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The role of high molecular weight hyaluronic acid in mucoadhesion on an ocular surface model

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ABSTRACT

Hyaluronic acid (HA) is frequently formulated in eye drops to improve the stability of the tear film by hydration and lubrication. Mucoadhesion is related to the ocular residence time and therefore to the effectiveness of the eye drops. The ocular residence time of the HA formulation is correlated with the ability of HA to create specific strong interactions in the ocular surface with the mucus layer, mainly composed of a mixture of secreted mucins (MUC; gel forming MUC5AC and MUC2) and shed membrane-bound soluble mucins (MUC1, MUC4, and MUC16). Dry eye disease (DED) is a multifactorial pathology of the preocular tear film with possible damage to the ocular surface classified in two types: (1) aqueous-deficient dry eye and (2) evaporative dry eye, caused by a decrease in goblet cell density that reduces MUC expression and/or by meibomian gland dysfunction, that results in a drop in the lipidic fraction of the tear film.

In this work, the binding affinity between HA and MUC2 has been evaluated with three complementary approaches because the secreted MUCs play a pivotal role in the viscoelastic properties of the tear film: 1. Rheological analysis, measuring the mucoadhesive index and the complex viscosity in relation to MM (Molecular Mass) and concentration; 2. Fluorescence analysis, using a fluorescent hydrophobic probe, to investigate the conformational change of MUC2 during the interaction with the HA polymer; 3. Surface plasmon resonance analysis, used to measure the affinity between MUC2 (immobilized on the surface of a sensor chip) and the HA polymers that flowed on it at the molecular level.

For all these tests, the mucoadhesive performance of the natural HA linearly increases with the MM, whereas cross-linked HA and other emollient and gelling agents (formulated in artificial tears) do not show the same mucoadhesive properties (with the exception of xanthan gum). The mucoadhesive performance of high MM HA has also been confirmed in conditions that simulate the pathological condition of the tear film during DED by decreasing the MUC2 or oleic acid concentration. Physico-chemical analysis of a series of marketed artificial tears confirms the linear correlation between the MM of the HA used in the products and the mucoadhesive index measured on the ocular surface model.

1. Introduction

Hyaluronic acid (HA) is a ubiquitously expressed linear glycosaminoglycan composed of repeating units of D-glucuronic acid and N-acetylglucosamine, which plays the main structural role in the formation of extracellular matrix (ECM), a structural network that comprises the bulk of the tissues (Sainio and Järveläinen, 2020). The biological and biophysical features of HA are mostly determined by its molecular size (Garantziotis and Savani, 2019). Depending on their molecular mass (MM), HA fragments can enhance or attenuate HA receptor-mediated signaling pathways, especially those involving CD44 and RHAMM (Wolny et al., 2010). Indeed, unlike high MM HA, HA oligomers do not possess multivalent sites to bind CD44; consequently, they can act as antagonists, reducing the affinity between high MM HA and the receptor.

HA has a variety of applications in medicine, including viscosupplementation for osteoarthritis treatments, scaffolding for tissue engineering, wound healing, and ophthalmologic and cosmetic treatment

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(Fakhari and Berkland, 2013). However, to develop more effective HA formulations, we need to better understand the interaction between HA and the ECM macromolecular receptor in the specific microenvironment.

In the ophthalmic field, HA is exploited as a principal component in eye drops to improve the stability of the tear film by hydrating and lubricating. These HA solutions have a mean half-life on the ocular surface of 321 s (Snibson et al., 1992), significantly longer than other demulcents formulated in the artificial tears on the market (Kathuria et al., 2021) (*e.g.*, hydroxypropyl methylcellulose and polyvinyl alcohol: 44 s and 39 s, respectively). The ocular residence time of the HA formulation is correlated with the ability of HA to create specific strong interactions in the ocular surface with the mucus layer (Černohlávek et al., 2021; Menchicchi et al., 2015), mainly composed of mucin (MUC), immunoglobulins, urea, salts, glucose, leukocytes, cellular debris and enzymes (Davidson and Kuonen, 2004). MUC comprise a family of large, highly glycosylated, hydrophilic proteins; the mucin component in tears is a mixture of secreted (MUC5AC and MUC2, which play a pivotal role in the rheological properties of the tear film) and shed membrane-bound soluble mucins (MUC1, MUC4, and MUC16) (Baudouin et al., 2019). Recently it was reported in literature that tear secretory mucins and mucomimetic polymers like hyaluronic acid (Georgiev et al., 2019; Eftimov et al., 2021) may also contribute to the uniform spreading and structure of the tear film lipid layer probably via interactions with the polar lipid headgroups. Thus, secretory mucins and mucomimetic polymers may serve as a connection between the lipid and aqueous layer of the tear film.

Dry eye disease (DED) is a multifactorial pathology of the preocular tear film with possible damage to the ocular surface. Epidemiological studies carried out in the United States identified the condition in 5%–30% of the population, where gender, contact lenses, use of computers, thyroid abnormalities, hypertension, antidepressants, and antihistamines were identified to be the strongest and most common risk factors (Hasan ZA, 2022). DED is classified into two types: (1) aqueous-deficient dry eye and (2) evaporative dry eye (Craig et al., 2017), where the latter is caused by meibomian gland dysfunction (MGD). Aqueous-deficient dry eye is associated with alterations in the expression pattern and glycosylation degree of mucin components: a decrease in goblet cell density, which are epithelial cells responsible for mucin secretion, is accompanied by a decrease in the gel-forming mucin MUC5AC and MUC2 production; however, especially in those with mild to moderate dry eye, compensatory mechanisms of mucin production and glycosylation have been reported in patients. To confirm this evaluation, in a mouse DED model (Portal et al., 2019), the absence of MG induced an increase in MUC5AC and MUC5B expression, suggesting a different response of goblet cells.

