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Characterization of the Molecular Weight of Hyaluronan in Eye Products Using a Novel Method of Size Exclusion High-Pressure Liquid Chromatography

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Purpose: Hyaluronan (HA) exists in two forms, high molecular weight HA (HMWHA) and low molecular weight HA (LMWHA), which have distinct physiological functions. Therefore it is imperative to know the form of HA within pharmaceutical products, including eye products. This study developed an accurate, sensitive, and quantitative method to characterize the form of HA in eye products. Thereafter, the effects of the HA-containing eye products on corneal wound healing were investigated.

Methods: The MW distributions and concentrations of HA in over the counter eye products were determined by size exclusion chromatography (SEC) high-pressure liquid chromatography (HPLC). The effects of the eye products containing HA on corneal wound healing were characterized both in vitro and in vivo using the scratch assay and the debridement wound model, respectively.

Results: The concentrations and MWs of HA were successfully determined within a range of 0.014 to 0.25 mg/mL using SEC HPLC. The concentrations of HA in the ophthalmic products varied from 0.14 to 4.0 mg/mL and the MWs varied from ~100 kDa to >2500 kDa. All but one HA-containing eye product had an inhibitory effect on corneal wound healing, whereas pure HA promoted corneal wound healing.

Conclusions: A novel SEC-HPLC method was developed for quantifying and characterizing the MW of HA in eye products. Although HA promoted corneal wound healing, HA-containing eye products inhibited corneal wound healing, likely caused by preservatives.

Translational Relevance: SEC-HPLC could be implemented as a routine method for determining the form of HA in commercially available ophthalmic products.

Introduction

Hyaluronan (HA) is a nonsulfated, high molecular weight glycosaminoglycan made of repeating disaccharide units composed of D-glucuronic acid and N-acetylglucosamine, linked alternately by β-1,3 and β-1,4 glycosidic bonds.[1] Enriched during the early stages of development and disease, HA is a ubiquitous component of extracellular matrices. HA is naturally synthesized by hyaluronan synthases (HASs), a class of integral membrane proteins, of which vertebrates have three types: HAS1, HAS2, and HAS3.[2] HA is nonsulfated, and therefore it does not exist with the same structural variability as other glycosaminoglycans. HA has extremely high biocompatibility and is widely distributed throughout the body. The human dermis and epidermis are among the tissues with the highest abundance of HA, ranging from 0.20 to 0.50 and 0.10 mg/g, respectively.[3] Other HA-rich tissues

include the human umbilical cord, which contains 4.10 mg/mL of HA, the vitreous humor containing 0.14 to 0.34 mg/g, and the synovial fluid containing 1.40 to 3.60 mg/mL.[3] Within the synovial fluid and cartilage, HA is synthesized by synovial cells, fibroblasts, and chondrocytes.[4] HA is naturally present in all structures of the eye, of note, the tear film, limbal stem cell niche, Meibomian glands (MG), trabecular meshwork, retina, and the aqueous and vitreous humor.[5–10] HA is highly expressed in the cornea within the provisional matrix after injury.[11,12] In the cornea, HA maintains limbal epithelial stem cells and regulates lymphangiogenesis and stiffness.[6,7,13,14] In the MGs, HA has been shown to surround the basal cell layer of the glands and regulate morphogenesis and homeostasis.[8,9] HA is also present within the niche surrounding MG progenitor cells.[8,9] HA is also an active component in various other stem cell niches, including the stem cell niche of umbilical cord mesenchymal stem cells and bone marrow mesenchymal stem cells.[6,15–22]

The natural abundance and physiochemical properties of HA, such as high viscosity, have made it a useful adjunct in various clinical applications. For example, pharmaceutical companies have targeted HA for age-related skincare products since moisture loss is a hallmark of aging skin.[23] In patients with knee pain and reduced joint mobility, weekly intra-articular injections of HA have proven to decrease symptoms within one to two months.[24] Over the years, the pharmaceutical application of HA in ophthalmology has also grown significantly. HA is now commonly used as a viscoelastic tool in ophthalmic surgeries to protect ocular structures.[24,25] During surgery, HA protects the structural integrity of the eye and prevents it from collapsing while also protecting the corneal endothelium from damage.[24–28] HA has also been studied in conjunction with contact lenses to alleviate the deleterious effects of contacts lens wear, improving both wearability and comfort, and patients experience less blurring between blinks and a more even distribution of tears.[25,29–31] Currently, various approaches are being explored to synthesize HA-loaded contact lenses that can release HA at a controlled rate.[32–36]

Recently, the pharmaceutical interest in HA-containing eye drops has grown exponentially. HA-containing eye drops have been used for the management of dry eye disease (DED), contact lens comfort, controlled release of drug, moisturization, reduction of mechanical stress and damage to cornea, and promotion of corneal damage repair and as a wetting agent.[25] The effects of HA eye drops at various concentrations (0.1%, 0.15%, 0.18% and 1.4%) have been evaluated in patients with mild to severe DED to study the efficacy and safety profile of HA.[37–42] These studies showed that HA resulted in improved tear film stability and provided an overall reduction of damage to the ocular surface. The natural viscoelastic properties of HA allow it to mimic the flow characteristics of natural tears, whereas the mucoadhesive properties aid in optimizing localized drug delivery.[43,44] Along with its well-accepted role in lubricating the ocular surface and stabilizing the tear film, HA can potentially aid in suppressing inflammation and ocular surface homeostasis to treat DED. Based on the reported benefits of HA and its extremely high safety profile with no known drug interactions, HA is now commonly found in multiple artificial tears, mostly listed as an inactive ingredient. However, details on the concentrations and forms of HA are rarely included in the formulations of these products.

The physiological activity of HA is directly dependent on its molecular weight (MW).[45,46] In tissues, primarily two forms of HA exist: high average molecular weight HA (HMWHA) of approximately 2000 kDa and low molecular weight HA (LMWHA) of approximately 200 kDa.[47] HMWHA has primarily been reported to have anti-inflammatory effects and to promote tissue integrity.[48] The higher the MW of HA, the higher its water-binding capacity, and higher its viscosity. Therefore HWMHA is also reported to have superior lubricating capabilities. In contrast, LMWHA is primarily reported to have proinflammatory effects and has been shown to be upregulated during pathogenesis.[48–52] Commercially-available HA is usually prepared by either purification or by chemical synthesis, such as microbial fermentation with *Streptococcus sp.*[53–56] Importantly, the current systems available for commercially synthesizing HA enable it to be manufactured at a variety of molecular weights, from an ultra-low molecular weight HA (ULMWHA) of $<$100 kDa to an extremely high molecular weight HA of $>$5000 kDa. Rooster comb and bovine nasal cartilage are the most common sources for animal-sourced HA.[3]

The main impediment for accurate information regarding the molecular weight of HA in various medical products is the lack of a simple, sensitive, and accurate method for characterizing its molecular weight.[25,45] Currently, the most widely available and most easily implemented method for characterizing HA is agarose gel electrophoresis.[57–59] Additionally, multi angle laser light scattering,[60] matrix-assisted laser desorption ionization mass spectrometry,[61] HPLC coupled with a refractive index detector,[25,62] and, more recently, a fabricated solid state nanopore platform using a nanometer scale aperture positioned between two electrolyte reservoirs[25,63] can be used to determine the molecular weight of HA. However, these

methods require either the purification of HA prior to its characterization, high concentrations of HA, expensive equipment, and/or highly trained personnel. Consequently, there is still a scientific need for an easy, accessible, and sensitive method for determining the molecular weight distributions of HA. Here we describe a sensitive method for characterizing the molecular weights of HA by size exclusion chromatography (SEC) using a high-pressure liquid chromatography system (HPLC). SEC-HLPC requires equipment that is standard or easily accessible to most laboratories, requires limited training, is easy and quick to run, and, other than the initial investment to purchase the chromatography columns, is very cheap to run.[64,65] Furthermore, we used this technique to characterize the molecular weights and determine the concentrations of HA in various eye product formulations and correlated these findings with the effects of the eye products on human corneal epithelial wound healing, both in vitro and in vivo.

Materials and Methods

HA Standards

Sodium hyaluronate standards ranging from 100 to 2670 kDa were used for the standardization of the separation profiles for HA. Specifically, the following HA standards from high to low molecular weight were obtained: 2670 kDa (Dried Sodium Hyaluronate, HA2M-5; Lifecore Biomedical, Inc., Chaska, MN, USA), 1550 kDa (Dried Sodium Hyaluronate, HA15M-5; Lifecore Biomedical, Inc.), 1200-1550 kDa (Organic Sodium Hyaluronate, 95%, EW-88252-56; Acros Organics, Geel, Belgium), 700 kDa (Dried Sodium Hyaluronate, HA700K-5; Lifecore Biomedical, Inc.), 500 kDa (Dried Sodium Hyaluronate, HA500K-5; Lifecore Biomedical, Inc.), 200 kDa (Dried Sodium Hyaluronate, HA200K-5; Lifecore Biomedical, Inc.), 100 kDa (dried sodium hyaluronate, lot no. 026472; Lifecore Biomedical, Inc.), and 4-8 kDa (Hyaluronan [Ultra Low MW], GLR003; R&D Systems, Minneapolis, MN, USA).

