Novel Starch Based Copolymers (DiHydroxyl Starch) as Ocular Lubricants: Synthesis and Characterizations

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ABSTRACT: Dry eye syndrome is an ocular tear deficiency disorder that affects millions of people in the United States. It has been recognized as a significant lifestyle issue and is among the most frequently established diagnoses in ophthalmology. Tear substitutes (or artificial tear formulations) are the mainstay of dry eye therapy. While many commercial products are available, their efficacy is limited because of their short retention time in the eye, and/or low patient acceptance. In this study, our objectives were to obtain water soluble products with opened glucose rings along the starch backbone, and tear substitute formulations based on these products with strengthened mucoadhesion, shear thinning behavior, improved tear film stability, and no irritancy to the eye. To this end we have synthesized a series of starch based copolymers, DiHydroxyl Starches (DHS). This modification of starch is not a new science, but the application of these polymers to tear substitutes is novel. These polymers were characterized as ocular lubricants utilizing both *in vitro* and *in vivo* testing. Specifically, mucoadhesion and rheological behavior were examined. Their performance as ocular lubricants was evaluated utilizing the tear-film break up times of rabbits before and after application of the copolymers. In addition, the effect of autoclaving and ocular toxicity were tested. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 343–351, 2010

Key words: biological applications of polymers; biopolymers; starch; dry eye; tear substitute

INTRODUCTION

Brewitt and Sistani[1] have defined dry eye as a disease of the ocular surface attributable to disturbance of the natural function and protective mechanisms of the ocular surface causing tear film instability during the open eye state. Clinically dry eye has two major causes: aqueous deficiency and increased evaporation. No matter the underlying cause, chronic desiccation of the ocular surface stimulates an inflammatory reaction, producing chronic ocular surface injury. The goals of dry eye therapy are a reduction of symptoms, an improvement of tear-film quantity and quality, and reversal of ocular surface damage.

Tear substitutes are the most commonly utilized therapeutic modality. They increase humidity at the ocular surface and improve lubrication. An effective tear substitute formulation should meet the following criteria: (1) it should have adequate retention time in the eye, contributed by either increased viscosity and/or adhesion with the mucin in the eye, (2) it should improve the tear film stability, and (3) it should cause no irritancy to the eye. A variety of components are used to formulate a considerable number of commercially available preparations. Cellulose ethers are the most commonly utilized agents in tear substitutes because of their retention times on the ocular surface.[2] Sodium hyaluronate has been found particularly beneficial for corneal wound healing.[3] Carbomers provide excellent adhesive behavior and increase retention time.[4] Recently, lipid containing formulations have been created to restore the lipid layer.[5]

In this study, our objectives were to obtain water soluble products with opened glucose rings along the starch backbone, and tear substitute formulations based on these products with strengthened mucoadhesion, shear thinning behavior, improved tear film stability, and no irritancy to the eye. To this end we have synthesized a series of starch based copolymers, DiHydroxyl Starches (DHS). These polymers were characterized as ocular lubricants utilizing both *in vitro* and *in vivo* testing. Specifically, mucoadhesion and rheological behavior were examined. Their performance as ocular lubricants was evaluated utilizing the tear-film break up times of

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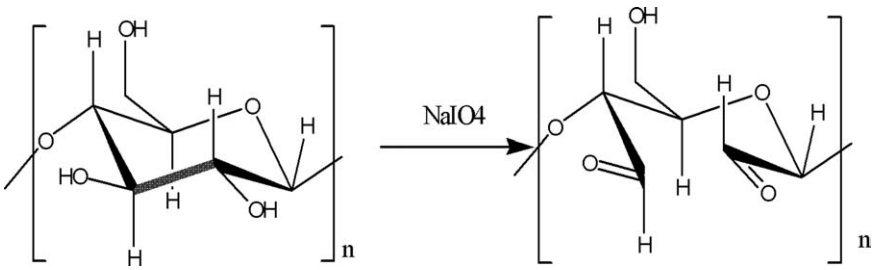

Figure 1 Periodate oxidation of starch.

rabbits before and after application of the copolymers. In addition, the effect of autoclaving and ocular toxicity were tested.