HA in ophthalmic solution is widely used in the composition of artificial tears for DED treatment, and recently, the efficacy of high MM HA (in comparison to low MM HA) has been reported in an *in vivo* mouse model of dry eye stress (EDES) that mimics the office work environment (Kojima et al., 2020).

In this work, to better understand the mucoadhesive property of HA, the binding between HA and MUC2 was measured with three different approaches: 1. Rheological analysis, measuring the mucoadhesive index in relation to the MM; 2. Fluorescence analysis, using a fluorescent hydrophobic probe non-covalently linked to MUC2 to show the stability of interactions between mucin and polymer; 3. Surface plasmon resonance (SPR) analysis, where MUC2 was covalently immobilized on the surface of a sensor chip to demonstrate the dose-dependent interaction between mucin and polymer. The effect of linear HA at different MMs, cross-linked HA, and other demulcents and gelling agents, commonly employed in the production of artificial tears, have been analyzed. The mucoadhesive performance of HA was also evaluated by simulating the pathological condition of the tear film during DED in detail by decreasing the mucin (MUC2) or polar lipid fraction (oleic acid) concentration. Finally, a series of marketed artificial tears were analyzed and compared in terms of HA MM, rheological properties, and mucoadhesiveness.

2. Experimental

2.1. Materials and methods

Hyaluronic acid sodium salt (HA) at different MMs was provided by Fidia Farmaceutici S.p.A. (Abano Terme, Italy). HA cross-linked with 1,4-butanediol diglycidyl ether (BDDE) polymer (HBC) was synthesized from 700 kDa HA at a nominal BDDE derivatization degree of 10% (*vs.* HA r.u.) as previously reported (Guarise et al., 2021). At the end of the chemical reaction, the HBC polymer was purified by precipitation in EtOH, washed three times in an aqueous alcoholic solution (EtOH/H_2O 8:2), dried, rehydrated in PBS at 10 mg/mL, and sterilized by steam for 15 min at 121 °C. All the other reagents were supplied by Sigma and were used without further purification.

The marketed dry eyes were purchased from different suppliers: Thealoz Duo (Théa, Laboratories Théa, Clermont-Ferrand, France); Hyalistil synfo (SIFI, Catania, Italy), Visu XL (VISUfarma, Roma, Italy); Artelac Splash MDSC (Bausch & Lomb, Dr. Gerhard Mann, Berlin, Germany); Relys (Oftagest, SILDEHA Swiss SA, Paradiso, Switzerland); Optive fusion (Allergan, Allergan Pharmaceutical International Limited, Dublin, Ireland); Systane idra (Alcon, Alcon Laboratories Belgium, Puurs-Sint-Amands, Belgium); Blu yal A free, Trium free and Iridium A free (Fidia, Fidia Farmaceutici, Abano Terme (PD), Italy).

Type 2 mucin from porcine stomach (Merck; code M2378) was solubilized at 40 mg/mL in PBS pH 7 and centrifuged for 20 min at 4000 RPM; the supernatant was filtered (through a Gooch funnel Por. 4), transferred to a dialysis tube (Biotech CE Tubing, MWCO: 100 kDa, code 131414) and dialyzed against pure water (5 L) for 3 days at RT. Finally, the solution inside the dialysis tube was withdrawn, filtered with a nylon filter at 0.45 μm, and lyophilized (Freeze dryer: Martin-Christ Epsilon 2-6D LSC plus). The purified MUC2 lyophile, at the time of use, was dissolved at a concentration of 8% w/v in simulated Lachrymal fluid (SLF; aqueous solution composed of 1.8 g/L of KCl, 6.3 g/L of NaCl, 2.2 g/L of $NaHCO_3$, 44.4 mg/mL of $CaCl_2$ and $MgCl_2$ of 47.6 mg/mL at pH 7.4) (Ceulemans et al., 2002).

2.2. SEC-TDA analyses

The linear and cross-linked HA reconstituted samples as well as the eye drops products were diluted in PBS to the nominal final concentration in HA of 0.5 mg/mL. All samples were prefiltered with 0.2 μm nylon syringe filters and analyzed using an Omnisec Resolv and Reveal (Malvern) equipped with four detectors (RI, UV/VIS, LALS-RALS, and a differential viscometer). Two Viscogel GMPWxl columns were eluted at 40 °C with a buffer composed of 0.1 M $NaNO_3$ and 3 mM NaN_3 at a flow rate of 0.8 mL/min; an injection loop of 350 μL was used. All the acquired chromatograms were processed with OmniSec 11.35 (Malvern) software using a refractive index increment (dn/dc) of 0.155.

The GPC injection of the product: Optive fusion gives two peaks (due to the presence of 2 different polymers: HA and carboxymethylcellulose) to define and integrate only the signal related to HA, the same sample was also analyzed after treatment with 5 U/mL bovine testes hyaluronidase (Merck, H3506) and reinjected into SEC after deactivating the enzyme by heating the solution at 100 °C for 10 min (only the HA peak moved itself to a higher retention time).

The lyophile purified Type II mucin powder (from porcine stomach) (see paragraph 2.1) was solubilized in PBS at 0.5 mg/mL in the presence of D,L-dithiothreitol (0.2 mg/mL) to reduce the cystine bridge and analyzed by SEC-TDA Viscotek to measure the MM. The dn/dc of the purified MUC2 was experimentally measured (see S.I.) by injection of different concentrations of MUC2 solution (from 0.09 to 0.45 mg/mL) and calculated as 0.1195 (n=6; standard deviation= 0.0017).