Preparation of HA Standards

The standard solutions of various molecular weights were prepared by dissolving sodium hyaluronan in 15 mL of nano-purified H_2O to create 2.5 mg/mL concentrations of HA samples. These standard solutions were filtered by 0.22 µM syringe filters before analysis. They were then diluted with nano-purified H_2O to create various diluted concentrations of each standard (ranging from 2.5 µg/mL to 2.5 mg/mL) to determine the range of detection for HMWHA, mid-MWHA, and LMWHA. We determined that the maximum concentration of HA that could be injected into the HPLC system was 2.5 mg/mL. Above this amount HMWHA became too viscous, leading to nonreproducible profiles. All standards were stored at −80°C before runs.

HA-Containing Eye Products

Nine commercially available over-the-counter (OTC) eye drops or ophthalmic products were included. All products listed HA as an inactive ingredient on the label at the time of the study. The products included Biotrue MPS (Bausch + Lomb, Rochester, NY, USA); Blink Contacts, Blink Gel Tears, and Blink Tears (Johnson & Johnson Vision, Santa Ana, CA, USA); Clear Eyes Pure Relief (Prestige Consumer Healthcare, Irvington, NY, USA); CVS Moisturizing Eyelid Spray, CVS Refreshing Eyelid Spray, CVS Soothing Eyelid Spray (CVS Health, Woonsocket, RI, USA); and Refresh Repair (Allergan, Dublin, Ireland). During this study, Refresh Repair was discontinued and replaced with Refresh Relieva. Before analysis, samples were diluted 1:10 and 1:20 in nano-purified H_2O. The products were not purified or filtered before analysis. The elution profile was monitored using UV-vis. To confirm the presence of HA in the products, elution profiles were compared for each product, with and without hyaluronidase (HYAL) digestion. The HYAL was prepared from *Streptomyces hyalurolyticus* (H1136 Sigma) with 300 µL of 20 µM acetate buffer pH 6.0 containing 0.01% BSA to make 1 U/mL. The digestion was carried out using 90 µL of sample with 10 µL of HYAL incubated overnight at 37°C. A control sample was then prepared with 90 µL of sample with 10 µL of the acetate buffer without the addition of the enzyme. Both the digested and non-digested samples were filtered through an Amicon Ultra-0.5 Centrifugal Filter Unit with a 3000 kDa cutoff (Millipore Sigma, Burlington, MA, USA) to remove digested products and then run sequentially through the size exclusion HPLC system.

Analysis of the Molecular Weight of HA by HPLC

The molecular weights of HA were analyzed by SEC using two Advanced Bio SEC 300 Å 4.6 × 300 mm columns (Agilent Technologies, Santa Clara, CA, USA) and one Zorbax GF-250 4.6 × 250 mm column (Agilent Technologies) set in tandem in a 3000

UltiMate Dionex system (Thermo Fisher Scientific, Waltham, MA, USA). A 0.1 M sodium phosphate buffer containing 20 µM ethylenediamine tetra-acetic acid (EDTA), pH 6.8, was used as the mobile phase with a flow rate of 0.300 mL/min. Temperature was maintained at 35°C throughout the run using a column oven (Dionex UltiMate 3000 TCC-3000SD Standard Thermostatted Column Compartment; Thermo Fisher Scientific), and UV detection was set at 204 nm and 280 nm using a VWD-3100 UV detector. All samples were loaded in a total volume of 25 µL per run. Each standard or eye product sample was run in triplicate, and the runs were repeated on two separate occasions.

Quantification of HA was done by calculating the area under the peaks in comparison to size-matched standard controls of known concentrations. The different HA peaks were identified both automatically using the Chromeleon Chromatography Data System (CDS) Software (Thermo Fisher Scientific) and confirmed by two investigators in a blinded manner based on the retention times using the linear equation determined from the standard calibration curves. With this, the concentrations and compositions of HA were determined for each ophthalmic product.

Analysis of the Molecular Weight of HA by Agarose Electrophoresis

The molecular weights of the different HA standards were additionally confirmed by agarose gel electrophoresis, as previously shown.[58,59] Approximately 7 µg of the HA standards used in this study were lyophilized and then suspended in 10 µL of 10 M deionized formamide (S4117, Millipore Sigma) and thereafter maintained at 4°C overnight. HA of 500 kDa and 50 kDa (Echelon Biosciences, Salt Lake City, UT, USA) and HA of 2500 kDa, 1000 kDa, 600 kDa, 250 kDa, 150 kDa, and 100 kDa were used as HA ladder mixes at 0.7 µg/µL. A 1% agarose gel (Omnipur Agarose, 2125; Calbiochem, San Diego, CA, USA) was prepared in 1× TAE buffer (A0033; Biotech USA, Minneapolis, MN, USA). The eye products were concentrated in a SpeedVac (Eppendorf, Hamburg, Germany) to reach an estimated concentration of HA of approximately 2.5 µg/mL. The gels were pre-run for six hours at a constant voltage of 80 V (OWL EasyCast systems, Thermo Fisher Scientific) to remove impurities. The next day, 2 µL of 0.2% bromophenol blue loading solution (Sigma-Aldrich) was added to the samples for a final volume of 12 µL and loaded onto the gel. The electrophoresis was run at a constant voltage of 100 V until the tracking dye migrated to about two-thirds the length of the gel. The gels were then immersed in 30% ethanol with gentle rocking for one hour at room temperature, followed by overnight staining with 6.25 µg/ml Stains-All solution (no. H32127; Alfa Aesar, Haverhill, MA, USA) at room temperature, protected from light. The next morning, the staining solution was replaced with water, and the gels were de-stained by exposure to light for approximately 20 minutes and imaged using a Bio-Rad imager (Bio-Rad chemidoc MP imaging system, Universal Hood III; Bio-Rad Life Science, Hercules, CA, USA) with white light.

Maintenance of Human Telomerase-Immortalized Corneal Epithelial Cells

Human telomerase-immortalized corneal epithelial cells (hTCEpi)[66] were maintained in serum-free keratinocyte culture medium (DermaLife Basal media kit; Lifeline Cell Technology, Frederick, MD, USA), supplemented with supplements provided in the DermaLife Basal media kit (DermaLife Basal media kit; Lifeline Cell Technology, Frederick, MD, USA), with final concentrations of 6 mM L-glutamine, 0.4% bovine pituitary extract, 1.0 µM epinephrine, 0.50 ng/mL recombinant human transferring growth factor-α, 100 ng/mL hydrocortisone hemisuccinate (no. H2270; Sigma-Aldrich Corp., St. Louis, MO, USA), 5 µg/mL rh-insulin, and with 5 µg/mL Apo-Transferrin, 30 mg/mL gentamicin, and 15 µg/mL Amphotericin B in 100 mm cell culture dishes (Nunc, no. 172931; Thermo Fisher Scientific). The cells were sub-cultured when reaching 70% to 90% confluence with 0.25% trypsin-EDTA (no. 25200-056; Gibco, Thermo Fisher Scientific). Cells were maintained in a humidified environment at 37°C and 5% CO_2.

In Vitro Scratch-Wound Assay

HTCEpi were seeded in 24-well plates (Nunc Cell-Culture Treated, no. 142475; Thermo Fisher Scientific) at a density of 92,000 cells/well and left at 37°C and 5% CO_2. When the hTCEpi achieved confluence, cells were mechanically removed from the center of the confluent monolayer by dragging a 200 µL pipette tip linearly down the middle of the well. The position and length of the scratches were standardized by using a guide placed under the multi-well plate. Loose cells and debris were removed with two sequential phosphate-buffered saline solution (PBS) washes, and, thereafter, fresh medium was placed into the well. To study the effects of HA-containing eye products on the rate of wound closure, the eye products were added to the wells at a dilution factor of 1:5 or 1:10. HA standards (HMWHA,

mid-MWHA, and LMWHA) were also included as positive controls. Both media and media containing PBS at a dilution factor of 1:5 or 1:10 were used as the vehicle controls yielding comparable results. The microplates were placed into a 24-well plate heated insert (PM 2000 RBT incubator; Zeiss, Oberkochen, Germany) and maintained at 5% CO_2 and 37°C. The scratched edges were imaged every four hours for 24 hours under a ZEISS LSM 800 Confocal microscope (Zeiss) using the time-elapse module. The wounded areas were measured and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), as described previously.[67] Each wounded area was calculated as the cell-free area at a certain time point divided by the cell free area at 0 hours × 100. This experiment was carried out three times in triplicate.

