



Optimization of hyaluronan-based eye drop formulations



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ABSTRACT

Hyaluronan (HA) is frequently incorporated in eye drops to extend the pre-corneal residence time, due to its viscosifying and mucoadhesive properties. Hydrodynamic and rheological evaluations of commercial products are first accomplished revealing molecular weights varying from about 360 to about 1200 kDa and viscosity values in the range 3.7–24.2 mPa s. The latter suggest that most products could be optimized towards resistance to drainage from the ocular surface. Then, a study aiming to maximize the viscosity and mucoadhesiveness of HA-based preparations is performed. The effect of polymer chain length and concentration is investigated. For the whole range of molecular weights encountered in commercial products, the concentration maximizing performance is identified. Such concentration varies from 0.3 (wt%) for a 1100 kDa HA up to 1.0 (wt%) for a 250 kDa HA, which is 3-fold higher than the highest concentration on the market. The viscosity and mucoadhesion profiles of optimized formulations are superior than commercial products, especially under conditions simulating *in vivo* blinking. Thus longer retention on the corneal epithelium can be predicted. An enhanced capacity to protect corneal porcine epithelial cells from dehydration is also demonstrated *in vitro*. Overall, the results predict formulations with improved efficacy.

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1. Introduction

Topical applications currently represent the main route of administration of drugs used to treat many eye disorders, including dry eye, conjunctivitis, post-operative inflammation, etc. and eye drops are the formulation of choice for the delivery (Almeida, Amaral, Lobão, & Lobo, 2013; Davies, 2000; Di Colo, Zambito, Zaino, & Sansò, 2009; Van Santvliet & Ludwig, 2004). One of the main challenges associated with the use of conventional topical ophthalmic formulations is the short retention time of the components on the ocular surface. After instillation, there is drainage of the exogenous substances, mainly due to blinking and lachrymation that lowers the efficacy. Frequent instillations would be necessary to maintain the drug concentration in the tear film at a pharmacological level; although, this would worsen patient compliance and lead to ocular and systemic side effects (Almeida et al., 2013; Davies, 2000; Davies, Farr, Hadgraft, & Kellaway, 1991; Di Colo et al., 2009; Ludwig, 2005;

McKenzie & Kay, 2015; Séchoy et al., 2000; Snibson et al., 1990; Van Santvliet & Ludwig, 2004). Introduction of mucoadhesive polymers is one of the most used strategies to prolong the contact time of the preparation with the corneal/conjunctival epithelium (Davies et al., 1991; Davies, 2000; Di Colo et al., 2009; Ludwig, 2005; Séchoy et al., 2000; Snibson et al., 1990). The incorporation of macromolecules increases the formulation viscosity; therefore, the drainage rate from the pre-corneal area is reduced. Moreover, mucoadhesive macromolecules are able to intimately interact with the mucin layer, covering the corneal and conjunctival surfaces of the eye. This adhesive capacity further prolongs precorneal retention, improving the ocular bioavailability of the active agent (Davies et al., 1991; Davies, 2000; Di Colo et al., 2009; Ludwig, 2005; Séchoy et al., 2000; Snibson et al., 1990).

Both the mucoadhesiveness and viscosity of the preparations are mainly dependent on polymer molecular weight and concentration; therefore, these parameters have to be adjusted for optimal performance (Di Colo et al., 2009). When tuning the formulation, limits concerning viscosity must be considered. It has been reported that final viscosity should not exceed 30 mPas; otherwise, discomfort due to blurred vision and foreign body sensation occurs, resulting in a faster elimination due to reflex tears and blinks (Oechsner & Keipert, 1999; Pires et al., 2013). Thus, an ideal

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preparation should have the maximal contrast to drainage without excessively increasing viscosity.

Hyaluronic acid sodium salt (hyaluronan, HA) is commonly used as a bioavailability-enhancer in eye drops (Ludwig, 2005; Liao, Jones, Forbes, Martin, & Brown, 2005; Tong, Petznik, Yee, & Tan, 2012; Zambito & Di Colo, 2011). In the presence of HA, the precorneal residence times of pilocarpine, timolol, aceclidine, tropicamide, arecoline, gentamicin, and tobramycin were prolonged (Bernatchez, Tabatabay, & Gurny, 1993; Liao et al., 2005). In addition to its viscosifying and mucoadhesive properties, HA has other beneficial effects on the corneal epithelium, including: 1) protection against dehydration, 2) reduction of healing time, 3) reduction of the inflammatory response caused by dehydration, and 4) lubrication of the ocular surface (Aragona, Di Stefano, Ferreri, Spinella, & Stilo, 2002; Di Colo et al., 2009; Guillaumie et al., 2010; Ludwig, 2005; Tong et al., 2012; Zambito & Di Colo, 2011; Zheng, Goto, Shiraishi, & Ohashi, 2013;). Due to this clinical efficacy, HA is largely used in ophthalmology not only as an excipient but also as the main component of the artificial tear substitutes commonly prescribed for the treatment of dry eye disease (Aragona et al., 2002; Johnson, Murphy, & Boulton, 2006; Ludwig, 2005; McDonald, Kaye, Figueiredo, Macintosh, & Lockett, 2002; Snibson et al., 1990; Zheng et al., 2013). High-performing HA-based eye drop formulations are of great clinical interest.