2.3. Rheological analyses

The demulcents, humectants, and gelling agents were formulated in PBS pH 7 at the concentration commonly formulated in the artificial tears on the market. In detail, HA at MMs of 188, 606, 1317, and 1867 kDa and HBC were formulated at 2.8 mg/mL, PEG 400 (Merck; code 06855) (Springs, 2010), Guar Gum (Merck; code G4129 (Favuzza et al., 2020) and glycerol (Riedel de Haen; code 15524) were formulated at 4.0, 1.8, and 9.0 mg/mL, respectively; CMC (Microcrystalline cellulose Sodium carboxymethylcellulose; Biopolymer; code CL611FMC) and Xanthan gum (Merck; code G1253) were formulated at 5 and 8 mg/mL, respectively (Ceulemans et al., 2002), treated at 70 °C under stirring for 30 min and stored at 5 °C overnight before use. The eye drop products were used without any further treatment.

Before the rheological analysis, each solution was diluted 1/1 in MUC2 solution at 8% (or in SLF as control), vortexed, and sonicated for 5 min at RT (Ceulemans et al., 2002).

To measure the mucoadhesive index of HA formulation varying the HA concentration, linear HA solution at MM of 188 or 1867 kDa at 4 mg/mL was diluted at 3 mg/mL, 2 mg/mL and 1 mg/mL in PBS and each solution was diluted 1:1 with MUC2 solution at 8% (or in SLF as control), vortexed and sonicated for 5 min at RT.

To measure the mucoadhesive index of HA formulation varying the MUC2 concentration, MUC2 at 8% was diluted at 6%, 4%, 2%, and 1% in SLF, and each solution was diluted 1:1 with linear HA solution at MM of 188 or 1867 kDa (at 2.2 mg/mL in PBS), vortexed and sonicated for 5 min at RT.

To measure the mucoadhesive properties of the HA/MUC2 formulation in the presence of oleic acid at different concentrations, 20 mg of pure oleic acid was added to a test tube containing 2 mL of 8% MUC2 in SLF, and the solution was vortexed and sonicated for 15 min at 40 °C (the amount of oleic acid solubilized was experimentally quantified by GC analysis; see S.I.) and diluted in pure MUC 8% in SLF at the theoretical final concentration of oleic acid of 4, 3, 2 and 1 mg/mL. Each solution was diluted 1:1 with linear HA solution at MM of 1867 kDa or 188 kDa (at 2.2 mg/mL in PBS), vortexed, and sonicated for 5 min at RT.

Approximately 1 mL of each sample was analyzed by using an Anton Paar MCR92 Rheometer at 35 °C equipped with a stainless-steel blasted plate (Ø: 50 mm) and a cone plate (CP50-1). The G' (elastic modulus) and G" (viscous modulus) were measured (in Pa) from 0.07 to 30.0 rad/s at a fixed strain value of 10% (an initial sweep strain with an oscillatory shear strain of increasing amplitude at the constant frequency of $\omega = 1$ Hz was applied to determine the region of linear response of the sample: at 10%, the viscoelastic range is linear). The Dynamic viscosity was measured from 0.01 to 1000 s^{-1}. The complex viscosity is given by $|\eta^*| = [(G'/\omega)+(G''/\omega)]^{1/2}$, while the percentage of elasticity is given by % elasticity$= [G'/(G'+G'')] \times 100$. All the samples analyzed were processed with Anton Paar Rheo Compass 1.21 software, and the data collected were processed using Origin 8SR4 and Microsoft Excel. All samples were tested in three replicates. The mucoadhesive index is given by ($|\eta^*|$ of mixture: Sample + Mucin) - ($|\eta^*|$ of mixture: Sample + Buffer) - ($|\eta^*|$ of mixture: Buffer + Mucin), where $|\eta^*|$ was sampled at 1 Hz (expressed as the mean ± standard deviation).

2.4. Fluorescence analyses

Nile Red was dissolved in acetone to obtain a solution of 0.2 mg/mL. Each sample of linear HA (at different MMs) was dissolved in PBS at pH 7 at 10 mg/mL and diluted from 4.5 to 0.5 mg/mL. After the addition of MUC2 at a constant final concentration of 5 mg/mL, Nile Red solution (20 μL) was added to each solution (1 mL of HA/MUC2 sample) to a final concentration of 0.004 mg/mL. The solutions were sonicated for 20 min and then transferred into a 96-well plate. The total volume in each well was 100 μL, and the fluorescence emission (Ex/Em = 510/600 nm) was measured in duplicate (expressed as the mean ± standard deviation).

2.5. Surface plasmon resonance (SPR) binding affinity to MUC2

SPR measurements were carried out at 25 °C on a dual flow cell Biacore-X100 instrument (GE Healthcare). Mucin was immobilized (255 RU) on a carboxymethylated dextran chip (CM5) using amide coupling chemistry. Briefly, the chip was first activated by EDC (0.05 M)/NHS (0.2 M) followed by an injection of the protein (400 μg/mL) dissolved in 10 mM sodium acetate pH 4.0 (10 μL/min) using HBSEP + as the running buffer. After the immobilization of mucin, any unreacted NHS esters were deactivated by injecting an excess of ethanolamine hydrochloride pH 8.5 (1 M). Increasing concentrations of HA analogs (0-1 mg/mL) were injected over the MUC2-coated sensor chip at a flow rate of 10 μL/min, with a contact time of 180 s, in PBS. The regeneration step was performed using 0.5% SDS for 20 s. Each binding curve was subtracted from the corresponding baseline obtained on the reference flow cell.