Viability Assay

The effect of selected eye drops on the viability of hTCEpi were assessed using Cell Counting Kit-8 (CCK-8, APExBIO Technology LLC, USA# K1018). For such, cells were treated with eye drops prepared at a 1:10 dilution in complete media for 24 hours. The CCK-8 assay assesses mitochondrial activity by the formation of a water-soluble formazan dye upon reduction of tetrazolium salt (WST-8) catalyzed by dehydrogenases in live cells. The hTCEpi cells were trypsinized and seeded in 96-well plates at a density of 3000 cells/well. Medium only was used as a blank, and cells in the media were used as a control. After 44 hours, 10 µL of the CCK8 reagent was added to each well and incubated for four hours at 37°C. Thereafter, the formazan products were analyzed by reading absorbance at 450 nm using a spectrophotometer microplate reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). The viability of the cells was calculated using the formula: viability = (experimental condition-blank)/(control − blank) × 100. This experiment was carried out four times in triplicate.

Proliferation Assay

The effect of selected eye drops on the proliferation of hTCEpi were assessed using a BrdU (5-bromo-2′-deoxyuridine) Cell Proliferation Assay Kit (no. 2750; EMD Millipore, Burlington, MA, USA), according to the manufacturer's instructions. In short, hTCEpi cells were trypsinized and seeded in a 96-well plate at a density of 3000 cells per well. After 24 hours, cells were treated with eye drops prepared at a 1:5 or 1:10 dilution in complete media and left for 48 hours at 37°C and 5% CO_2. At 40 hours, BrdU was added to the culture media, and plates were incubated for an additional eight hours, after which the cells were fixed using the provided fixing solution. Incorporated BrdU was detected using the provided anti-BrdU monoclonal antibody, followed by the provided IgG-peroxidase conjugated secondary antibody, substrate and stop solution. Absorbance was measured as OD at 450 nm using a microplate reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). Medium only was used as a blank, and cells in the media were used as a control. This experiment was carried out three times with five replicates per experimental point.

Animal Maintenance

C57BL/6J mice between seven to eight weeks old were subjected to debridement wounds. Mice were randomized into the different groups based on the type of eye drops used. All mice were bred and housed in a temperature-controlled facility with an automatic 12-hour light-dark cycle at the Animal Facility of the University of Houston. All experimental procedures for handling the mice were previously approved by the Institutional Animal Care and Use Committee at the University of Houston under protocol 16-044.

Debridement Wounds and Eye Drop Treatments

In preparation for the corneal debridement wounds, seven- to eight-week-old mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg) (no. 07-890-8598; Vedco Inc., Saint Joseph, MO, USA) and xylazine (10 mg/kg) (no. 07-808-1947; Akorn Pharmaceuticals, Gurnee, IL, USA) before wounding. Corneal epithelium was demarcated using a trephine (1.0 mm in diameter), and the epithelium within the demarcated area was removed using an AlgerBrush II with a 0.5 mm rotating burr (Alger Company, Inc., Lago Vista, TX, USA). Thereafter, polyvinyl acetal eye spears (no. AX10086; Accutome Inc., Malvern, PA, USA) combined with sterile PBS were used to wash out the debrided cells. The wounded areas were determined immediately after wounding and 12, 16, and 24 hours after the injury by instilling 1.5 µl of a 1 mg/mL fluorescein solution (GloStrips, no. A01-33E; Amcon Laboratories, St. Louis, MO, USA) onto the cornea. The eyeballs were imaged using a Zeiss Discovery V12 Stereo Microscope (Zeiss), and the injured areas were calculated using the ImageJ software. For the treatments, each eye was instilled with eye drops continuously every 15 minutes for the first

2 hours, and a single dose was administered at 12 and 16 hours after injury. PBS was used as a control. After the first two-hour eye drop instillation, terramycin (NADA no. 8-763; Zoetis, Parsippany-Troy Hills, NJ, USA) ointment was applied. At least five mice were included for each experimental condition.

Quantification and Statistics

Standard calibration curves of the peak area of HA of certain MW versus its concentrations were obtained from linear regression analysis of the data. The regression equation, coefficient of determination (r^2), and linear range were reported. Correlation coefficients were used to ascertain the linearity of the standard calibration curve. Student's *t*-tests and analysis of variance with post-hoc by Holm-Bonferroni correction were used to analyze the statistical difference between two or more groups, respectively. Differences are considered statistically significant when $p \leq 0.05$.

Results

Characterization of Gel Filtration Chromatography for Characterizing the Molecular Weights of HA

Our system used two Advanced Bio SEC 300 Å 4.6 × 300 mm columns (Agilent Technologies) and one Zorbax GF-250 4.6 × 250 mm column (Agilent Technologies) that were set in tandem to efficiently separate ULMWHA, LMWHA, mid-MWHA, and HMWHA. A 0.1 M sodium phosphate buffer containing 20 µM EDTA, pH 6.8, was selected as the buffer to ensure the best separation, reproducibility, and lowest background. Flow rates ranging from 0.250 to 0.750 mL/min achieved comparable results. However, the flow rate of 0.300 mL/min flow rate was selected to ensure that the system maintained a pressure ≤ 25 MPa. Temperatures ranging from 30° to 60°C also achieved similar results. However, below 30°C the separation profiles were compromised, and the pressure in the system tended to rise.

The elution profile of HA was monitored using a UV detector at 204 nm. For the assignment of peaks to HA of different molecular weights, retention times were determined using 0.50 mg/mL standards. The retention time for HMWHA was determined to be above 14.548 ± 0.074 minutes. The retention time for mid-range HA was determined as 15.349 ± 0.038 minutes, and for LMWHA was determined as 15.829 ± 0.197 minutes (Table 1). Once the separation of the peaks was accomplished, the HA standards were administered at different concentrations to determine the resolving differences in the molecular weight of HA at different concentrations. Standard calibration curves for HMWHA ($r^2 = 0.991$, n = 4), mid-range HA ($r^2 = 0.994$, n = 5), and LMWHA ($r^2 = 0.991$, n = 10) were determined based on the concentration (mg/mL) plotted versus area (mAU) (Figs. 1A–C). The linear range for HMWHA was the smallest, ranging from 0.0050 to 0.25 mg/mL (0.00050–0.025% w/v) (Fig. 1A). Mid-range MWHA had a linear range from 0.0050 to 0.50 mg/mL (0.00050–0.050% w/v) (Fig. 1B) and LMWHA had a largest linear range, from 0.0050 to 1.0 mg/mL (0.00050–0.10% w/v) (Fig. 1C). The lowest detectable concentration was 0.0050 mg/mL (0.00050% w/v) for all forms of HA.

Table 1. Retention Times for the Separation of HA of Different Molecular Weights by Size Exclusion HPLC

HA Standards (kDa)	Retention Times (Min)	Standard Deviation	Relative Standard Deviation (%)
2670	14.1320	0.030	0.214
1550	14.2860	0.024	0.172
1200–1550	14.5480	0.021	0.151
700	15.4630	0.035	0.248
500	15.6480	0.020	0.137
200	15.8290	0.038	0.247
100	16.0180	0.008	0.051

Finally, we verified the reproducibility of our method. For such, all the standards within each molecular weight range were administered consecutively at a concentration of 0.50 mg/mL in six separate runs, successfully demonstrating the reproducibility of both separation and retention times (results not shown). The relative standard deviation was calculated by dividing the standard deviation of the retention time by the average of the retention times to estimate the reproducibility of the method. The relative standard deviation of the HA standards' retention times ranged from 0.050% to 0.25%, indicating good reproducibility of this method (Table 1).