There are limited scientific data on HA-containing products for topical ophthalmic use. These include the HA concentration range generally used (0.1–0.4 wt%; the HA concentration is also indicated in the package inserts of the commercialized products) and the biopolymer weight average molecular weight (M_w), which varied from 155 to 1400 kDa in 11 commercial products (Guillaumie et al., 2010; Johnson et al., 2006; Liu, Harmon, Maziarz, Rah, & Merchea, 2014; McDonald et al., 2002). No viscosity or mucoadhesiveness data are reported. To address this, we performed hydrodynamic and rheological characterizations on six additional products in this study and found most available formulations do not exhibit optimal viscosity. Therefore, we aimed to determine novel, optimized formulations by varying the HA M_w and concentrations (considering the range of molecular weights commercially used) to maximize the mucoadhesiveness and viscosity while maintaining the latter within suitable limits. Such formulations are expected to exhibit the maximum practical retention on the corneal epithelium *in vivo*. We determined the viscosity and mucoadhesion profiles of selected preparations and their capacity to protect the corneal epithelium against dehydration *in vitro* using porcine corneal epithelial cells. The preparations were also compared with commercial products.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt, lot. N. 02622 (HA1100) and hyaluronic acid sodium salt, lot. N. 11004 (HA250) were kindly provided by Altergon srl (Italy). Hyaluronic acid sodium salt (HA800 and HA500) were produced as described below. Six commercial HA-based formulations indicated for the treatment of dry eye syndrome were evaluated in this work: *Bluyal* (SOOFT italia S.p.A., Fermo, Italy, multi-dose bottle, 8 mL, HA 0.15%), *Blugel* (SOOFT italia S.p.A. Fermo, Italy, multi-dose bottle, 8 mL, HA 0.30%), *Hyabak* (Laboratorios Thea, Barcelona, Spain, multi-dose bottle, 10 mL; HA 0.15%), *Artelac Splash* MDSC (Fabrik GmbH, Berlin, Germany multi-dose bottle, 10 mL HA 0.24%), *Hyalistil Bio* (S.I.F.I S.p.A., Catania, Italy, multi-dose bottle, 10 mL, 0.2%), and *Octilia Natural* (C.O.C. Farmaceutici S.r.l., Bologna, Italy, 10 single-dose vials x 0.5 mL). Mucin (from porcine stomach type II, cat. N. M2378), Na_3PO_4 (cat. N. 342483), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, cat. N. 71505, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (cat. N.

71643), EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate, cat. N. E5134), and sodium hydroxymethylglycinate (Cat. N. CDS003712) were all purchased from Sigma-Aldrich (Milan, Italy). Dulbecco's Phosphate Buffered Saline (PBS) without calcium and magnesium was purchased from Lonza Sales Ltd, (Switzerland, cat. N. BE17-516F).

2.2. HA800 and HA500 preparation

HA800 and HA500 samples were prepared by hydrolysing a HA powder (lot. N. 08748 $M_w = 1584 \pm 100$ kDa; $M_w/M_n = 1.70$) under heterogeneous acid conditions, as reported elsewhere with slight modifications (D'Agostino et al., unpublished). In brief, a certain amount of the HA powder was dispersed in ethanol (93% v/v) (ethanol/HA 10 mL/g). The dispersion was pre-warmed at 65 °C and HCl (37 wt%) was added under vigorous stirring, resulting in a 0.2 M HCl final concentration. The hydrolysis was carried out for 50 min to obtain HA800 and for 110 min to obtain HA500. Reactions were stopped by adding Na_3PO_4 (0.35 M) until neutralized, while cooling in an ice/water bath. Products were purified by washing in ethanol/water (8/2 v/v) to remove phosphate salts. Purification was monitored using conductivity measurements: a conductivity in the range of 30–40 $\mu\text{S}/\text{cm}$ was the target value. Samples were then treated with pure ethanol and dried under vacuum at 40 °C. The resulting sodium hyaluronate powders will be referred to as HA800 and HA500.

2.3. Hydrodynamic characterization of HA using a SEC-TDA system (Viscotek)

The HA samples and commercial products were characterized using SEC-TDA (Size Exclusion Chromatography-Triple Detector Array) equipment by Viscotek (Lab Service Analytica, Italy). A detailed description of the system and its analytical conditions were reported elsewhere (La Gatta, Schiraldi, Papa, & De Rosa, 2011; La Gatta, De Rosa, Marzaioli, Busico, & Schiraldi, 2010). The molecular weight (M_w , M_n , M_w/M_n), molecular size (hydrodynamic radius- R_h), and intrinsic viscosity ($[\eta]$) distributions of samples were derived. Each sample was analyzed in triplicate; results were reported as means \pm SD. The Mark-Houwink-Sakurada (MHS) curves ($\log [\eta]$ vs $\log M_w$) were also directly obtained (La Gatta et al., 2010, 2011).

2.4. Rheological evaluation

Rheological measurements were carried out using a Physica MCR301 oscillatory rheometer (Anton Paar, Germany) equipped with a coaxial cylinders geometry (CC27-SN7969; measuring cup diameter/measuring bob diameter: 1.0847 according to ISO 3219; gap length 39.984 mm; sample volume 19.00 mL) and a Peltier temperature control.

2.4.1. Viscosity measurements

The HA1100, HA800, HA500, and HA250 powders were dissolved at different concentrations (in the range 0.15–1.5 wt%) in $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (2.2 g/L), $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (9.5 g/L), sodium hydroxymethylglycinate (0.04 g/L), EDTA (1.1055 g/L), and NaCl (4.3 g/L) in H_2O . This was the composition of the buffer (pH 7.4) in commercial products. The dynamic viscosity of the samples was registered as a function of shear rate ($1\text{--}1000 \text{ s}^{-1}$) at 35 °C, using 50 measuring points and no time setting. From each flow curve, the value of zero-shear viscosity (η_0 , viscosity in the range of Newtonian plateau) was obtained. Each solution was prepared in triplicate and each resulting sample was analyzed once, therefore, three flow curves were registered for each HA solution. The η_0 values reported were the mean values of the three measurements. For all solutions tested,

values of each measurement presented a maximal standard deviation from the mean value lower than 3%. For each HA sample, the dependence of η_0 (mean value) on concentration was derived.

Flow curves for commercial products were collected under the same conditions. For each determination, samples were taken from diverse bottles/vials of the same batch in order to reach the volume needed for the measurement (19 mL). Three flow curves of different samples from the same batch were registered for each product and the zero-shear viscosity values reported are the mean. For each formulation, the maximal standard deviation registered was less than 5%.

2.4.2. Mucoadhesion measurements

The mucoadhesiveness of the HA solutions was evaluated by means of viscosity measurements as previously described with modifications (Hassan & Gallo, 1990; Oechsner & Keipert, 1999; Uccello-Barretta et al., 2010). In particular, the following samples were prepared for each determination:

- 1) a suspension of mucin (10 wt%) in the buffer indicated in section 2.4.1;
- 2) a HA solution in the same buffer at a certain concentration; and
- 3) a suspension containing mucin (10 wt%) and the polymer under investigation at the same concentration as in the sample 2. A flow curve in the range of $1\text{--}1000\text{ s}^{-1}$ was registered at 35°C for each sample (50 measuring points, no time setting). At each value of shear rate, the mucoadhesiveness of sample 2 was expressed as:

$$\Delta(\%) = [\eta_{\text{muc+HA}} - (\eta_{\text{muc}} + \eta_{\text{HA}})] / (\eta_{\text{muc}} + \eta_{\text{HA}}) \times 100$$

where $\Delta(\%)$ is the mucoadhesion index, η_{muc} is the dynamic viscosity of sample 1, η_{HA} is the dynamic viscosity of sample 2, and $\eta_{\text{muc+HA}}$ is the dynamic viscosity of sample 3.