3. Results and discussion

3.1. Evaluation of the mucoadhesive index by rheological analysis

Mucoadhesion is related to the ocular residence time and therefore to the effectiveness of eye drops (Salzillo et al., 2016). Mucin is a cysteine-rich protein with numerous O-linked glycans on threonine and serine hydroxyl groups. At physiological pH, the interaction between this glycoprotein and the polyanionic polymer may be electrostatic, involving patches of positively charged amino acids in the mucin protein backbone, but the hydrogen bonds between the heavily glycosylated region of the MUC and the hydroxyl groups of the HA backbone, in addition to hydrophobic and van der Waals bonds, can also be responsible for this interaction (Graça et al., 2018).

To measure the mucoadhesive index, several in vitro models have been proposed in the literature: the analysis of rheological synergism is probably the most common technique (Menchicchi et al., 2014; Černohlávek et al., 2021; Ceulemans et al., 2002). Gel-forming MUC2 has been used as a MUC model because it is expressed in tears, has already been used in ocular models (Ceulemans et al., 2002), and is commercially available. It has to be considered that the most prominent tear secretory mucin is MUC5AC and that the mucoadhesion is also determined by the interaction of polysaccharides with membrane-bound soluble mucins (MUC1, MUC4, and MUC16). All these mucins have somewhat different glycosylation profile (Baudouin et al., 2019) compared with MUC2, however only rarely the interaction between polysaccharides and mucin have been shown driven by specific linkages (Menchicchi et al., 2015). Partially purified Type II mucin powder (from porcine stomach) was further purified as described in 2.1. The MM of the purified MUC2 was estimated to be approximately 1256 kDa (MM/Mn=2.46), while the protein content in the lyophile (quantified by bicinchoninic acid assay) was approximately 3% w/w. To simulate the physiological condition in the precorneal tear film after the instillation of an eye drop, MUC2 was solubilized at 8% w/w in simulated lachrymal fluid (SLF), mixed 1:1 with the HA formulation (Ceulemans et al., 2002) and analyzed by rheometer at 35 °C. In the literature, the mucoadhesive effect on the rheology of samples is evaluated by different methods, measuring the intrinsic viscosity (using an Ostwald viscometer (Graça et al., 2018) or automated rolling ball microviscometer (Menchicchi et al., 2014)), the dynamic viscosity at 33.9 s^{-1}, to simulate physiological blinking (Černohlávek et al., 2021) and the elastic modulus at 1 rad/s (Ceulemans et al., 2002). After an initial assessment in rotational and oscillatory mode, we selected to sample the complex viscosity at 1 Hz: in this condition, the measure is sensitive and reproducible; at lower frequencies, physiological blinking is not simulated (Černohlávek et al., 2021), while at higher frequencies, the measure is less reproducible due to the slippage of the rheometer plates (see S.I. for details).

As reported in Fig. 1, the mucoadhesive index of linear HA at different MMs, cross-linked HA, and other demulcents and gelling agents solubilized in PBS, at the concentration commonly formulated in the

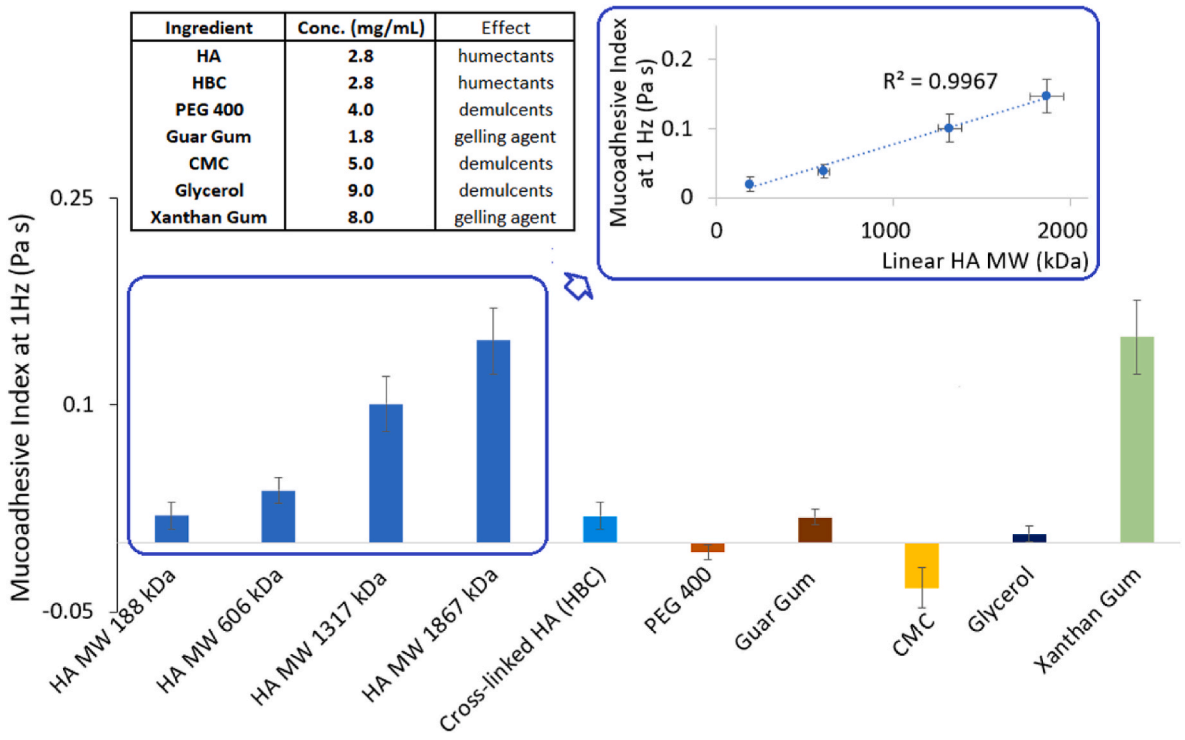

Fig. 1. (Above) Table with concentration and effect of tested ingredients for the rheological properties after dilution 1:1 with 8% MUC2. (Below) Mucoadhesive properties of a series of humectants, demulcents, and gelling agents at the concentration commonly formulated in the artificial tears on the market and linear correlation between linear HA MM (at 1.4 mg/mL) and the mucoadhesive index.