Identification, Quantification, and Determination of the Molecular Weight of HA in OTC Eye Products by HPLC

The elution profile of HA was monitored in nine different OTC eye products, including five eye drops, one multipurpose solution (MPS), and three eyelid sprays by size exclusion HPLC. We identified three out of the nine eye products contained a major HA peak in the HMWHA range, while three contained a major HA peak in the mid-MWHA range, and lastly

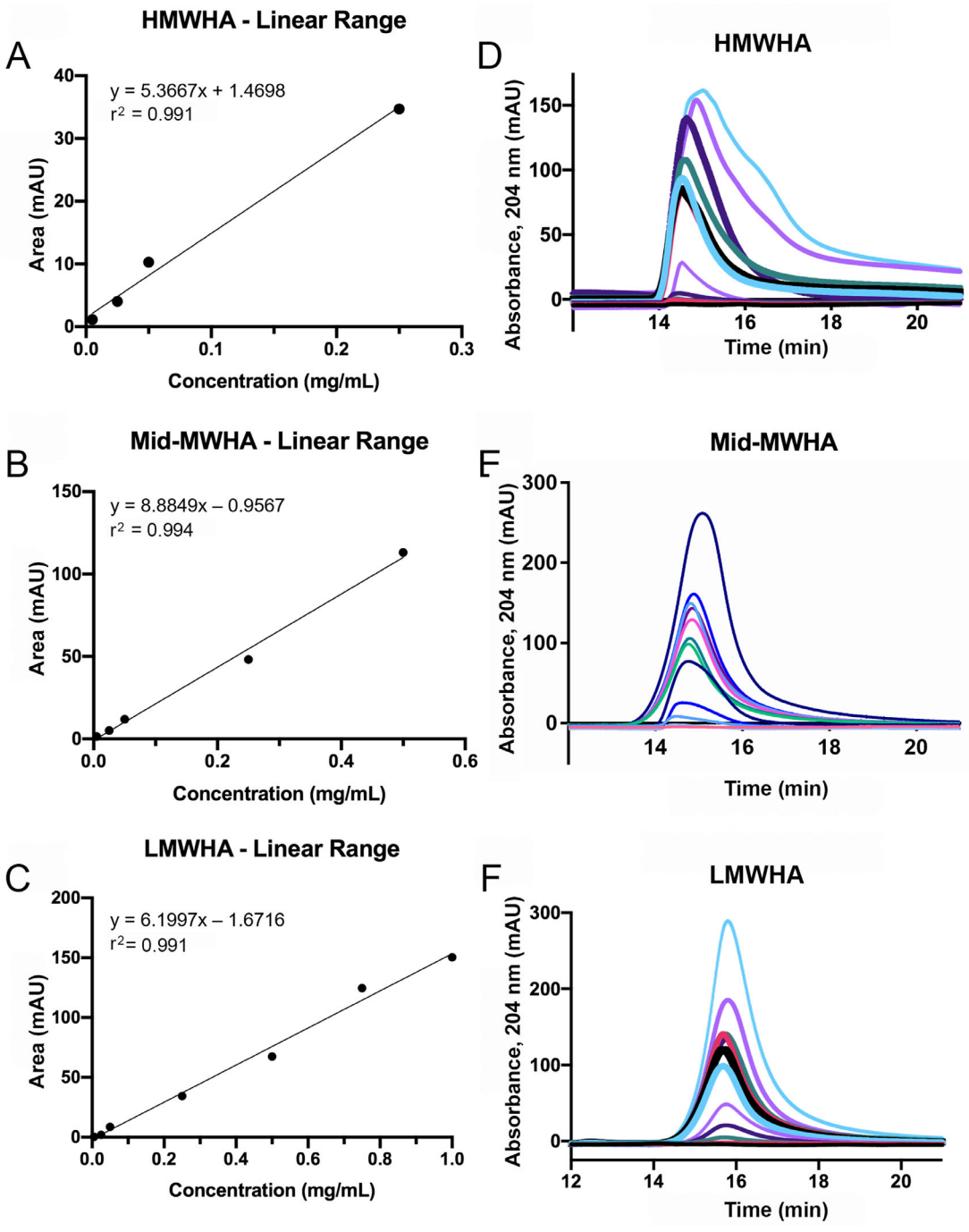

Figure 1. Standardization of the chromatographic profile for HMWHA, LMWHA, and ULMWHA by size exclusion HPLC. HMWHA, mid-MWHA, and LMWHA were applied to a size exclusion HPLC at increasing concentrations starting from 0.0025 mg/mL to 2.5 mg/mL, and the concentration and molecular weight of HA were successfully determined within a range of 0.014 to 0.25 mg/mL. (A–C) Linear regression equations and r^2 were calculated for HMWHA (A), mid-MWHA (B) and LMWHA (C). (D–E) Chromatographic profiles for HMWHA, mid-range MWHA, and LMWHA at increasing concentrations, specifically 0.0025, 0.005, 0.025, 0.05, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, and 2.5 mg/mL, are shown.

one contained a major HA peak in the LMWHA range (Fig. 2, Table 2). One eye product (Refresh Repair) contained major peaks in both the HMWHA and Mid-MWHA ranges (Fig. 2, Table 2). One of the eye products (CVS Moisturizing Eyelid Spray) contained negligible levels of HA that fell below the detection threshold (Table 2). Our data showed significant variability in the concentrations and molecular weights of HA between the different eye products (Table 2). The peak concentrations of HA varied

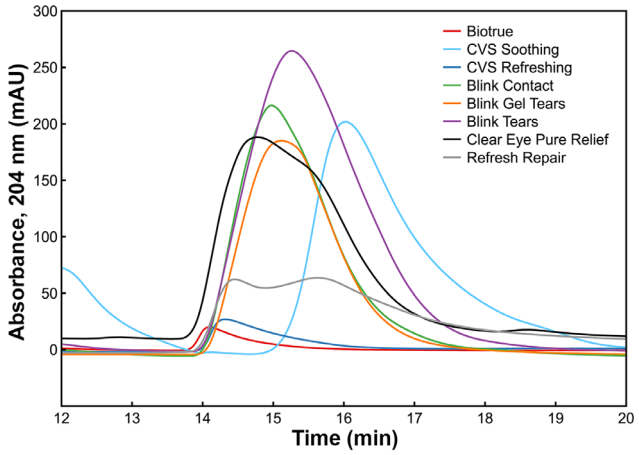

Figure 2. Chromatographic profile for HA in different HA-containing eye products by size exclusion HPLC. Eight HA-containing eye products were administered to size exclusion HPLC and monitored at 204 nm, and an overlay of their chromatographic profiles is shown.

from 0.14 to 4.0 mg/mL (0.014%–0.40% w/v), thus a 30-fold difference in the concentration of HA was identified among the different eye products (Table 2). More importantly, most eye products contained HA with a large range of molecular weights. The different forms of HA present in each eye product are summarized in Table 3. Finally, HYAL digestion was used to confirm that the identified peaks represented HA (Fig. 3).

Determination of the Molecular Weight of HA in OTC Eye Drops by Agarose Gel Electrophoresis

The molecular weights of the OTC eye products were also determined by agarose gel electrophoresis (Fig. 4). For such, the different eye drops had to be concentrated using a refrigerated vacuum concentrator between five- to 40-fold to fall within the limits of detection. The analysis of the OTC eye products by agarose electrophoresis showed that the Biotrue MPS Solution contained primarily HMWHA, whereas the remaining eye products contained a range of HA of different molecular weights. Blink Contacts, Blink Gel Tears, Blink Tears, and Clear Eyes Pure Relief contained HA ranging from HMWHA to LMWHA (Table 4, Fig. 4). CVS Refreshing Eyelid Spray contained HA ranging from HMWHA to ULMWHA (Table 4, Fig. 4). CVS Soothing Eyelid Spray contained HA ranging from LMWHA to ULMWHA (Table 4, Fig. 4). Finally, Refresh Repair contained HA ranging from mid-MWHA to ULMWHA (Table 4, Fig. 4). Overall, the analysis by agarose electrophoresis confirmed the identified molecular weights of the different HA standards that were established by size exclusion HPLC Tables 3 and 4. During this study,

Table 3. Identification of the Range of Molecular Weights of HA Within OTC Eye Drops by Size Exclusion HPLC

Eye Care Product	Range of HA
Biotrue MPS Solution	HMWHA
Blink Contacts	HMWHA-LMWHA
Blink Gel Tears	HMWHA-LMWHA
Blink Tears	HMWHA-ULMWHA
Clear Eyes Pure Relief	HMWHA-LMWHA
CVS Moisturizing Eyelid Spray	—
CVS Refreshing Eyelid Spray	HMWHA-LMWHA
CVS Soothing Eyelid Spray	LMWHA-ULMWHA
Refresh Repair	HMWHA-ULMWHA

Table 2. Identification of the Retention Time and Molecular Weight HA Within the Major Peaks and Concentration of HA Within OTC Eye Products by Size Exclusion HPLC

Ophthalmic Products	Retention Time (min)	Standard Deviation	Relative Standard Deviation (%)	Molecular Weight of Major HA Peak	Concentration, mg/mL	%, w/v
Biotrue MPS Solution	14.092	0.041	0.276	HMWHA	0.139	0.014
Blink Contacts	14.974	0.050	0.351	Mid-MWHA	2.898	0.184
Blink Gel Tears	15.254	0.042	0.272	Mid-MWHA	1.843	0.403
Blink Tears	15.333	0.041	0.281	Mid-MWHA	4.032	0.290
Clear Eyes Pure Relief	14.744	0.032	0.210	HMWHA	1.930	0.193
CVS Moisturizing Eyelid Spray	No HA detected	—	—	—	—	—
CVS Refreshing Eyelid Spray	14.273	0.021	0.141	HMWHA	0.177	0.018
CVS Soothing Eyelid Spray	16.123	0.032	0.191	LMWHA	1.774	0.177
Refresh Repair	14.437, 15.654	0.200	1.368	HMWHA, Mid-MWHA	0.321	0.032