For a mucoadhesive polymer, $\eta_{\text{muc+HA}}$ is higher than $(\eta_{\text{muc}} + \eta_{\text{HA}})$ due to the interaction occurring between the polymer and mucin, and $\Delta(\%)$ is a measure of the mucoadhesion strength (Hassan & Gallo, 1990; Oechsner & Keipert, 1999; Uccello-Barretta et al., 2010).

Samples 1, 2, and 3 were prepared as follows. Sample 1): mucin was hydrated with sterile water to 15 wt% final concentration (10 h, 300 rpm, room temperature). The pH of the resulting suspension was 3.8–4.0. Then, Na_3PO_4 (0.35 M) was added to bring the pH to 7.0–7.6. Water was added to a 10 wt% final mucin concentration. Sample 2): HA was dissolved at the desired (wt%) concentration in a phosphate buffer with the same pH and salt concentration as sample 1. Sample 3): a mucin suspension, 15 wt%, was buffered using Na_3PO_4 (0.35 M). Then a small volume of a highly concentrated HA solution in pure water was added to a final HA concentration equal to that of sample 2. Water was added to the final volume. The final pH and conductivity values were in the range 7.0–7.6 and 12.0–14.0 mS/cm, respectively, for all samples. The pH and conductivity variations within these ranges did not affect viscosity.

For each HA solution, the protocol described above was performed in triplicate and the mucoadhesion index was reported as the mean value. The maximal standard deviation registered was less than 5%.

2.4.3. Oscillatory measurements

Oscillatory measurements were performed to ascertain the nature of the interaction of the formulations with mucin (Ceulemans & Ludwig, 2002). Dispersions containing mucin and the HA sample, at the selected concentration, prepared as described in the paragraph 2.4.2 (sample 3), were measured. The dynamic moduli of the mixtures were evaluated as functions of the oscillation

stress and frequency. In particular, strain sweep tests were performed at a constant oscillatory frequency of 0.1 s^{-1} over a strain amplitude range of 0.01–100%, with no time setting at 35°C . Oscillation frequency sweep tests were then carried out over a frequency range of $0.1\text{--}10\text{ s}^{-1}$ at a constant strain selected within the linear viscoelastic range (0.045%), with no time setting at 35°C .

Three different samples were analyzed for each dispersion; the resulting curves overlapped.

2.5. In vitro evaluation of corneal (epithelial cells) protection against dehydration

2.5.1. Cell culture and growth conditions

Primary porcine corneal epithelial cells (PCECs) were a gift from A.O.R.N. Antonio Cardarelli, Centre of Biotechnologies (Naples). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 15% (w/v) foetal bovine serum (FBS), 10 ng/ml human epidermal growth factor (EGF), and 40 $\mu\text{g/ml}$ gentamicin, in a humidified atmosphere of 5% CO_2 –95% air at 37°C (Zheng et al., 2013). All materials were purchased from Invitrogen (Milan, Italy) except for gentamicin, which was purchased from Fisiopharma S.r.l. (Salerno, Italy).

2.5.2. Evaluation of cell viability after dehydration

The protective effect of the selected formulas against dehydration was evaluated using previously reported protocols, with modifications (Hill-Bator, Misiuk-Hojlo, Marycz, & Grzesiak, 2014; Matsuo, 2001; Rangarajan, Kraybill, Ogundele, & Ketelson, 2015; Zheng et al., 2013). Specifically, cells were seeded in 24-multiwell plates (5×10^4 /well) and in DMEM containing 15% FBS until 70% confluence was reached. The medium was then replaced with selected HA formulations (HA1100-0.28%, HA500-0.67%, and HA250-1.03% (wt% solutions prepared in cell culture medium) and with the same solutions diluted 1:3, 1:10, and 1:30. For the positive and negative controls, the medium was replaced with fresh medium not containing HA. Cells were incubated under cell culture conditions for 2 h. Cells treated with the HA samples and not treated (negative control, NC) were then dehydrated: the medium was removed and the multiwells were incubated at 37°C without the lid until a stress response (morphological change) was evident in the NC (about 20 min). The positive control (CTR, not treated with HA), was not dehydrated (cells were kept in the presence of the medium during all experiments). Cell viability was then evaluated using the Presto Blue assay (Cat. N. A13261, Invitrogen, GIBCO) according to manufacturer's instructions. When added to cells, the cell-permeable PrestoBlue reagent, resazurin, is modified into resofurin by the reducing environment of viable cells. The conversion is proportional to metabolically active cells and was quantitatively determined by absorbance measurements. Cell viability (%) was calculated with respect to the positive control (100% viability). Each sample was tested in triplicate. Results were reported as means \pm SD. A Student *t*-test was used for statistical analysis and *p* values <0.05 were considered statistically significant differences.

3. Results and discussion

3.1. Hydrodynamic and rheological characterization of commercial formulations

In this study, we first evaluated commercially available HA-based eye-drops. The results of the hydrodynamic and rheological characterizations are reported in Table 1 and in Fig. 1. In particular, the sample molecular weight (M_w , M_n , M_w/M_n), molecular size (hydrodynamic radius- R_h), intrinsic viscosity ($[\eta]$), and the biopolymer concentration derived from SEC-TDA analyses, are shown in Table 1. The molecular weight of HA varied from ~ 360

Table 1
Hydrodynamic and rheological data for the commercial formulations. The values of weight average molar mass (M_w), numeric average molar mass (M_n), polydispersity index (M_w/M_n), intrinsic viscosity ($[\eta]$), hydrodynamic radius (R_h), and HA concentration (wt%) derived from the SEC-TDA analyses are reported. The values of zero-shear viscosity (η_0) derived from the flow curves (Fig. 1b) are also reported.