artificial tears on the market (Ceulemans et al., 2002; Springs, 2010; Favuzza et al., 2020), has been analyzed. It has been demonstrated that linear HA at different MMs has a higher mucoadhesive index than cross-linking HA (HBC) and other polymers, except for xanthan gum versus low MM HA, which, according to the literature (Ceulemans et al., 2002), confirms its mucoadhesivity. Fig. 1 also shows that there is a linear correlation between the HA MM and the mucoadhesive index. In Fig. 2a, the relationship between mucoadhesivity and HA concentration was investigated at fixed concentration of MUC2: the mucoadhesive index increases exponentially with the concentration of high molecular weight HA.

In Fig. 2b, the relationship between mucoadhesivity and mucin concentration was investigated at fixed HA concentration. With the High MM HA, the mucoadhesive index *vs.* mucin concentration fits with a sigmoidal correlation, while the same correlation is linear for the Low-MM HA. This means that also reducing the mucin concentration (*e.g.*, from 40 to 20 mg/mL) as often happens in a moderate DED (Baudouin et al., 2019), the presence of high MM HA in the artificial tear formulation guarantees high viscosity (see also S.I.) due to its mucoadhesive property, which is not the case when low MM HA is present. On the other hand, DED can also be associated with MGD that leads to altered tear film composition, ocular and eyelid discomfort, and evaporative dry eye (Chhadva et al., 2017). The bulk of the polar lipid fraction produced by the MG consists of 28 distinct species, where oleic acid (C18:1) represents the predominant fatty acid found in this class of lipids, followed by C16:1, C18:2 and a small amount of C18:0 (Lam et al., 2011). The non-polar lipid classes of cholesteryl ester, wax ester and triacylglyceride that comprise the bulk of human meibum lipids (Lam et al., 2011) were not considered because they are technically not compatible with the solution models used for the assessment of mucoadhesion.

In Fig. 2c, the effect of decreased fatty acid concentration on the mucoadhesive properties of high MM HA is shown, using oleic acid (OA) as a polar lipid model in the tear film. OA is not soluble in PBS; however, in the presence of MUC2, it can be solubilized at the concentration reported in the graph (see S.I.: GC analysis of OA in MUC2 solution). In summary, at lower OA concentrations (which can mimic the DED associated with MGD), the mucoadhesive effect of high MM HA is well maintained; in contrast, increasing the OA concentration decreases the mucoadhesive effect of HA following a sigmoidal fitting, probably because OA competes with HA for the same binding site in the hydrophobic core of MUC2.

3.2. Evaluation of the HA-Mucin interaction using a hydrophobic fluorescent probe

The study of the interaction between polyanionic polymers and mucin using fluorescence quenching has already been described in the literature, with a decrease in the fluorescence emission intensity of mucin (at 339 nm) following the addition of alginate polymers (Menchicchi et al., 2015). However, in a series of preliminary tests, this analysis showed poor sensitivity, probably due to the low protein content in the purified MUC2 sample. To overcome this problem, we performed the same experiment using the hydrophobic probe Nile Red. This solvatochromic fluorophore is nearly insoluble in water but is very soluble and shows a high fluorescence in non-polar organic solvents (Guarise et al., 2022). Consequently, the incorporation of the dye in the hydrophobic core of the amphiphilic MUC2 structure allowed us to observe conformational changes when MUC2 is perturbed by binding with the HA backbone.

Fig. 3 shows the decrease in the fluorescence increasing the HA concentration, maintaining fixed MUC2 and Nile Red amounts. This effect is linearly dependent on HA MM, meaning that by increasing the MM of HA, the interactions between MUC2 and the polymer become more stable, causing important conformational changes of the MUC2 core with the consequent release of the fluorescent probe in a hydrophilic environment. These results agree with the rheological analysis.