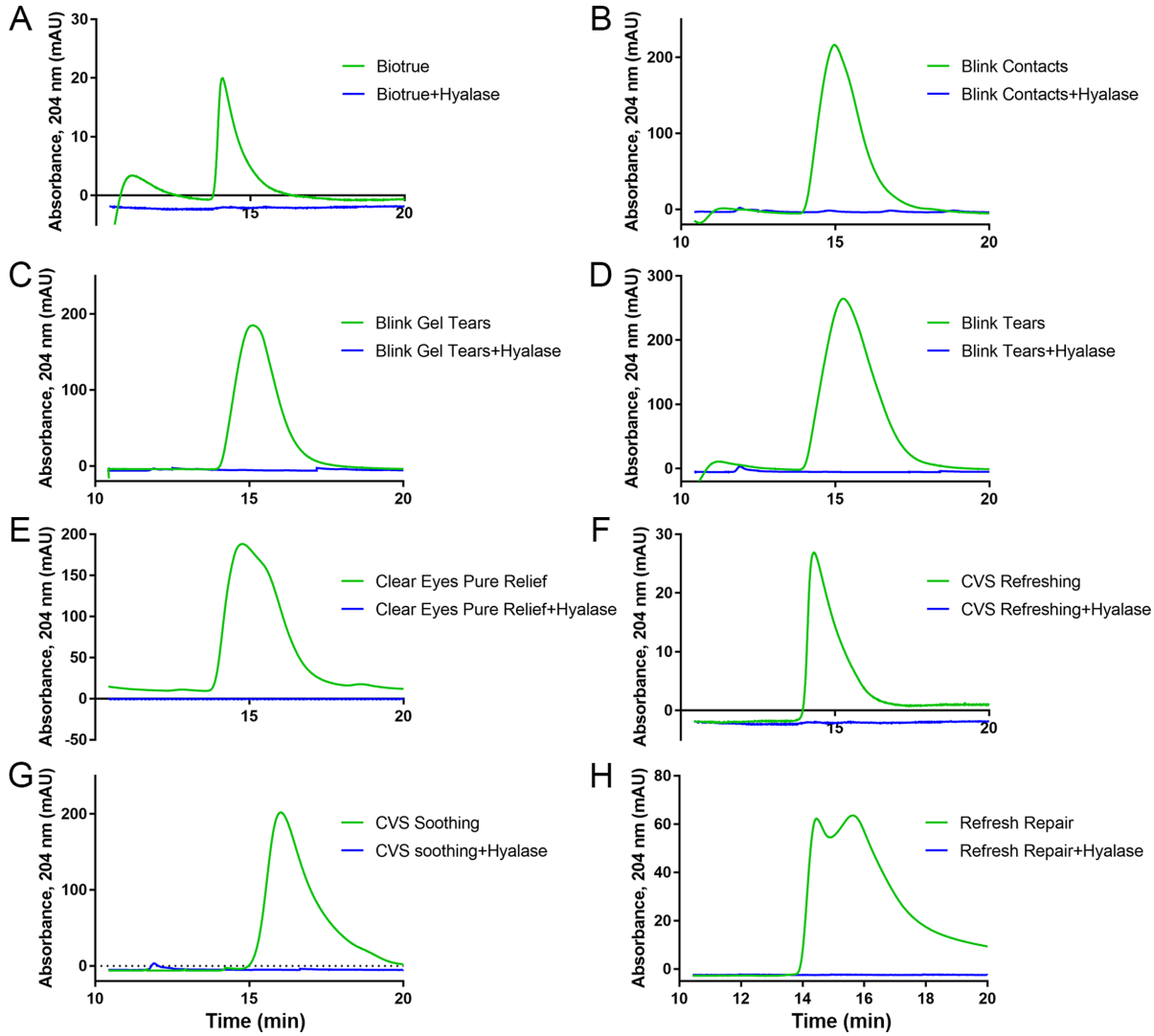

Figure 3. Identification of HA in HA-containing eye products by analyzing susceptibility to hyaluronidase (Hyalase) digestion. Eight HA-containing eye products were treated with Hyalase or not and then administered to size exclusion HPLC and monitored at 204 nm. An overlay of the chromatographic profiles of the HA-containing eye products that were treated with Hyalase or with enzyme buffer in the absence of Hyalase is presented.

Refresh Repair was discontinued and was substituted by Refresh Relieva. Our analysis by agarose gel electrophoresis revealed that both Refresh Repair and Refresh Relieva contained comparable populations of HA (Fig. 4).

HA-Containing OTC Eye Products Inhibit Corneal Epithelial Cell Migration In Vitro

Using a scratch assay model with human immortalized corneal epithelial cells, we investigated the effect of HA-containing eye products on corneal epithelial wound healing in vitro. First, the effects of HMWHA, mid-MWHA, and LMWHA alone on corneal epithelial cell migration were determined (Fig. 5A, Supplementary Fig. S1A). Our data showed HMWHA, mid-MWHA and LMWHA all promoted corneal epithelial cell migration when compared to PBS; however, only reaching significance for mid-MWHA at 16 and 24 hours (Fig. 5A, Supplementary Fig. S1A). Eye products containing HMWHA were then assayed and compared to the effects of HMWHA alone (black dashed line—Fig. 5B, Supplementary Fig. S1B). Both Clear Eyes Pure Relief and HMWHA promoted corneal epithelial cell migration, with Clear Eyes Pure Relief significantly promoting cell migration compared to control (Figs. 5A, 5B, Supplementary Figs. S1A, S1B). In comparison, all other OTC eye products containing HMWHA significantly inhibited corneal

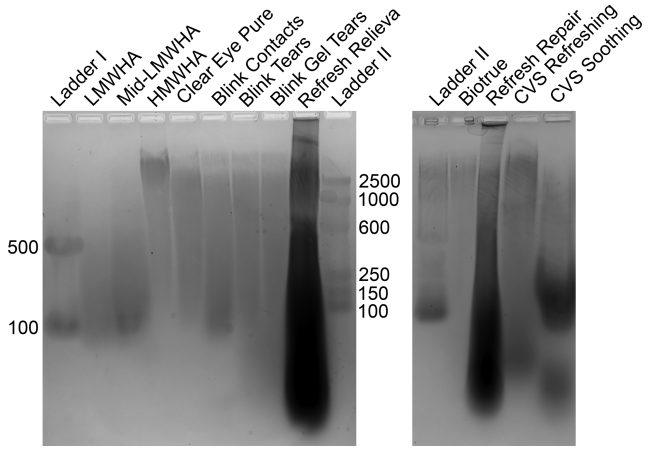

Figure 4. Characterization of the molecular weight of HA by agarose gel electrophoresis. HA standards and OTC eye products were subjected to agarose gel electrophoresis, and molecular weight was determined based on the electrophoretic profile of two HA ladders that contained HA standards of known molecular weights. The molecular weights of HA (kDa) in each ladder (I and II) are indicated next to each ladder.

Table 4. Identification of the Range of Molecular Weights of HA Within OTC Eye Drops by Agarose Gel Electrophoresis

Eye Care Product	Form of HA
Biotrue MPS Solution	HMWHA
Blink Contacts	HMWHA-LMWHA
Blink Gel Tears	HMWHA-LMWHA
Blink Tears	HMWHA-LMWHA
Clear Eyes Pure Relief	HMWHA-LMWHA
CVS Moisturizing Eyelid Spray	—
CVS Refreshing Eyelid Spray	HMWHA-ULMWHA
CVS Soothing Eyelid Spray	LMWHA-ULMWHA
Refresh Repair	Mid-MWHA-ULMWHA

epithelial cell migration, with slower wound closure when compared to both PBS and HMWHA (Fig. 5B, Supplementary Fig. S1B). When analyzing the eye products containing Mid-MWHA compared to the effects of Mid-MWHA (black dashed line), we found that all mid-MWHA-containing eye products inhibited corneal epithelial cell migration (Fig. 5C, Supplementary Fig. S1C). When analyzing the OTC eye products containing LMWHA compared to the effects of LMWHA (black dashed line), we found that CVS Soothing inhibited corneal epithelial cell migration (Fig. 5D, Supplementary Fig. S1D). Thus, with the exception of Clear Eyes Pure Relief eye, all HA-containing eye products inhibited corneal epithelial cell migration, whereas all pure forms of HA promoted migration.