EXPERIMENTAL SECTION

Synthesis

Dialdhyde starch (DAS) was prepared by oxidizing 8.1 g native waxy maize starch powder from Cargill, suspended in 400 mL water with sodium periodate at ambient temperature in the dark (Fig. 1). The characterizations of native waxy maize starch have been provided by Cargill, the sample supplier. The native waxy maize starch has 1–5% of amylose (linear chains) and 95–99% of amylopectin (branched chains). The molecular weight of amylose is less than 0.5 million, and the molecular weight of amylopectin is between 50 and 500 millions. Solubility (2.5% db) at 60, 75, and 90°C for waxy starch was 0.6, 5.2, and 8.8 respectively. Sodium periodate was added at 1.2 times the theoretical amount (12.8 g) for preparation of completely oxidized starch (100DAS). Partially oxidized starch was prepared by adding sodium metaperiodate at 0.6 and 0.2 times the theoretical amount (2.1 and 6.4 g respectively). The oxidant concentration of each solution prepared was 0.025, 0.075, and 0.15*M*, respectively. The oxidation was performed with magnetic stirring for 6 h. Oxidized products were recovered by filtering and washing at least three times with 400 mL distilled water to remove inorganic salts.

Oxidized product (DAS) was added to a solution containing sodium borohydride at 1.5 times the theoretical amount (Fig. 2). The reduction was performed at room temperature with magnetic stirring for 2 h. The excess borohydride was destroyed with acetic acid. Both 100DHS and 60DHS were completely soluble in water. The 20DHS was only partially soluble and thus was separated through filtration into a supernatant solution and an undissolved portion. All solutions were dialyzed against distilled water though cellulose membrane with 12,400 MWCO (molecular weight cut off) to remove inorganic salts, then concentrated and dried to recover the products. The large molecular weight cut off membrane (MWCO = 12,400) was chosen to remove not only inorganic salt but also produced short chains during starch modification. To perform as a tear substitution ingredient, the polymer should be able to obtain desired viscosity and mucoadhesion, so its molecular weight should be higher than certain level. This is why we used the membrane with large MWCO in purpose to remove short chains. The undissolved portion of 20DHS was suspended in distilled water, filtered, thoroughly washed with distilled water, and then dried to a constant weight.

Carbonyl content determination

Measurement of the aldehyde groups in DAS was achieved by quantitative reduction with sodium borohydride. This relatively rapid method used by Lindberg and Misiorny[6] for estimation of reducing monosaccharides was found to be convenient and applicable for the analysis of the DAS samples.

A DAS sample of 0.10 g was placed in a flask with 0.0280 g sodium borohydride, which was more

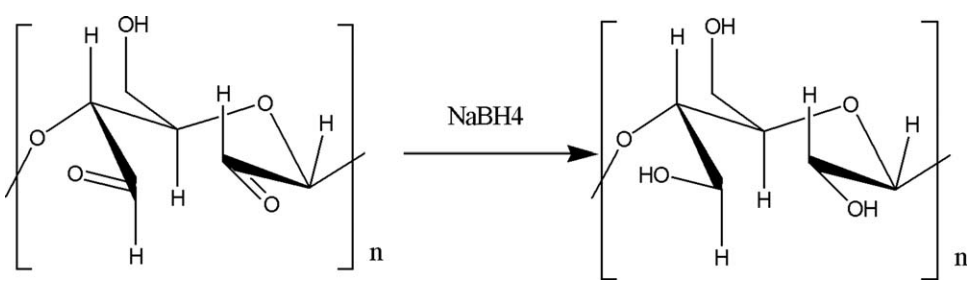

Figure 2 Borohydride reduction of DAS.

than the theoretical amount. A 100 mL buret filled with water was connected to a flask to measure the volume of evolved hydrogen. The reduction was performed at room temperature with magnetic stirring for 2 h. The stirring was stopped and the hydrogen volume recorded before it was released. Excess acetic acid was then added to the flask to destroy unreacted sodium borohydride, and the volume of evolved hydrogen was recorded. The sum of both recorded hydrogen volumes was the volume of hydrogen generated by the portion of sodium borohydride, which did not react with DAS. In a separate flask, a blank experiment without DAS was performed under the same conditions. The difference in total hydrogen volume between the sample and the blank was the amount of sodium borohydride reacted with DAS, and therefore the carbonyl content.

FTIR

A Perkins Elmer System 2000 FTIR was used to characterize all samples. Samples were pressed in KBr pellets and run for various numbers (16–100) of scans to achieve high quality spectra. The wavelength range was between 4000 cm^{-1} and 450 cm^{-1}.

Molecular weight

The molecular weight distribution of all synthesized polymers was determined by gel permeation chromatography (GPC) at room temperature. DMSO was the solvent and Pullulan was the standard.