Sample	SEC-TDA analysis						Rheological analysis
	HA (wt%)	M_w (kDa)	M_n (kDa)	M_w/M_n	$[\eta]$ (dL/g)	R_h (nm)	η_0 (mPa s)
Bluyal	0.14 ± 0.01	1130 ± 30	690 ± 60	1.6 ± 0.1	18.6 ± 0.7	67 ± 2	6.7
Blugel	0.29 ± 0.01	1070 ± 10	640 ± 40	1.6 ± 0.1	17.7 ± 0.2	64 ± 1	24.2
Hyabak	0.15 ± 0.01	360 ± 20	230 ± 50	1.6 ± 0.2	8.8 ± 0.5	35 ± 3	2.3
Artelac Splash	0.23 ± 0.01	890 ± 20	570 ± 40	1.6 ± 0.1	16.3 ± 0.3	59 ± 2	9.8
Hyalistil Bio	0.21 ± 0.01	1050 ± 10	500 ± 40	2.1 ± 0.1	17.5 ± 0.6	63 ± 1	11.6
Ocilia Natural	0.10 ± 0.01	1170 ± 10	610 ± 60	1.9 ± 0.2	18.5 ± 0.4	67 ± 2	3.7

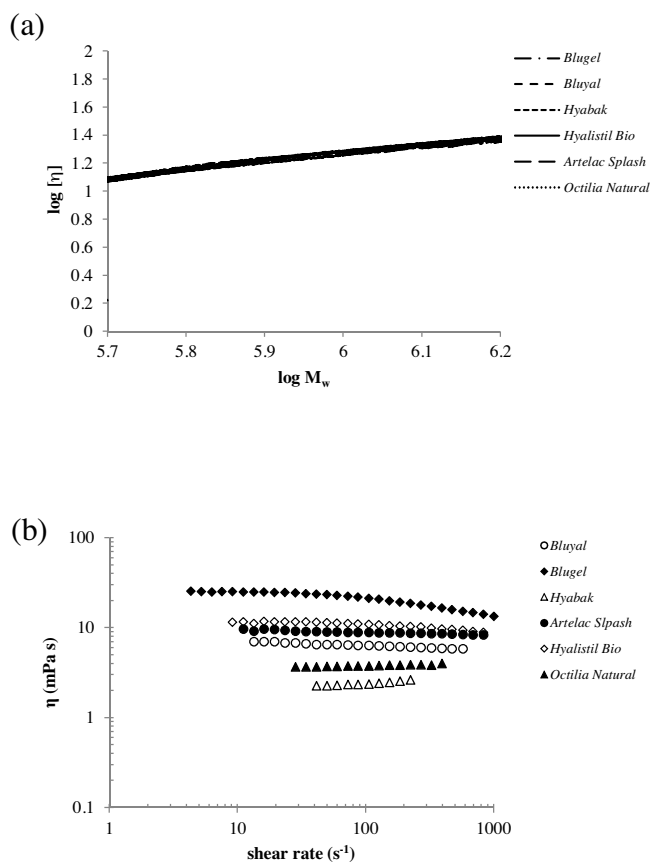


Fig. 1. Results of commercial HA-based eye drops characterization. (a) superimposition of the MHS curves derived from the SEC-TDA analyses; (b) flow curves (dynamic viscosity as a function of the shear rate) registered at 35 °C.

to ~1200 kDa. Most of the products (*Bluyal*, *Blugel*, *Hyabak*, *Artelac Splash*) had equivalent distribution widths (M_w/M_n 1.6), and *Hyalistil Bio* and *Ocilia Natural* showed higher polydispersity (M_w/M_n of 1.9 and 2.1, respectively). Intrinsic viscosity ($[\eta]$) values coherently decreased with the decrease in chain length. The same trend was found for the hydrodynamic radius. The values of HA concentration were consistent with the ones indicated in the packaging inserts. Conformational information could also be derived from the SEC-TDA analyses. Actually, as it is shown in Fig. 1a, the MHS curves ($\log [\eta]$ vs $\log M_w$) of the commercial samples evidently overlap. This means that chains from the diverse samples having the same molecular weight, also exhibit the same intrinsic viscosity, and therefore, the same hydrodynamic volume, the same arrangement (conformation) in solution (Harding, Rowe, & Horton, 1992; Hokputsa, Jumel, Alexander, & Harding, 2003; La Gatta et al., 2011; La Gatta et al., 2010). Finally, hydrodynamic analyses corroborated the limited data reported about the ranges of polymer molecular

Table 2
Results of SEC-TDA analyses of the HA samples used for the optimization study: values of weight average molar mass (M_w), numeric average molar mass (M_n), polydispersity index (M_w/M_n), intrinsic viscosity ($[\eta]$), hydrodynamic radius (R_h) are reported.

Sample	SEC-TDA analysis				
	M_w (kDa)	M_n (kDa)	M_w/M_n	$[\eta]$ (dL/g)	R_h (nm)
HA1100	1120 ± 100	730 ± 70	1.5 ± 0.1	18.7 ± 1.8	67 ± 7
HA800	800 ± 10	490 ± 9	1.6 ± 0.0	14.5 ± 0.3	54 ± 1
HA500	470 ± 20	240 ± 10	1.9 ± 0.1	9.8 ± 0.2	40 ± 1
HA250	250 ± 3	160 ± 6	1.6 ± 0.1	6.7 ± 0.1	29 ± 1

weights and concentrations generally encountered in the products on the market (Guillaumie et al., 2010; Johnson et al., 2006; Liu et al., 2014; McDonald et al., 2002).

The flow curves of the preparations (dynamic viscosity as a function of the shear rate) are reported in Fig. 1b and the zero-shear viscosity values, derived from these curves, are indicated in Table 1 (last column). The samples greatly differed in viscosity: η_0 in the range 3.7–24.2 mPa s. A crucial finding emerged. As expected, commercial combinations of molecular weights/concentrations resulted in proper viscosities that were within the limit reported for the specific use; however, the η_0 values of most formulations (Table 1) were much lower than the limit. This indicates these are not optimized products since the drainage can be improved. *Blugel* had the most optimized drainage based on its zero-shear viscosity (24.2 mPa s); therefore, this clinically accepted viscosity was chosen as the target value for the following optimization studies.

The rheological characterization highlighted that commercial preparations also differ for viscosity dependence on shear rate (Fig. 1b). Most of the products exhibited a viscosity almost constant with the shear rate predicting a same contrast to drainage at rest (low shear rates) and during blinking (high shear rates); while, a pseudoplastic behaviour was observed for the most viscous sample (*Blugel*) indicating a resistance to removal diminishing under blinking conditions.