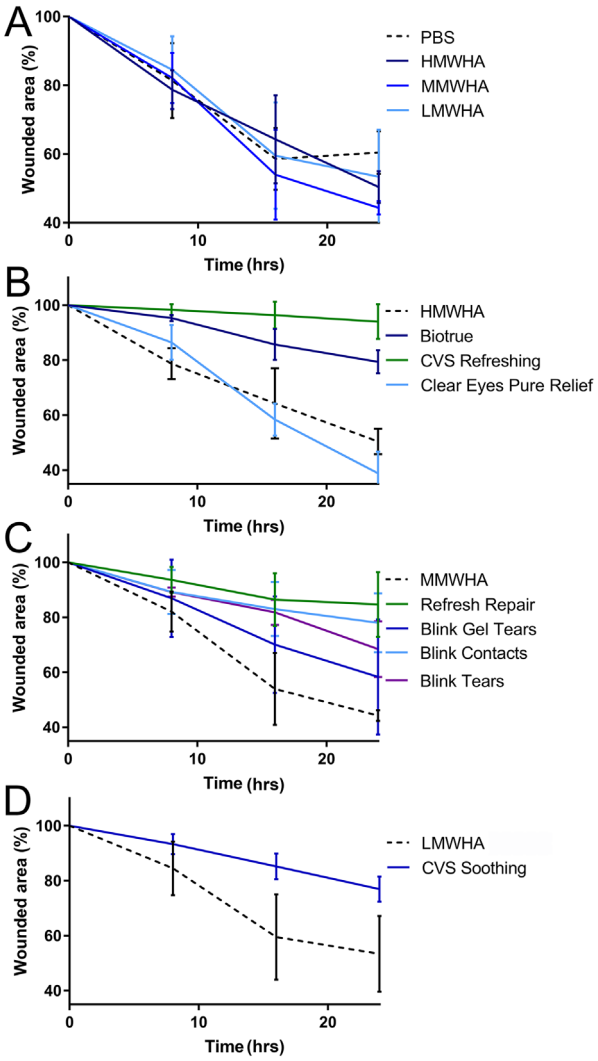

Figure 5. Effects of HA of different molecular weights and HA-containing OTC eye drops on corneal epithelial wound healing were investigated using a scratch assay. Confluent hTCEpi were subjected to scratch wounds and the cells allowed to migrate into the cell free area for 24 hours in presence of HMWHA, mid-MWHA, and LMWHA at a concentration of 0.05 mg/mL or various HA-containing OTC eye products diluted 1:10 in media. Images were captured at zero, six, 18, and 24 hours under an EVOS microscope or an LSM 800 confocal microscope (Carl Zeiss Microscopy LLC), and the cell-free area was calculated using the plugin in Image J designed by Suarez-Arnedo et al.[67] that is specific for in vitro wound healing assays. The cell-free area was calculated at each timepoint, and the wounded area was calculated as a percentage compared to zero hours. (A) Indicates the migration profile of corneal epithelial cells in the presence of HMWHA, mid-MWHA, and LMWHA compared to PBS control. (B) Indicates the migration profile of corneal epithelial cells in the presence of HA-containing eye drops that contained HA primarily in the HMWHA range compared to the HMWHA standard. (C) Indicates the migration profile of corneal epithelial cells in the presence of HA-containing eye drops that contained HA primarily in the mid-MWHA range compared to the mid-MWHA standard. (D) Indicates the migration profile of corneal epithelial cells in the presence of HA-containing eye drops that contained HA primarily in the LMWHA range compared to the LMWHA standard.

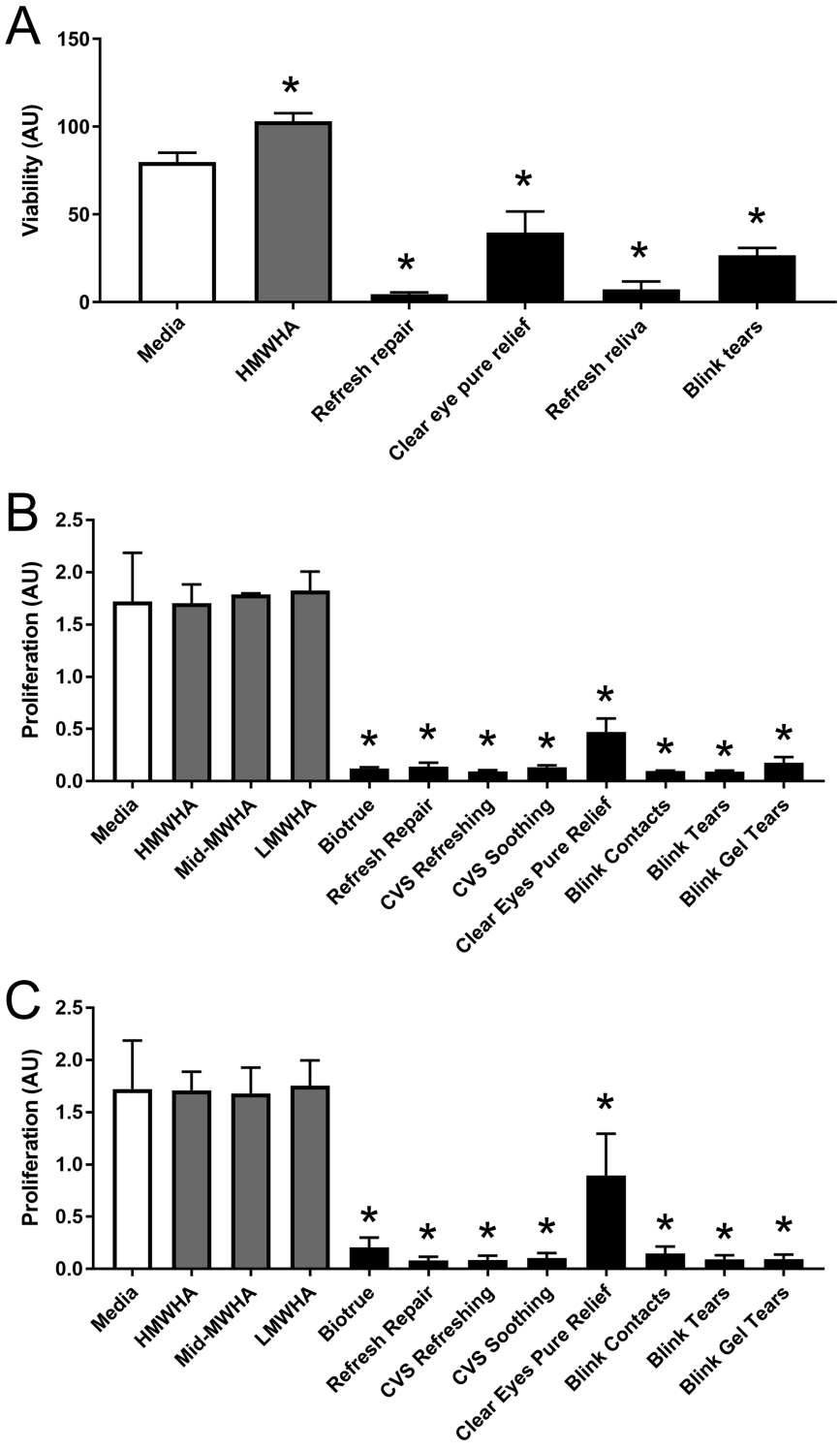

Figure 6. The effects of HA-containing OTC eye drops on corneal epithelial cell viability (A) and proliferation (B) was investigated using the CCK-8 assay. For such, corneal epithelial cells were treated with selected HA-containing OTC eye products at 1:10 and compared to HMWHA and PBS (vehicle control). *Error bars:* SD. *Asterisk* represents $P \leq 0.05$.

The Effect of HA-Containing OTC Eye Products on Corneal Epithelial Cell Viability

Given that seven of the eight eye products used in the cell migration assay significantly inhibited corneal epithelial cell migration; we proceeded to verify whether the eye products have an effect on corneal epithelial cell viability. The effects of selected HA-containing eye products on corneal epithelial cell viability were investigated using a CCK-8 assay and

compared to HMWHA and PBS. HMWHA significantly promoted the viability of corneal epithelial cells compared to the PBS control (Fig. 6A). All HA-containing eye products investigated, specifically Refresh Repair, Clear Eyes Pure Relief, Refresh relive and Blink Tears, at a concentration of 1:10, significantly inhibited the viability of corneal epithelial cells when compared to the PBS control (Fig. 6A). Refresh Repair and Refresh relive had the most significant effect on decreasing the viability of corneal epithelial cells, whereas Clear Eyes Pure Relief had the most subtle effect by only decreasing the viability of corneal epithelial cells by ~50%.