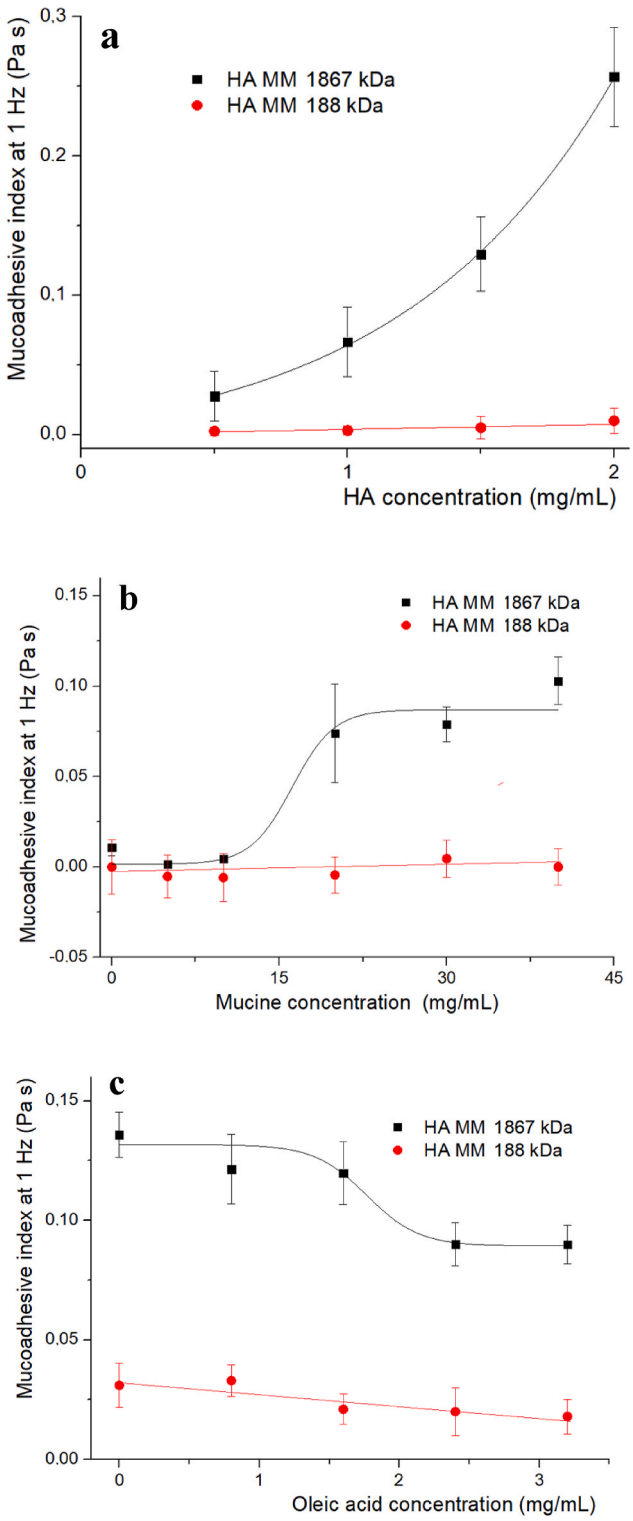

Fig. 2. (a): Correlation between HA concentration and mucoadhesive index in the presence of MUC2 at fixed concentrations of 4% in SLF/PBS (1/1). High MM HA was fitted with an Exponential plot (R^2=0.999). (b) Correlation between mucin concentration and mucoadhesive index in the presence of Low MM or High MM HA at 1.1 mg/mL in SLF/PBS (1/1). High MM HA was fitted with a Boltzmann plot (R^2=0.891). (c) Correlation between the mucoadhesive index and oleic acid concentration in the presence of Low MM or High MM HA at 1.1 mg/mL and MUC2 at fixed concentrations of 4%, in SLF/PBS (1/1). High MM HA was fitted with a Boltzmann plot (R^2=0.934).

3.3. Evaluation of the affinity of HA derivatives for MUC2 by SPR analysis

The binding specificity of linear HA at low or high MM and cross-linked HA to the MUC2 glycoprotein was evaluated using SPR. In this study, solutions of HA at 188 and 1867 kDa or BDDE cross-linked HA (derivatization degree =5% mol/mol *vs.* HA repeat unit) were flowed for predetermined times and at different concentrations over carboxymethylated chips functionalized with MUC2 glycoprotein to evaluate their binding capacity. High MM HA (1867 kDa HA) showed a very moderate positive dose-dependent SPR signal, indicating a direct interaction between HA and MUC2 (Fig. 4). However, the modest meaningful signal observed does not allow us to estimate a reliable affinity constant.

As expected, the SPR data (Fig. 4d) showed no interaction with MUC2 for low MM HA (188 kDa HA) and cross-linked HA (HBC), under the same experimental conditions. These data showed the dose-dependent interaction of linear high MM HA with MUC2, confirming the mucoadhesive property of high MM HA in contrast with low MM HA and crosslinked HA (HBC).

3.4. Correlation between HA MM and mucoadhesive index: assessment on marketed DED treatment

A series of DED treatments on the market, containing HA as the primary component, have been analyzed to investigate mucoadhesive performance. For each marketed eye drop, the HA MM and concentration are reported, and the sample viscosity is investigated in terms of elasticity, zero shear viscosity, and complex viscosity.

The HA in these formulations was analyzed by SEC-TDA, and for all the tested items, the recovery in concentration was between 90 and 110% compared to the declared content (see S.I. for details). The depolymerization of HA during the shelf life of the product was also considered: to minimize this effect, all products were analyzed 14±3 months before the expiration date (see S.I. for details).

As shown in Fig. 5, a linear correlation has been reported between the MM of the HA formulated in the eye drops and the mucoadhesive index; the product Hyalistil Synfo has been excluded from this correlation because it contains xanthan gum, a well-known mucoadhesive agent (Ceulemans et al., 2002). No linear correlation was shown between the mucoadhesive index and HA concentration, sample elasticity, or viscosity (see S.I. for details). These results agree with the previous evaluation with HA humectants, demulcents, and gelling agents alone, confirming the mucoadhesive property of high-MM HA.

4. Conclusion

Hyaluronic acid (HA) is frequently formulated in eye drops to improve the stability of the tear film by hydration and lubrication. Mucoadhesion is related to the ocular residence time and therefore to the effectiveness of the eye drops.