3.2. Hydrodynamic characterization of the HA samples used in this work

The results of the hydrodynamic characterization of the HA samples used for the optimization study are reported in Table 2. The four samples significantly differed in molecular weight with M_w values equal to 1120 ± 100, 800 ± 10, 470 ± 20, and 250 ± 3 kDa for HA1100, HA800, HA500, and HA250, respectively. The HA1100, HA800, and HA250 samples had the narrowest distributions (M_w/M_n 1.5 and 1.6) while the polydispersity was slightly higher (M_w/M_n 1.9) for HA500. The intrinsic viscosity and hydrodynamic radius coherently varied with molecular weight and were consistent with literature data (La Gatta et al., 2013; La Gatta, Papa, Schiraldi, & De Rosa, 2016). The MHS curves of the linear HA samples overlap with the ones of commercial formulations (data not

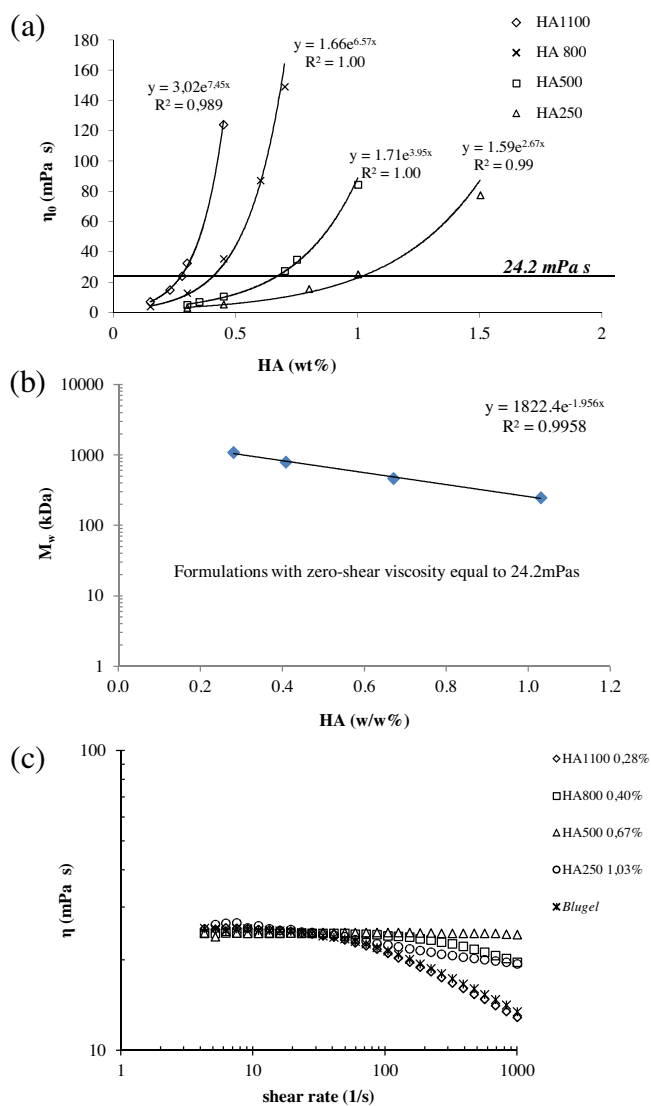


Fig. 2. (a) Zero-shear viscosity as a function of polymer concentration for diverse molecular weight samples; the target value of η_0 (zero-shear viscosity for *Blugel*) is evidenced; (b) relationship between M_w and concentration for formulations exhibiting ideal η_0 (24.2 mPa s); (c) flow curves for selected formulations and *Blugel*.

shown) indicating, as discussed above, equivalent conformation and, therefore, the presence of linear, naturally occurring HA in commercial products (Harding et al., 1992; Hokputsa et al., 2003; La Gatta et al., 2010; La Gatta et al., 2011). Overall, results in Table 2 indicate that the HA samples used cover the range of molecular weights found on the market. In addition, a comparison of the data in Table 2 and in Table 1 indicates similarities between HA1100 and *Bluyal*, *Blugel*, and *Octilia Natural*.

3.3. Viscosity measurements

Flow curves were determined for HA1100, HA800, HA500, and HA250 at several concentrations (data not shown). The dependence of the zero shear viscosity (η_0) on the biopolymer amount in solution was derived for each HA sample (Fig. 2a). As shown, regardless of chain length, viscosity exponentially increased with concentration; however, this dependence became more marked with the increase in molecular weight. This is consistent with the highly viscosifying properties of HA being mainly due to physical entanglements between polymer chains that are favoured by the presence of long chains and/or high concentrations.

The curves in Fig. 2a were used to derive, for each molecular weight, the biopolymer amount needed to have a η_0 value equal to *Blugel*, which was the most viscous among the commercial products analyzed. The amounts were: HA1100-0.28%, HA800-0.40%, HA500-0.67%, and HA250-1.03%. When HA1100 is dissolved, the desired viscosity is reached at a low HA content (0.28 wt%), similar to the commercial products. When molecular weight decreases, higher polymer amounts, up to about 3.3-fold more than found in *Blugel*, have to be considered to maintain optimal viscosity.

The relationship found between M_w (in the range 250–1100 kDa) and the concentration of the preparations exhibiting zero-shear viscosity equal to 24.2 mPa s is reported in Fig. 2b. This relationship is expected to be valuable for designing topical ophthalmic preparations containing HA. Actually, all the formulations identified by the curve in Fig. 2b can be considered “optimum” since they exhibit a viscosity very close to the maximum value for the intended application and already exploited clinically. Among the optimal formulations, HA1100-0.28%, HA800-0.40%, HA500-0.67%, and HA250-1.03%, which cover the whole range of molecular weights considered, were selected for further characterization.

The flow curves of the selected preparations and of the best performing on the market (*Blugel*) are reported in Fig. 2c to better compare viscosity dependence on the shear rate. As expected, the HA1100 formulation and *Blugel* behave similarly; therefore, they were considered equivalent. When considering the other formulations, it is evident that, although having the same (maximized) η_0 , they behave differently when the shear rate increases. In particular, the reduction in viscosity lessens with the decrease of molecular weight with HA250, which maintains a constant viscosity under the conditions applied. Consequently, when at high shear rate values, the molecular weight was inversely proportional to the viscosity of the formulation. Such differences in the capacity to maintain viscosity at high shear rate values, more closely simulating *in vivo* blinking conditions, are significant during application. The lower the molecular weight (the higher the concentration), the more likely the formulation will be retained *in vivo* during blinking. Additionally, when considering the similarities between HA1100-0.28% and *Blugel*, the curves in Fig. 2c suggest all the formulations containing HA with molecular weights <1100 kDa should have bioavailability values that are superior to the commercially available preparations.

