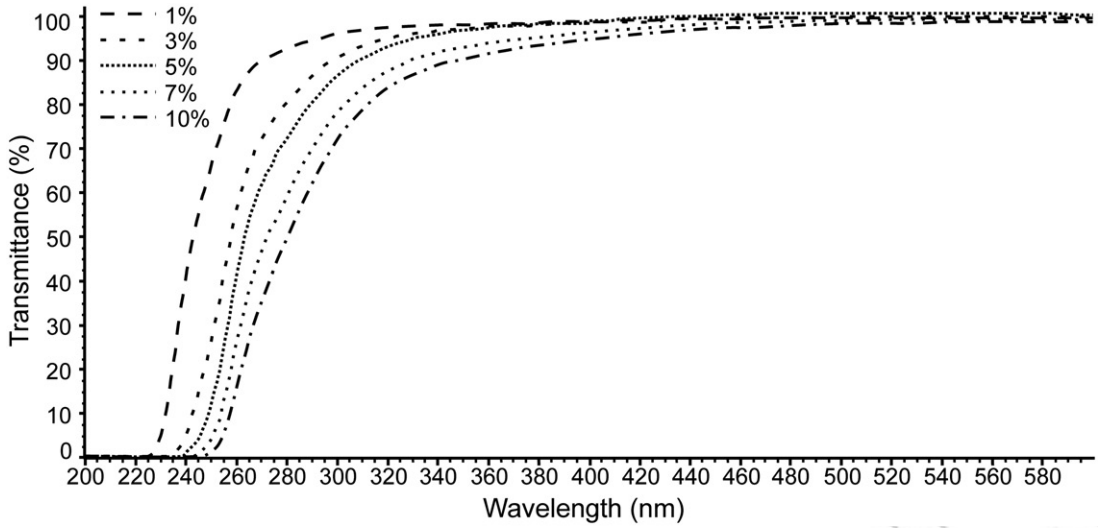

Figure 1. Spectral transmittance curve of ThioPASP solution at five concentrations.

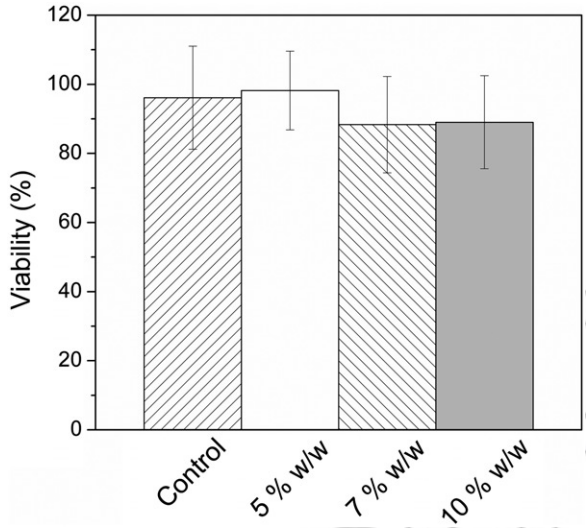

Figure 2. Cell viability after contact with ThioPASP solutions.

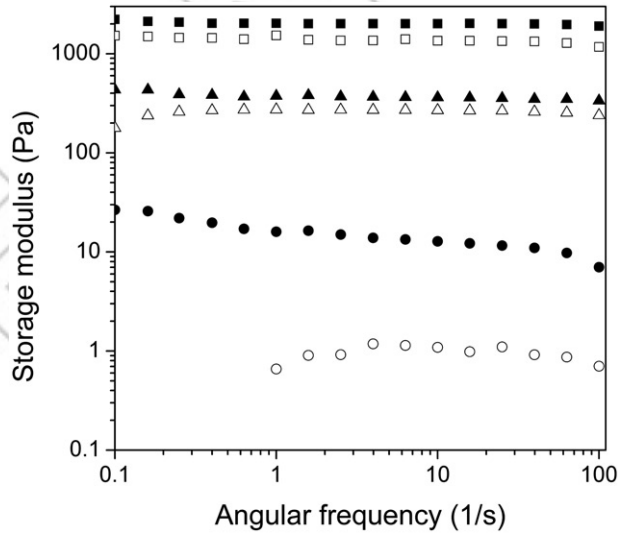

Figure 3. Frequency sweep test of 10% w/w (◨), 7% w/w (◭) and 5% w/w (◑) ThioPASP gels with (filled symbols) and without mucin (empty symbols).

Table 2. Gelation time determination.