Mucoadhesion

Determination of mucoadhesive bond strength is important in the development of ophthalmic solutions, because it can quantitatively compare different mucoadhesive materials. Hassan and Gallo[7] are considered to have pioneered the work on the rheological assessment of mucin-polymer bioadhesive bond strength. They observed a synergistic increase in apparent viscosity, when a mucoadhesive polymer and mucin were mixed together. The apparent viscosity of a dispersion containing mucin and a bioadhesive polymer is determined by the contribution of the different components:

$$\eta_t = \eta_m + \eta_p + \eta_b$$

where η_t: apparent viscosity of the system, cP η_m: apparent viscosity of mucin, cP η_p: apparent viscosity of polymer, cP η_b: bioadhesion apparent viscosity, viscosity component because of bioadhesion, cP

Therefore, the bioadhesion apparent viscosity (or mucoadhesion apparent viscosity) can be obtained by rearranging the above equation:

$$\eta_b = \eta_t - (\eta_m + \eta_p)$$

For these two equations to be valid, η_t, η_m and η_p should be measured at the same concentration, temperature, time, and shear rate. In this study, steady state controlled rate flow curves, with shear rate varying from 15 to 300 1/s, of polymer solutions, 5% mucin dispersion, and the mixture of polymer and mucin were collected. η_b was calculated and used as a direct estimate of the strength of mucoadhesion for ophthalmic solutions.

Rheology

The flow properties of the samples were examined using a Haake RS100 RheoStress (Thermal Fisher Scientific, Waltham, MA) equipped with a Haake circulation bath and temperature controller. All experiments were performed at 25°C. Steady state flow curves were recorded automatically. Although the shear rate associated with the eye has been estimated to range from 0 to as high as 28,500 L/s,[8] we tested the apparent viscosity at shear rates varying from 15 to 300 L/s because of internal limitations of this rheometer. The limitation is typical for rotational rheometers.

Tear break-up time (TBUT)

All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee, and were performed in compliance with the The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. The synthesized polymer solutions were tested at varied concentrations. Commercial products (Advanced Eye Relief™ from Bausch & Lomb and Systane™ from Alcon) and solutions of commonly used commercial polymers (carboxymethylcellulose, hydroxylpropyl methylcellulose, and sodium hyaluronate) were also tested. There were 10 female, New Zealand white rabbits in each group that initially received balanced salt solution (BSS) and then a test solution. Each rabbit was anesthetized by mask administration of isoflurane and oxygen. One drop (50 μL) of BSS containing 1.25 mg/mL of fluorescein sodium was administered to one eye of each rabbit once. The lid was manually closed twice to distribute the fluorescein stain. The lids were then manually held open for measurement. Immediately after the second blink a timing instrument was started. The tear film was scanned using a broad beam slit lamp (Kowa SL-15) in a darkened room using a cobalt blue filter. The timer was

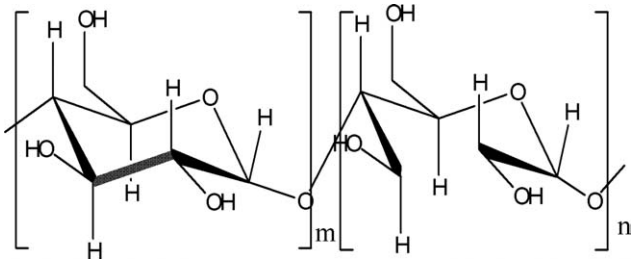

Figure 3 Chemical structure of glucose-*c*-DHS.

stopped when the film disruption first occurred. The ocular surface was flushed with BSS and the test polymer formulation was then tested utilizing the same procedure. The final TBUT was reported as the TBUT of the test substance minus the TBUT of BSS to minimize interindividual differences.

Effect of autoclaving

The influence of sterilization by autoclaving was studied to test the stability. In this study, viscosity was used to assess the solutions' stability. To test the effect of autoclaving, viscosities of the same solutions were recorded before and after autoclaving and compared thereafter.

EpiOcular™ test

The effects of the polymers on epithelial cell viability was determined using an MTT (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay and the EpiOcular™ Tissue Model (MatTek Corporation, Ashland, MA). The EpiOcular™ Tissue Model consists of a multilayered structure of stratified human keratinocytes on cell culture inserts. All testing was performed in triplicate by a masked investigator. The 20DHS and 100DHS polymers were tested at concentrations of 1, 3, 5, and 10%. The negative control was ultrapure water. The positive control was 0.3% Triton X-100. The polymers and the MTT solution were also combined and incubated for an hour at room temperature to ensure that the polymers could not directly reduce MTT leading to false viability measurements in the MTT assay.