It is worth underlying that no polymer degradation occurs at the high shear rate values experienced in this study: for all the samples analyzed, flow curves were registered also from high to low shear rate and they perfectly overlap with the ones obtained at the increase of the shear rate in the same interval (data not shown).

3.4. Mucoadhesion measurements

Mucoadhesiveness studies were performed to fully evaluate the potential for formulations to be retained on the ocular surface. Gastric mucin was employed due to its similarities to ocular mucin, which is not commercially available (Ceulemans & Ludwig, 2002). The biopolymer concentration used simulated the concentration in the corneo-conjunctival epithelium and the experimental conditions were carefully designed to simulate, as close as possible, the conformation of mucin in the eye (Ceulemans & Ludwig, 2002).

The results from mucoadhesion measurements are shown in Fig. 3. The HA samples were compared at the same concentration in Fig. 3a. The mucoadhesion index ($\Delta\%$) of the solutions, calculated as described in the paragraph 2.4.2, is reported as a function of the shear rate. When considering low shear rate values, HA1100 shows the highest mucoadhesion index, followed by HA800, HA500, and HA250. For all solutions, mucoadhesiveness decreases with the shear rate; however, the higher the molecular weight, the more marked the trend is. Thus, HA1100 is the most mucoadhesive until the shear rate reaches about 60 s^{-1} ; then, the

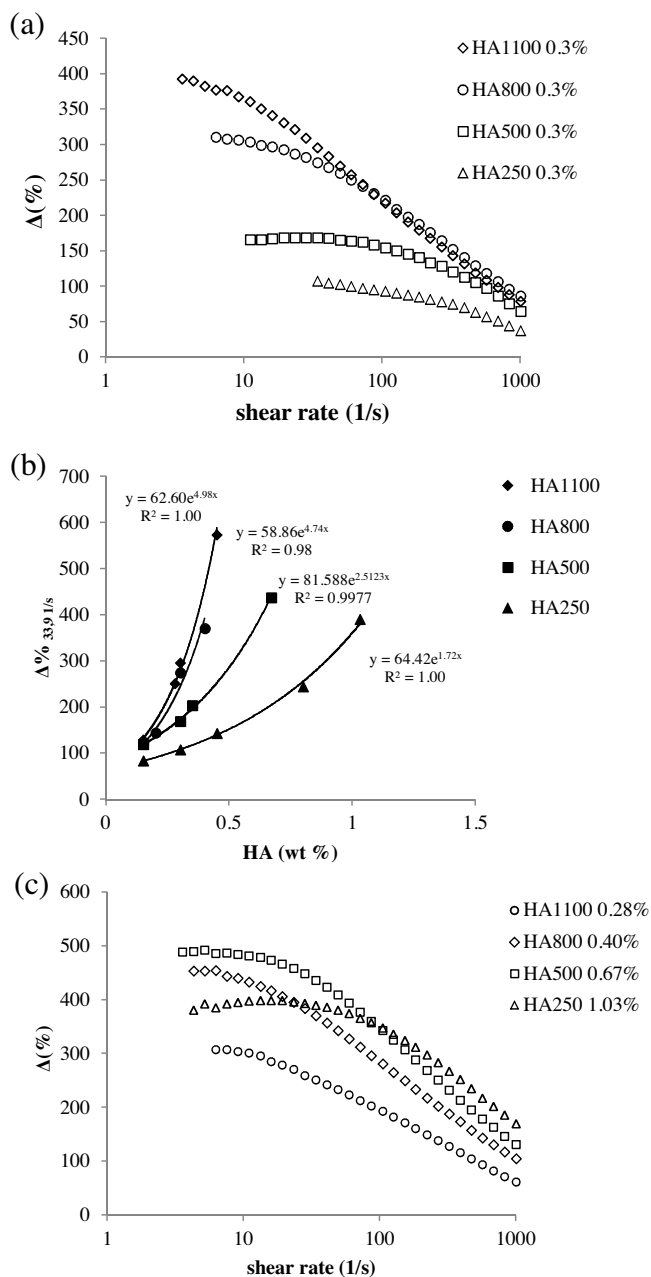


Fig. 3. (a) Mucoadhesion index as a function of the shear rate for HA1100, HA800, HA500 and HA250 formulations containing the same polymer amount (0.3 wt%). (b) Mucoadhesion index (calculated at 33.9 s^{-1} shear rate) as a function of polymer concentration for diverse molecular weight samples. (c) Mucoadhesion index as a function of the shear rate for the selected formulations.

mucoadhesion indexes of the diverse samples tend to become similar.

Mucoadhesion was evaluated for each molecular weight, at varying concentrations, and the dependence of Δ %, calculated at the 33.9 s^{-1} shear rate, on concentration is reported in Fig. 3b. The strength of the formulation/mucin interaction exponentially increases with HA concentration; the higher the molecular weight, the more notable the boost in mucoadhesiveness is with the increase in polymer amount.

Overall, the results shown in Fig. 3a and b indicate that the increase of both molecular weight and concentration positively affects the capacity of formulations to interact with mucin. However, for each molecular weight in the range considered, the maximum achievable mucoadhesiveness is that of the formula-

tions identified by the curves in Fig. 2b, with higher concentrations resulting in excessive viscosity. Therefore, the formulations identified as optimal in terms of viscosity were also the most advantageous for mucoadhesive properties.

In Fig. 3c the mucoadhesion profiles of the selected formulations are reported. In the region of low shear rate values, the rank in mucoadhesiveness is HA500-0.67% > HA800-0.4% > HA250-1.03% > HA1100-0.28%. The increase in shear rate affected the mucoadhesiveness of preparations differently. As a result, in the region of high shear rate values, mucoadhesion was inversely related to biopolymer molecular weight (increases with HA concentration): the shorter the chains (the higher the HA concentration), the stronger the interaction with mucin. Considering mucoadhesiveness dependence on molecular weight and concentration (Fig. 3a and b), the specific combinations HA molecular weight-amount within the preparations are rationally responsible for the relative mucoadhesiveness at rest conditions. The different effect of shear rate on mucoadhesiveness depending on polymer molecular weight (Fig. 3a) is reasonably at the basis of the predominant concentration effect registered at high shear conditions (the effect of molecular weight becomes negligible). Consequently, as with viscosity, under conditions simulating blinking, the interaction with mucin becomes stronger when moving from a preparation equivalent to *Blugel* to HA250-1.03%.