In this work, the mucoadhesive performance of linear, natural HA was robustly correlated with the MM and exponentially related to HA concentration. The binding affinity between HA and MUC2 has been characterized using three different approaches: rheological analysis, fluorescence analysis, and surface plasmon resonance (SPR) analysis. The results confirm a linear correlation between the MM of HA and the binding affinity with MUC2, while HBC and other emollient and gelling agents commonly formulated in artificial tears do not show the same mucoadhesive properties, with the exception of xanthan gum. The mucoadhesive performance of the high MM HA was also confirmed after simulating the pathological condition of the tear film during DED by decreasing the MUC2 or oleic acid concentration. The physical-chemical analysis of a series of marketed artificial tears on the market showed, as expected, a linear correlation between the MM of the HA formulated and the mucoadhesiveness.

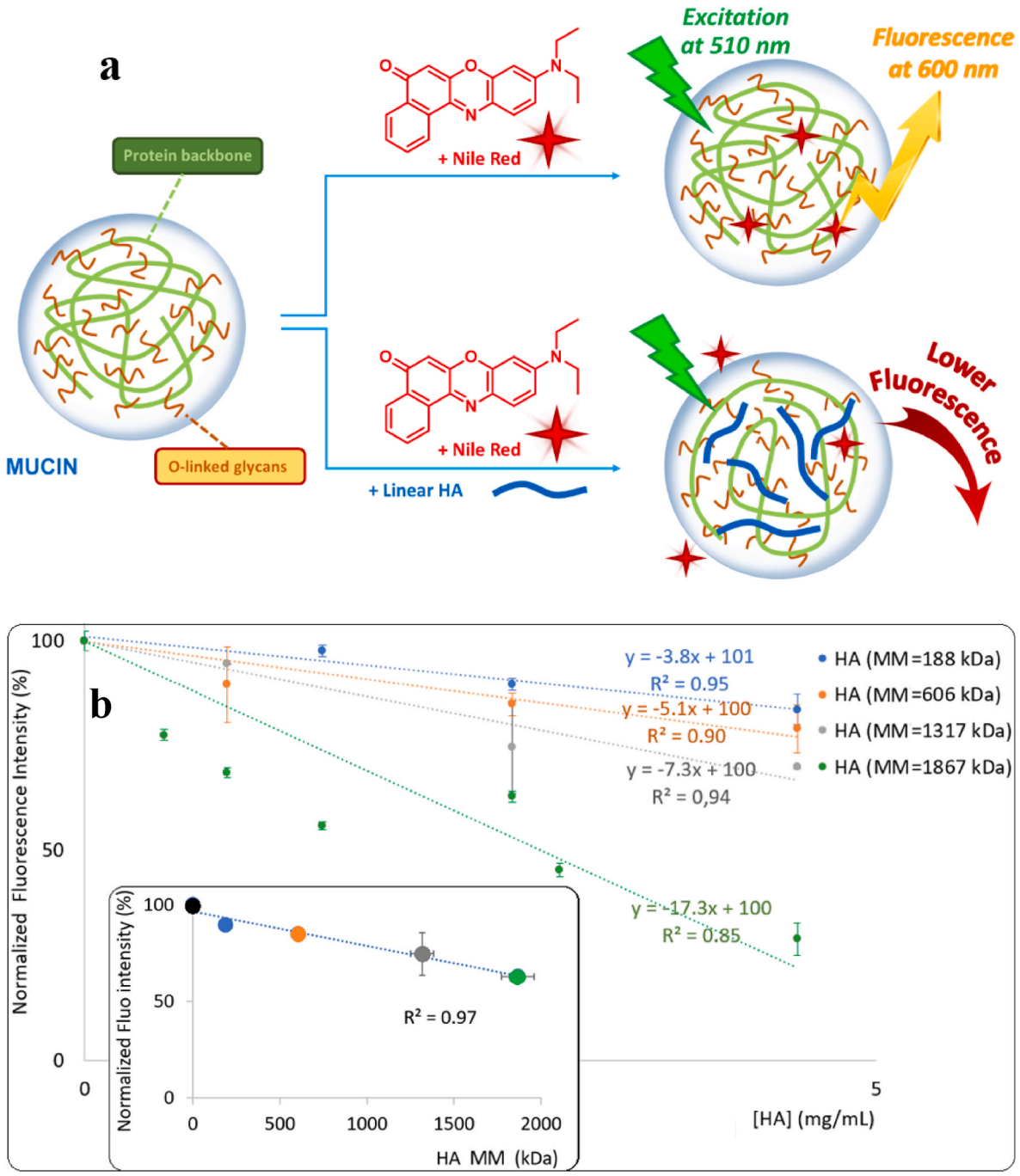

Fig. 3. (a) Schematic representation of the interaction between MUC2 and the dye with and without linear HA. (b) Correlation between HA concentration at different MMs and Nile Red fluorescence intensity, maintaining constant concentrations of MUC2 and Nile Red at 5 mg/mL and 0.004 mg/mL, respectively. The graph in the small box shows the linear correlation between HA MM and Nile Red fluorescence at a constant HA concentration (2.7 mg/mL).