100 μL of the test substance was added to a tissue culture insert, which was then incubated at 37°C and 5% CO_2 for 256 min. After exposure the test material was removed and the tissue culture insert rinsed with PBS. The tissue culture inserts were then placed in the MTT solution for 3 h at 37°C and 5% CO_2. The tissue culture inserts were removed from the MTT solution, rinsed in PBS, and immersed in 2.0 mL of extractant solution. The extraction took place overnight at room temperature in the dark in a sealed plastic bag. A total of 200μL of each extractant solution was then transferred to 3 wells of a 96-well plate. The optical density at 540 nm was determined using a 96-well plate reader. The 200μL of extractant solution was used as a blank.

The percentage viability was determined by the following equation:

$$\% \text{ viability} = 100 \times [\text{OD(sample)}/\text{OD(negative control)}]$$

where OD = optical density

The percent viability for each polymer was compared to the percent viability for ultrapure water (negative control) utilizing a Student's *t*-test with significance set at $p < 0.05$.

RESULTS AND DISCUSSION

The DHS was prepared through a two-step method. First, in the periodate oxidation step, the starch ring was opened between C-2 and C-3, which formed a dialdehyde structure (DAS). Then, the dialdehyde was reduced and hydroxyl groups were formed at C2 and C3 which increased water solubility. The product was denoted as DHS (Fig. 3). By varying the ratio between starch and oxidant in the first step, the content of dialdehyde groups was controlled. In this article, a number along with DAS or DHS represents products with different percentages of modification. For example, 20DAS means periodate oxidized starch with 20% of its ring open. The borohydride reduction product of 20DAS is called 20DHS.

By effectively controlling the periodate oxidation step, copolymers were formed that possess both the rigidity of the glucose ring and the flexibility of the open ring structures. Three final products: 20DHS, 60DHS, and 100DHS were synthesized. The carbonyl contents of periodate oxidized starches were measured to study how much anhydride glucose rings have been opened within 6 h. Also, the FTIR spectrums of final products were collected to make sure all aldehydic functions created by ring opening have been reduced within 2 h. They are described as follows.

Carbonyl content

The carbonyl content of the polymers is listed in Table I. The carbonyl contents of 20DAS and 60DAS

TABLE I
Dialdehyde Unit Content of DAS

Sample #	N(NaIO4)/ N(AGU)	% Dialdehyde	Std. Dev.
1	20	22.18	0.02
2	60	63.34	0.08
3	100	98.66	0.05

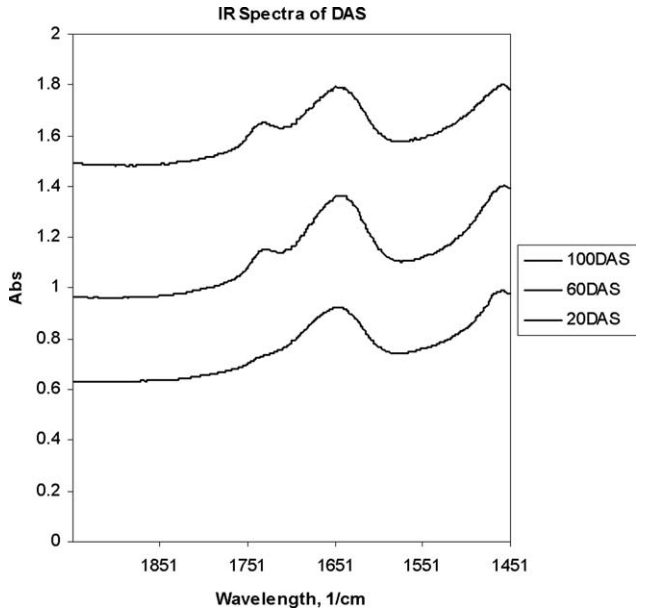

Figure 4 FTIR spectrum of DAS with increasing percentages of modification.

are slightly higher than the theoretical values because of moisture in the starch. However, the carbonyl content of 100DAS is slightly lower than 100%. This likely occurred because complete oxidation of the starch required longer than 6 h. However 98.66% yield is acceptable for this study.