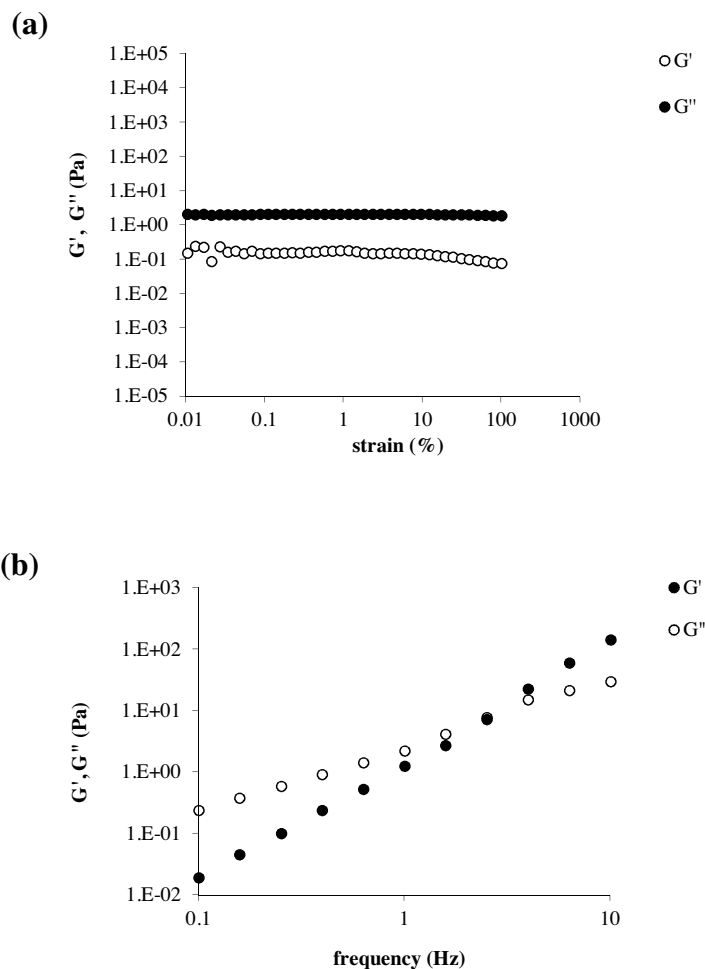


Fig. 4. Results of oscillatory measurements. (a) Dynamic moduli as a function of strain at 0.1 s^{-1} frequency and (b) dynamic moduli as a function of frequency at constant strain (0.045%) for HA500/mucin mixtures. Measurements were performed at 35°C . The trends shown are representative of all mixtures mucine/(selected HA sample).

Overall, based on the results of rheological measurements, the selected formulations should exhibit a retention on the ocular surface that is significantly improved over the commercially available products. Enhanced performance should be expected by decreasing molecular weight (increasing biopolymer concentration).

3.5. Oscillatory measurements (investigation of polymer/mucin interactions)

The interaction of a mucoadhesive polymer with mucin may occur by any of the following mechanisms: molecular interpenetration (physical entanglements), van der Waals bonds, electrostatic forces, hydrogen bonds, etc. (Ludwig, 2005). Oscillatory measurements of the HA formulations/mucin mixtures were performed in order to provide information about the type of interaction. Results are reported in Fig. 4. In particular, the results of the strain sweep test and of the frequency sweep test for the HA500-0.67%/mucin mixture, which is representative of all samples, are reported in Fig. 4a and b, respectively. G'' values were higher than G' values in the whole strain interval explored (Fig. 4a). This relative magnitude of the moduli is indicative of physical entanglements between the two biopolymers (Ceulemans & Ludwig, 2002). Such a structure was confirmed by the results of the frequency sweep. The mechanical

spectrum in Fig. 4b indicates the presence of an entangled network that behaves elastically with G' exceeding G'' at high frequencies (low relaxation time); while, at low frequencies (high relaxation times), chains can disentangle and a G'/G'' crossover was registered (Ceulemans & Ludwig, 2002; Cowman & Matsuoka, 2005). This type of interaction is consistent with the trend found for mucoadhesiveness as a function of HA molecular weight and concentration (Fig. 3a and b) and with the dependence of mucoadhesiveness on the shear rate (Fig. 3a and c).

3.6. In vitro evaluation of corneal (epithelial cells) protection against dehydration

Considering the massive use of HA-based eye drops for the treatment of eye dryness disorders, the more concentrated formulations (HA500-0.67%, and HA250-1.03%) were evaluated *in vitro* with respect to HA1100-0.28%, representative of the best performing product on the market, for their capacity to preserve the viability of PCECs during desiccation trials. The formulas were also tested after various dilutions (1:3, 1:10, and 1:30) to evaluate the HA dilution in the tear film that occurs *in vivo* immediately after instillation, and during progressive drainage of the formulation from the ocular surface.