CRediT authorship contribution statement

Cristian Guarise: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Laura Acquasaliente:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Gianfranco Pasut:** Writing – review & editing, Validation, Conceptualization. **Mauro Pavan:** Writing – review & editing, Writing – original draft, Conceptualization. **Matteo Soato:** Writing – review & editing, Formal analysis. **Giacomo Garofolin:** Writing – review & editing, Formal analysis. **Riccardo Beninatto:** Writing – review & editing. **Elena Giacomel:** Formal analysis. **Eleonora Sartori:** Writing – review & editing, Conceptualization. **Devis Galesso:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:At the time of the study: Cristian Guarise, Laura Acquasaliente, Gianfranco Pasut, Mauro Pavan, Matteo Soato, Giacomo Garofolin, Riccardo Beninatto, Elena Giacomel, Eleonora Sartori and Devis Galesso were full-time employees of Fidia Farmaceutici SpA. Elena Giacomel was in a

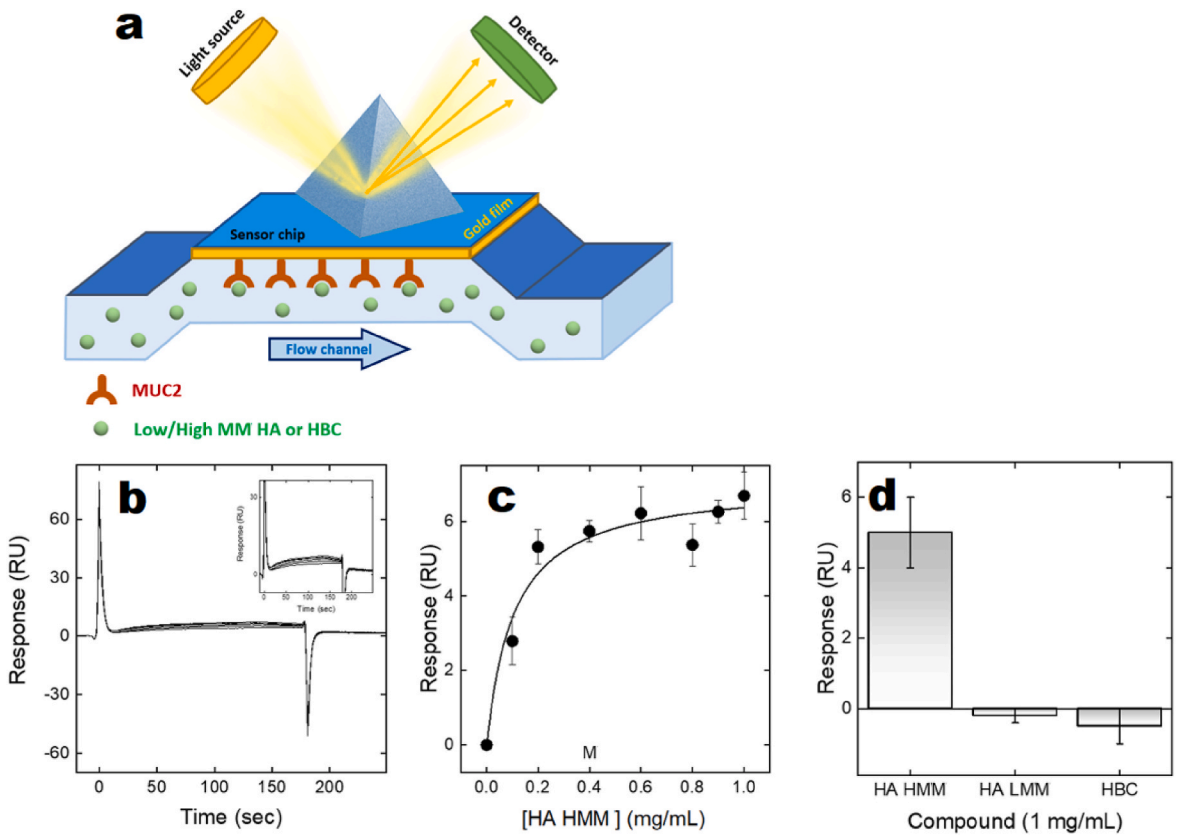

Fig. 4. Surface plasmon resonance (SPR) analysis of the HA derivative-MUC2 interaction. (a) Increasing concentrations of HA HMM were sequentially injected over the sensor chip at a flow rate of 10 μL/min at 25 °C using PBS as a running buffer. Each SPR trace was subtracted for nonspecific binding (*i.e.*, 2% RUmax). (b) The response units (RU) at the steady state plotted as a function of [HMM HA] indicate a dose-dependent interaction. (c) Comparison of the response units (RU) measured for the binding of HA derivatives (1 mg/mL) to immobilized MUC2. The data are the average of three independent measurements (n = 3), with error bars as ± SD.

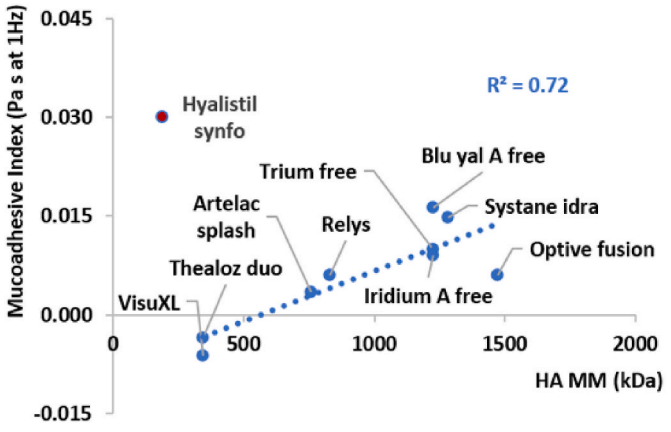

Fig. 5. (Above) Table reporting compositions and analysis performed on marketed eye drops. (Below) Linear correlation between the MM of the HA formulated in the dry eye and the mucoadhesive index. Note: Hyalistil Synfo has not been included in this correlation.

thesis internship at Fidia Farmaceutici SpA. The activities of Laura Acquasaliente and Gianfranco Pasut were paid by Fidia Farmaceutici SpA.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmbbm.2023.105908.

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