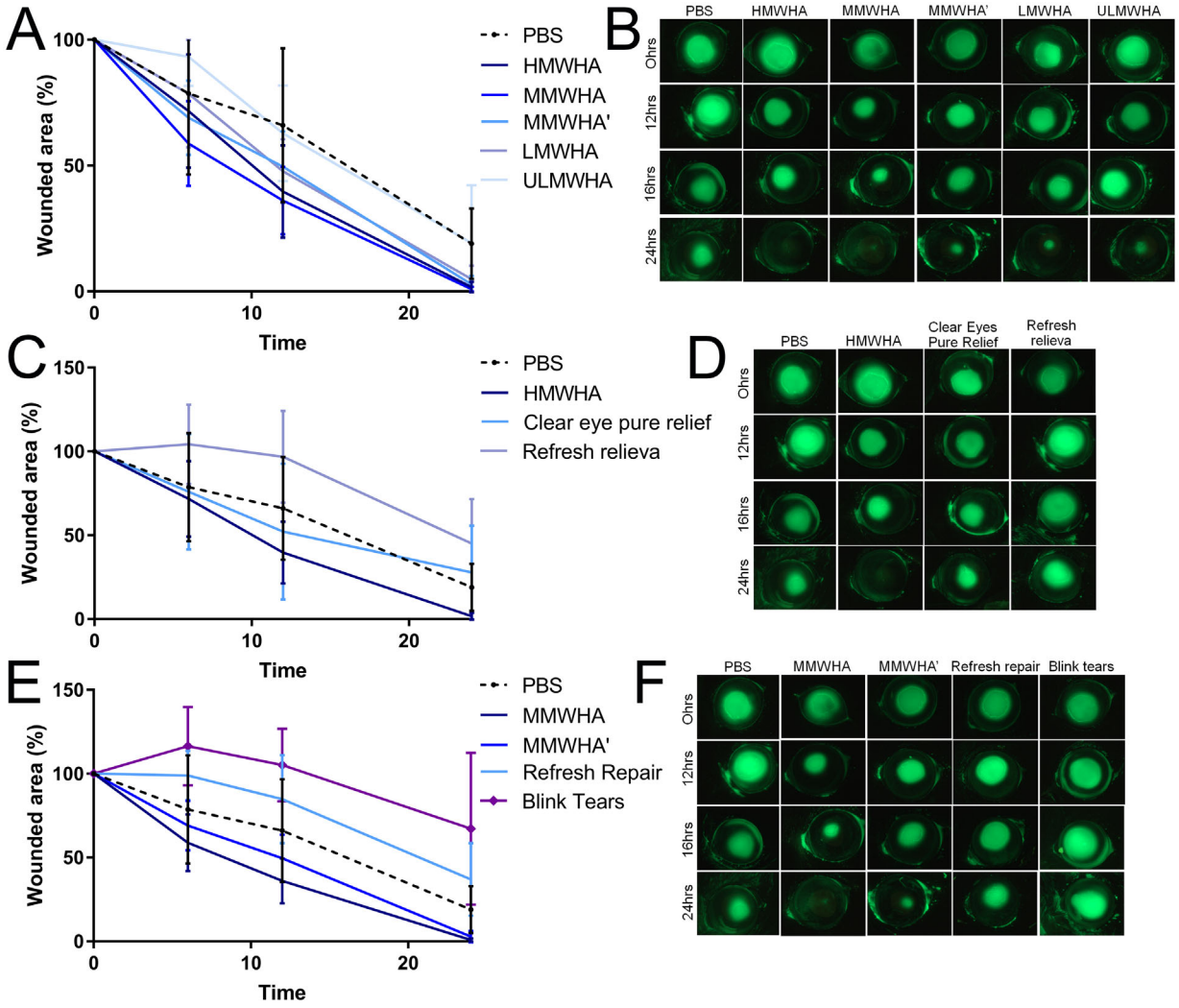

Figure 7. The effect of HA-containing eye drops on corneal epithelial wound healing in vivo. Effects of HA of different molecular weights and HA-containing OTC eye products on corneal epithelial wound healing were investigated in vivo using the debridement wounding model. For such, a 1 mm–diameter injury site was demarcated on the cornea of mice using a biopsy punch, and thereafter the corneal epithelial cells were removed using an AlgerBrush II rotating burr. Fluorescein stain was used to identify the injured area, and images were captured at zero, 12, and 24 hours. The injured area was calculated by manually drawing the wound edge as region of interest (ROI) using the polygonal selection function of the Image J software, and, thereafter, the area of the ROI was measured, and data were represented as the percentage of the wounded area remaining compared to zero hours. (A) Effects of HMWHA (2 mg/mL), mid-MWHA (2 mg/mL), mid-MWHA' (0.3 mg/mL), LMWHA (2 mg/mL), and ULMWHA (2 mg/mL) on corneal epithelial wound healing were investigated and compared to PBS, which served as the vehicle control. (B) Representative images of fluorescein-stained murine corneas that were quantified in panel A, imaged under a stereomicroscope at one, 12, 16, and 24 hours after injury. (C) Effects of eye products containing HA primarily in the HMWHA range compared to a HMWHA standard and PBS. (D) Representative images of fluorescein-stained murine corneas that were quantified in panel C, imaged under a stereomicroscope at one, 12, 16, and 24 hours after injury. (E) Effects of eye products containing HA primarily in the mid-MWHA range compared to a mid-MWHA standard at two different concentrations and PBS. (F) Representative images of fluorescein stained murine corneas that were quantified in panel E, imaged under a stereomicroscope at one, 12, 16, and 24 hours after injury.

The Effect of HA-Containing OTC Eye Products on Corneal Epithelial Cell Proliferation

The effects of the different HA-containing eye products at a dilution of 1:5 and 1:10 on corneal epithelial cell proliferation was also investigated (Figs. 6B, 6C, respectively). All forms of HA had no significant effect on corneal epithelial cell proliferation. In stark contrast, all HA-containing eye products significantly inhibited corneal epithelial cell proliferation when compared to untreated control (media) and when compared to all forms of HA. Clear Eyes Pure Relief had the most subtle effect only decreasing the proliferation of corneal epithelial cells by ~50%.

The Effect of HA-Containing OTC Eye Products On Corneal Wound Healing In Vivo

The effects of the different HA-containing OTC eye products on corneal wound healing were investigated in vivo using the mouse corneal debridement model (Fig. 7). First, the effects of HA of different molecular weights on corneal wound healing were investigated (Figs. 7A, 7B). For such, eye drops containing either HMWHA, mid-MWHA or LMWHA were maintained on the ocular surface of mice for the first two hours after a corneal debridement wound, and thereafter an eye drop was reapplied onto the ocular surface at 12 and 16 hours. PBS, HMWHA, mid-MWHA, LMWHA, and ULMWHA were used as controls. Images were collected at zero, 12, 16, and 24 hours, and the wounded area was calculated. HMWHA, mid-MWHA, LMWHA, and ULMWHA all significantly promoted the rate of wound healing when compared to the PBS control (Figs. 7A, 7B). When analyzing the effects of selected eye products containing HA in the HMWHA range on corneal wound healing, Clear Eyes Pure Relief was found to have no effect on corneal wound healing compared to PBS, whereas Blink Tears and Refresh Relieva significantly inhibited corneal wound healing (Figs. 7C, 7D). When analyzing the effects of selected eye products containing HA in the mid-MWHA range on corneal wound healing, Refresh Repair significantly inhibited corneal wound healing compared to mid-MWHA (Figs. 7E, 7F).

Discussion

A novel size exclusion HPLC method with UV detection was developed and successfully applied for characterizing the molecular weight of HA in OTC eye products listing HA as an inactive ingredient. This method provided a sensitive, accurate, and reproducible method for quantifying and characterizing the molecular weight of HA. Linear ranges were determined for HMWHA, mid-MWHA, and LMWHA and varied based on their molecular weights. HMWHA presented the most limited range, in particular, HMWHA at concentrations above 0.25 mg/mL could not be resolved using SEC HPLC in a reproducible manner. Studies have demonstrated that samples with high viscosity suffer a loss of separation performance by SEC HPLC.[68,69] This phenomenon is known as viscous fingering, or more formally, the Saffman-Taylor problem.[70] Therefore, in our study, with increasing concentrations of HMWHA, there was an intrinsic increase in viscosity, causing the chromatographic peaks to broaden and shift in retention time, resulting in poor separation in a nonlinear pattern. This phenomenon was most apparent for HMWHA and was only evident for LMWHA beyond 1.0 mg/mL. Viscous fingering does not preclude using size exclusion HPLC for characterizing the molecular weight of HMWHA; instead samples simply need to be diluted so that the concentration of HA falls within the linear ranges.