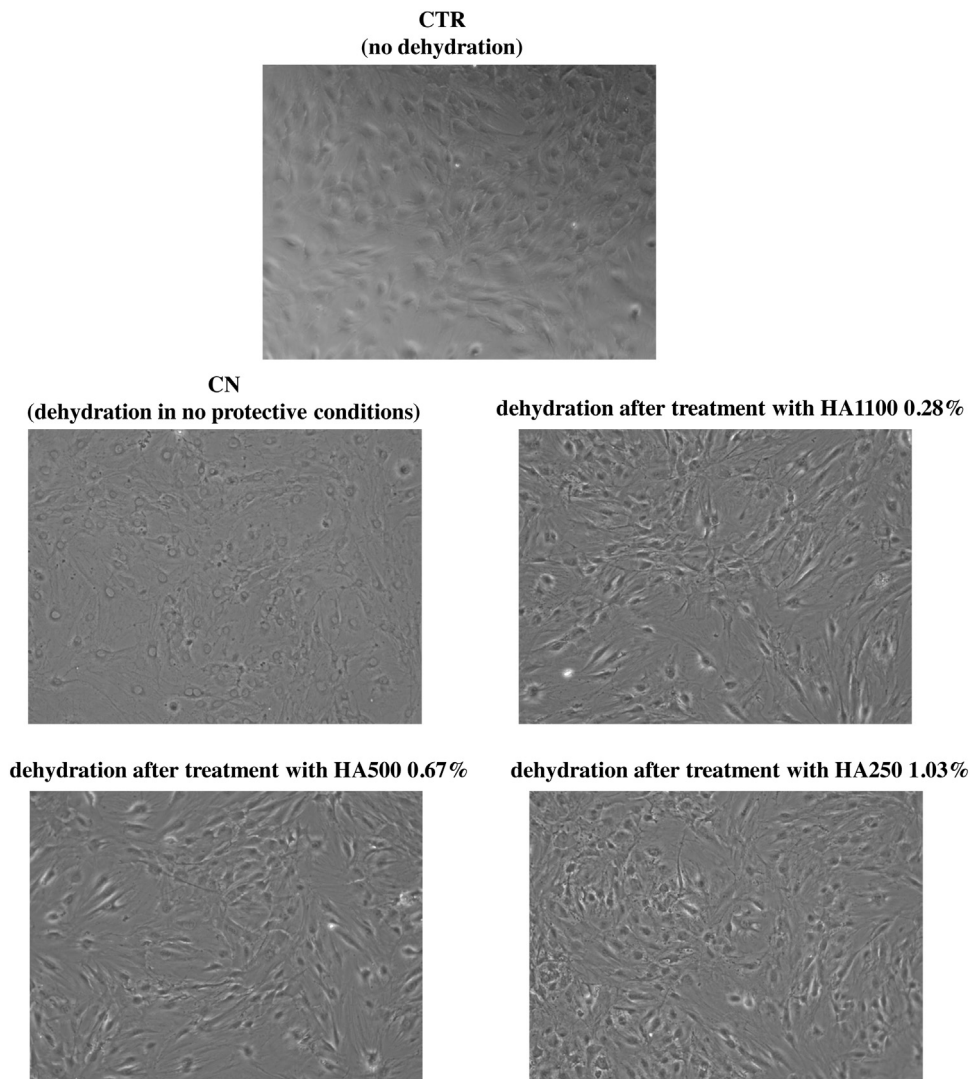


Fig. 5. Optical microscope images of PCECs after desiccation in no protective conditions (NC), after desiccation preceded by treatment with HA formulations (not diluted) and of cells not exposed to dehydration and to HA solutions (CTR).

Results are reported in Figs. 5 and 6. Fig. 5 shows optical microscope images of PCECs exposed to desiccation under no protective conditions (NC), or after being treated with the HA formulations (not diluted) and of cells that were not exposed to dehydration (CTR). It is evident that NC cells exhibited a “stressed” (non-typical) morphology and cell mortality with respect to CTR cells. In the samples pre-treated with HA prior to desiccation, typical morphology and a higher rate of survival can be observed regardless of the specific formula used. The same qualitative result was obtained when formulations were tested after a 1:3 dilution (data not shown). When the HA concentration was lowered 10- and 30-fold, the typical morphology and high survival rate could be still observed in cells pre-treated with HA500 and HA250; while, changes in morphology and mortality increased with the dilution in cells treated with HA1100 (data not shown).

The microscopic observation was confirmed by the results of quantitative analysis (Fig. 6). The applied stress was responsible for 50% cell mortality in the NC (no protective conditions) with respect to the CTR (not stressed cells). Under the same stress, 80–100% survival rates were estimated for cells pre-treated with selected formulations at 1:1 and 1:3 dilutions, confirming the same almost total protective effect displayed by all the HA forms evaluated. At a 1:10 dilution, there was about 90% cell viability with respect to the CTR measured for HA500 and HA250 samples; while, a lower (about 70% cell viability), but still evident protective effect, was registered for HA1100. When highly diluted (1:30), HA500 and HA250 were still able to protect PCECs from desiccation (about 70–75% survival rate), while no significant effect was found for HA1100.

These results are in line with the hypothesis that the protective effect displayed by HA-based preparations on corneal epithelium is related to the polymer water retaining capacity (Hill-Bator et al., 2014; Nakamura et al., 1993). The findings are important in view of application: with equal drainage (dilution) rates, higher HA amounts in the formulation correspond with longer-lasting efficacy *in vivo*. Since more concentrated formulations are expected to be retained longer *in situ*, the efficacy should be further improved.

4. Conclusions

In conclusion, we developed HA-based eye drop formulations that we expected will maximally reduce the drainage rate, while avoiding an excessive increase in viscosity. Comparisons with commercial products predicted there will be enhanced bioavailability

with the more concentrated formulation, which has the potential to exhibit the longest retention time in the tear film. This finding is valuable in the tuning of formulations, including HA, to extend the precorneal residence time of the active ingredient. The preparations also surpassed the commercially available products in their ability to protect the corneal epithelium from dehydration. This outcome, combined with the enhanced bioavailability, suggests the developed formulations may be promising medications for the treatment of dry eye disorders. Finally, this research provided more insight into the importance of the combined effect of polymer size and concentration on the rheological and mucoadhesive properties of topical ophthalmic preparations incorporating a bioavailability-enhancer; thus, it was a useful reference study for the optimization of similar products.

Acknowledgements

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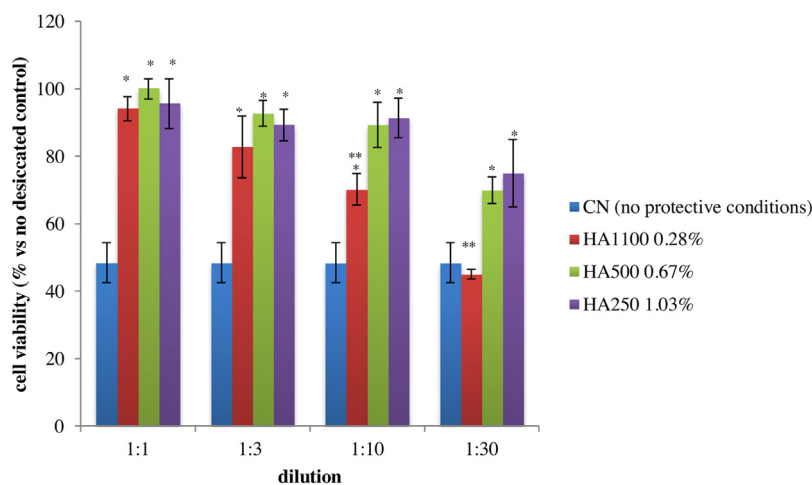


Fig. 6. Results of quantitative determination of the protective effect against dehydration: viability (%) with respect to CTR of PCECs exposed to desiccation in no protective conditions or after treatment with HA preparations, differently diluted.

*Indicates the formulations exhibiting a significant protective effect with respect to NC ($p < 0.05$).

**Indicates, significant differences among the formulations tested, the red bar, at 10 and 30 fold dilution, presents a lower protection respect to the others ($p < 0.05$).

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