FTIR

In Figure 4, the spectra of DAS with 20, 60, and 100% modification are shown. The top curve is the spectra of 100DAS, the middle curve is that of 60DAS, and the bottom curve is that of 20DAS. The different heights of the carbonyl peak around 1730 L/cm indicate the various degrees of modification were achieved as expected. The 20DAS shows a very weak carbonyl peak, because only 20% repeat units of starch were oxidized. The 60DAS shows a stronger carbonyl peak, and the 100DAS has the strongest carbonyl peak.

In Figure 5, the spectra of starch, 100DAS, and 100DHS are shown. The top curve is the spectra of starch, the middle curve is that of 100DAS, and the bottom curve is that of 100DHS. As aforementioned, a band around 1730 cm^{-1} in the 100DAS spectra represents stretching of the C=O group created by periodate oxidation. The disappearance of the band around 1730 cm^{-1} in the 100DHS spectra is caused by the change of aldehydic functions to alcoholic functions during borohydride reduction.

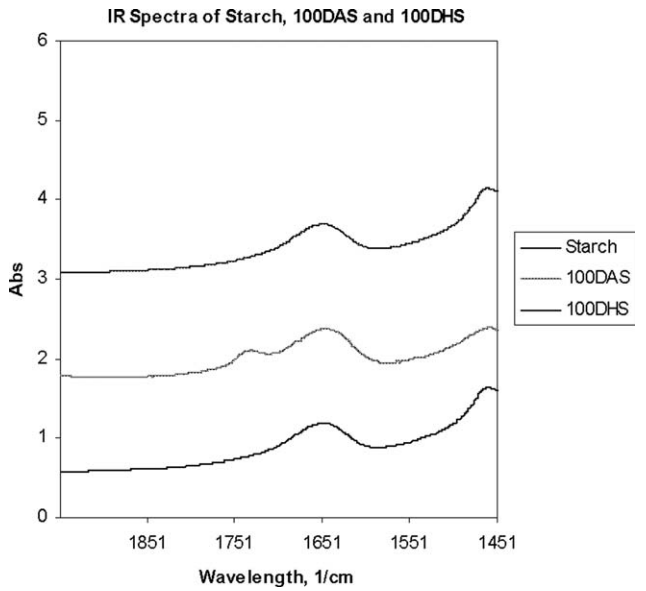

Figure 5 FTIR spectrum of starch, 100DAS, and 100DHS.

TABLE II
Molecular Weight as Determined by GPC

Sample	Mn, Da	Mw, Da	PDI
20DHS	878,000	1,047,400	1.19
60DHS	151,800	351,600	2.32
100DHS	139,000	371,600	2.67

Molecular weight

The calculated average molecular weights are shown in Tables II. Because the molecular weight of the native waxy starch is beyond the upper limit of the GPC column we used, only the results of DHS are shown in Table II. Among them, 20DHS has the highest molecular weight, followed by 60DHS and 100DHS, respectively. This is caused by degradation during starch modification. The more reactants used, the more starch was degraded. Therefore DHS products have different molecular weights as measured. Theoretically, 100DHS should have much lower average molecular weight than 60DHS because of the contribution of different amounts of degraded short chains during modification. A total of 100DHS should have much more low molecular weight short

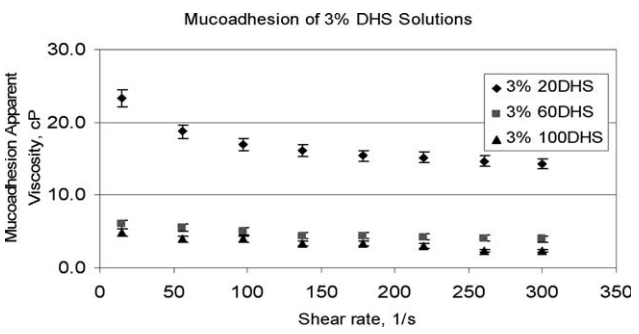

Figure 6 Mucoadhesion comparison of 3% DHS solutions.

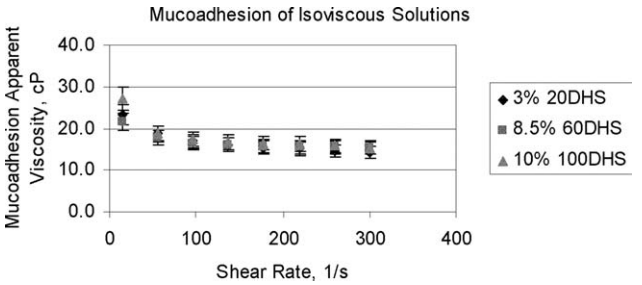

Figure 7 Mucoadhesion comparison of isoviscous DHS solutions.