For this study, we purchased various OTC ophthalmic products that reported sodium hyaluronan as an inactive ingredient, with the goal of verifying what form of HA is currently being used in ophthalmic products and to verify the concentrations currently being used. The presence of HA was detected in eight of the nine ophthalmic products analyzed, with the form and concentration of HA varying significantly among the products. Three of the eight eye products contained primarily HMWHA. However, the concentrations ranged from 0.139 to 1.930 mg/mL, showing an approximate 15-fold variability among the products containing HMWHA. Three of the eight products contained primarily mid-MWHA, with the concentrations varying from 1.843 to 4.032 mg/mL. One of the products contained LMWHA at a concentration of 1.774 mg/mL. Last, one eye product contained a major population of both HMWHA and LMWHA, with a total HA concentration of approximately 0.321 mg/mL. We were unable to identify HA in the ninth product, most probably because it contained such a low concentration of HA that it fell below the detection threshold for our method of analysis.

In our study, all forms of HA promoted corneal epithelial migration and wound healing, with HMWHA showing the most significant increase in corneal epithelial cell viability. Thus, based on our findings, the consensus is that the HMWHA form

of HA is the preferred form for maintaining ocular health and promoting wound healing. HA has previously been reported to promote corneal epithelial migration.[71–73] ULMWHA-LMWHA (4–100 kDa) have previously been shown not to promote rabbit corneal epithelial wound healing at a concentration of 0.40 mg/mL using an ex vivo debridement model, whereas mid-MWHA-HMWHA (680-2160 kDa) significantly promoted wound healing.[74] However, at 1.0 mg/mL, both LMWHA (90 kDa) and HMWHA (2800 kDa) have been shown to significantly promote rabbit corneal epithelium wound healing in vivo.[71] Thus the effects of HA on corneal wound healing have clearly been shown to depend on the concentration and form of HA. According to the US Code of Federal Regulations, companies are only required to supply an established concentration for active ingredients within ophthalmic products. Because all eye products included in this study listed HA as an inactive ingredient, there are no regulations or guidelines to address the form or concentration of HA added to these pharmaceutical formulations. In general, the regulatory process governing the control of the composition of OTC ophthalmic products does not require a diligent approval process. This lenient regulatory process explains the vast range in molecular weights and concentrations for HA within these products. Therefore further research is warranted to support the need to regulate the form and concentration of HA within these products.

The main impediment for accurate information regarding the molecular weight of HA in OTC eye drop formulations is likely the lack of a simple, sensitive, and accurate method to characterize the molecular weight of HA in nonpurified samples. Currently, the most widely available and easily implemented method for characterizing HA is agarose gel electrophoresis.[57–59] Although it is a relatively inexpensive system and does not require highly trained personnel, it does require HA to be purified before analysis. In addition, it lacks sensitivity, requiring at least 5 µg of HA per run. A method coupling HPLC with a refractive index detector has previously been used to identify the molecular weight of HA within a linear range of 270 kDa to 2000 kDa; however, a relatively high concentration of HA (0.1–2 mg/mL) is needed, and a refractive index detector is not standard with most HPLC systems.[25,62] Multi-angle laser light scattering[60] and matrix-assisted laser desorption ionization mass spectrometry[61] have also been previously used for estimating the molecular weight of HA; however, these methods are not quantitative and require expensive equipment and highly trained personnel. More recently, Rivas and colleagues[63] established a novel highly accurate method for quantifying and characterizing the molecular weight of HA, with the capability of identifying HA ranging from below 100 kDa to more than 5000 kDa using solid-state nanopore molecular weight analysis. However, although this method allows for extremely accurate separation of HA of a vast range of different molecular weights, it requires the purification of HA before characterization, requires high concentrations of HA, and requires expensive equipment and highly trained personnel.[25,63] Therefore there is still a scientific need for an easy, accessible, and sensitive method for determining the molecular weight of HA. Herein, we describe a sensitive method for characterizing the molecular weight of HA by size exclusion HPLC. Thereafter, we used this technique to characterize the molecular weight and to determine the concentrations of HA in various OTC eye product formulations and correlated these findings with the effects of the eye products on human corneal epithelial wound healing, both in vitro and in vivo.

Our study clearly demonstrates that HA, at all molecular weights, has a beneficial effect on corneal wound healing. However, we were unable to verify whether the addition of HA to the ophthalmic products had a favorable effect on wound healing. Most of the OTC ophthalmic products we tested contained preservatives, such as sodium benzoate and sodium chloride, except for Clear Eyes Pure Relief—which came in a preservative-free, multidose bottle (Supplementary Table S1). Topically applying solutions containing 0.005% to 0.02% benzalkonium chloride (BAC), a type of preservative, or 0.50% to 2.0% boric acid, an anti-fungal ingredient, on the cornea have been shown to delay corneal epithelial migration in a rat corneal abrasion model, compared with saline solution.[75] The concentration ranges of BAC and boric acid in this study are commonly used in OTC eye drops.[75,76] Repetitive topical application of 0.20% BAC has been shown to negatively affect the corneal epithelium by increasing apoptosis of corneal epithelial cells and increasing the expression of inflammatory cytokines, such as tumor necrosis factor-α.[77] When combining preservatives with HA in eye drops, the residence time of preservatives on the cornea may be prolonged since HA can significantly increase the viscosity of the fluid.[78] In fact, the viscosity of a 10 mg/mL HA solution is ~5000 times higher than H_2O.[78,79] As part of the tear film, HA would contribute to the tear film residence time.[80,81] Combining 0.002% BAC with HA has been shown to have a more significant effect on delaying corneal epithelial wound healing in vivo when compared to BAC solution alone, whereas eye drops containing HA without BAC significantly promote corneal epithelial wound healing.[79] In accor-

dance, we hereby show that applying eye drops that contain HA and preservatives reduced corneal epithelial viability, proliferation, migration in vitro, and corneal epithelial wound closure in vivo, whereas the application of HA in the HMWHA, mid-MWHA, and LWMHA forms all significantly promote corneal wound healing. Additionally, the product Clear Eyes Pure Relief, which claims to be "preservative free" was the only eye drop that promoted corneal epithelial migration in vitro, and did not delay corneal wound healing in vivo. Therefore the observed toxic effects of the ophthalmic products analyzed that culminated in delayed corneal wound healing were likely caused by the preservatives present within the products. Unfortunately, the addition of preservatives to the eye drops at unknown concentrations made it hard gauge the physiological properties of HA within them. An additional study comparing the preservative free options would help provide further insight.

In the United States, OTC artificial tears are regulated without requiring a diligent approval process.[45] If an eye drop is considered an "ophthalmic demulcent," it can be registered based on containing established concentrations of specified active ingredients, such as dextran and gelatin (US CODE OF FEDERAL REGULATIONS TITLE 21 CHAPTER I, SUBPART D PART 349).[45] However, details and concentrations for components listed as inactive ingredients, which include any substance in the ophthalmic demulcent other than the active ingredients, are not required to be listed. This results in substantial variations in the form and concentration of HA within the different OTC eye drops. However, as previously established, the physiological roles of HA differ drastically based on the molecular weight and physiological concentrations. Substantial research has shown that the therapeutic potential of HA formulations is highly dependent on using the correct molecular weight and concentration for each application.[24] For example, the American Academy of Orthopaedic Surgeons has observed that randomized placebo-controlled clinical studies using HMWHA provided an increased therapeutic benefit when compared to LMWHA, reinforcing the fact that distinctions must be made between LMWHA and HMWHA when developing HA based treatments. Our data supports the notion that the form and concentration of HA should be stated in OTC eye product formulations.

In conclusion, our study established a rapid, sensitive, accurate, and reproducible method for characterizing HA in pharmaceutical solutions, particularly eye care products. This quantitative method was then successfully used to characterize the molecular weights and concentrations of HA in various commercially available OTC eye products. This size exclusion HPLC method has the potential to simplify standardization of the HA added to ophthalmic products, offering an easy process for quality control for all HA-containing products. The physiological effects of these OTC eye products were tested in vitro and in vivo and compared to HA standards of the same molecular weight range, revealing detrimental effects of the ophthalmic products on corneal epithelial wound healing, irrespective of the type of HA in its formulation. The observed detrimental effects are likely related to the preservatives in the ophthalmic products, which warrant further studies. All forms of HA promoted corneal epithelial wound healing, with HMWHA having the most beneficial effect, demonstrating that preservative-free HMWHA-containing eye drops are a good option as supportive care for ocular surface conditions such as corneal abrasions.

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* LN and XL contributed equally.

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