	Gelation time (s)	
ThioPASP concentration (% w/w)	Without mucin	With mucin
5	n.g.	n.g.
7	780	600
10	360	300

n.g., no gelation.

The adhesion between the ocular formulation and the eye tissues is very beneficial, because such formulations provide better bioavailability; a lower API concentration and a lower administration frequency are sufficient for effective therapy because of the increased residence time[42–45].

The next important step was to perform the frequency sweep test on ThioPASP gels with and without mucin (Figure 3).

At higher polymer concentrations (7 and 10% w/w), *G′* proved to be independent of the frequency, which indicates strong chemical interactions between the polymer chains and a strong gel structure.

Figure 3 shows that mucin led to a higher *G′*, revealing the interaction between the mucin and the polymer. The most marked difference was at a polymer concentration of 5% w/w, while at 7 and 10% w/w ThioPASP stronger gel structure hindered interpenetration between the mucin and the polymer chains. The process of mucoadhesion involves interpenetration and chemical bond formation, leading to the increase of *G′*[16].

During the formulation of a mucoadhesive ocular drug delivery system, eye movements and blinking must be taken into consideration, because the gel on the surface of the eye is exposed to a continuous shear force, which may thin or dislodge the formulation[46]. As a result, the gel structure may be disrupted under this shear. The strength of the gel was investigated in cycling strain tests, simulating the real circumstances in the eye. One blinking cycle can correspond to 1 min of blinking.

Figure 4 depicts the changes in the structure during the blinking cycles. It can be observed that the moduli decreased only in the first two cycles and later became practically constant. The form applied

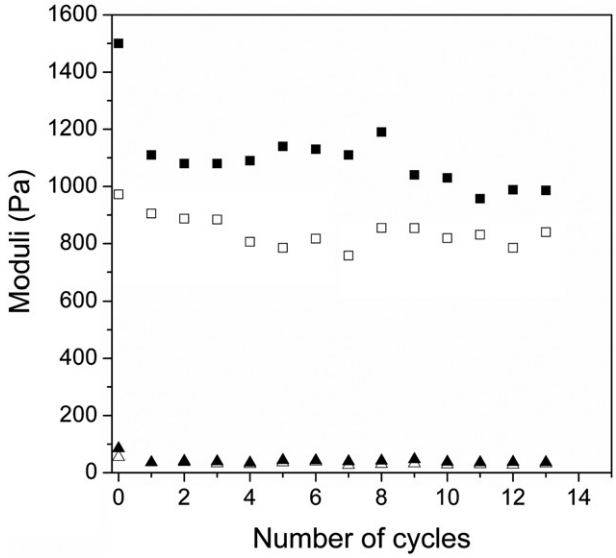

Figure 4. Modification of gel structure at 10% w/w ThioPASP during blinking cycles: storage modulus (◨) and loss modulus (◭) with (filled symbols) or without (empty symbols) mucin.

to the eye surface remained in a gel state during blinking, as indicated by the constant phase moduli. The large *G'* value and the difference between the moduli indicated the presence of the gel structure, which preserved its strength after several test cycles. There was no difference between the shapes of the curves in the cases of mucin-containing and mucin-free samples. The only difference was in the value of *G"*; the mucin-containing sample gave higher values, the mucoadhesivity being maintained under shear.

The rheological measurements showed that the ThioPASP gels retained their gel state during blinking, and also their mucoadhesivity. This advantageous property plays an important role in the increase of bioavailability by prolonging the residence time of the formulation on the ocular surface.

Conclusion

ThioPASP was characterized from ophthalmic formulation aspects. Preformulation measurements were performed (osmolality, surface tension, refractive index, transmittance and cytotoxicity) and on gels (rheological parameters, effects of blinking and mucoadhesion) in order to verify the suitability of these polymers for ophthalmic formulations. The results demonstrated that ThioPASP solutions are biocompatible and will not cause blurred vision. During eye drop formulation, ThioPASP facilitates adjustment of the optimum solution properties with other excipients (buffer, isotonizing agent, etc.), because it does not have a significant effect on the osmolality, refractive index, surface tension or pH. ThioPASP gels are resistant against blinking and, thanks to their mucoadhesive property, the bioavailability of the formulation can increase, leading to a decrease in administration frequency and, therefore, better patient compliance. Consequently, ThioPASP polymers are appropriate carriers in ophthalmic drug delivery formulations thanks to their biocompatibility, stable gel structure, inertness and mucoadhesive properties.

Declaration of interest

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