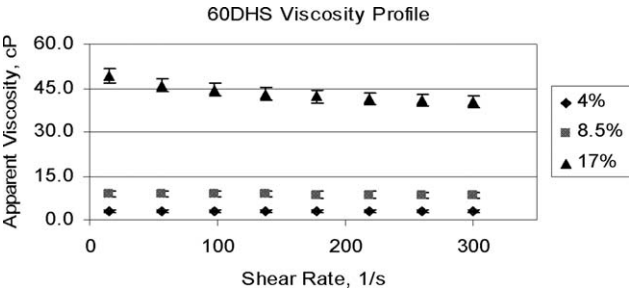

Figure 9 Rheogram of 60DHS.

chains than 60DHS, because the more reactants added, the more polymer chains degraded. However, since the membrane with large MWCO was used, so that lots of short chains were removed from the products. This explains why the Mw of 60DHS was so close to Mw of 100DHS.

Mucoadhesion

Bioadhesion, and more specifically mucoadhesion, is an important polymer property that impacts the adhesion of glycoproteins to epithelial surfaces. In this article, the mucoadhesion strength was measured with a rheological method pioneered by Hassan and Gallo,[7] which has been broadly applied by other researchers.[9] Hassan and Gallo observed that there was a synergistic increase in viscosity when a mucoadhesive polymer and mucin were mixed together. Determination of mucoadhesive bond strength allows quantitative comparisons between varied mucoadhesive materials. The mucoadhesive strength of our DHS solutions was determined by calculating the apparent bioadhesion viscosity, η_b. The mucoadhesive strength of 3% solutions of 20DHS, 60DHS, and 100DHS solutions are shown in Figure 6.

It is apparent from this figure that all DHS solutions exhibit mucoadhesion with positive η_b contributed by adhesion. This occurs because of the diffusion of DHS and mucin chains into each other and the hydrogen bonds formed between DHS and mucin, because of the substantial numbers of hydroxyl groups in both materials. The difference in mucoadhesive strength between 20DHS, 60DHS, and 100DHS solutions at the equal concentrations should be caused by the difference in molecular weight.

Since DHS solutions at equal concentrations exhibited different mucoadhesive strengths, the polymers were further tested as follows. Solutions of 8.5% 60DHS and 10% 100DHS were prepared to have viscosity similar to that of 3% 20DHS at certain shear rate range (20–300 L/s). The mucoadhesion of the three solutions are compared in Figure 7. It should be noted that upon matching the viscosity, solutions of 20DHS, 60DHS, and 100DHS exhibited the similar mucoadhesive strength.

Rheology

Viscosity is a measure of resistance to flow and is a function of the molecular attraction that resists flow. The relationship between the retention time of an ophthalmic solution and viscosity must be examined. Blinking causes a high shear rate to solution on the ocular surface. If the solution viscosity is too high, a high shear stress between the solution and the ocular surface occurs, which causes irritation and possibly damage. Between blinks the shear rate approaches zero, if the solution viscosity is too low, the ophthalmic solution will not be retained on the ocular surface. Therefore, how the apparent viscosity changes as a function of shear rate is an important rheological parameter. In this study, our goal was to

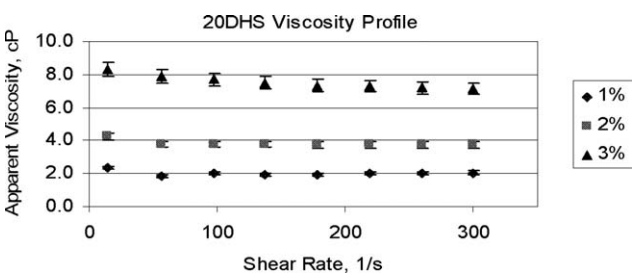

Figure 8 Rheogram of 20DHS.

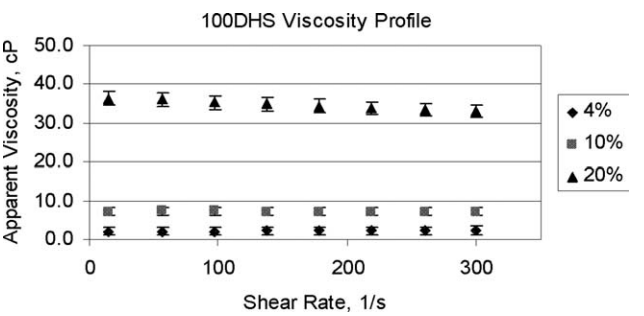

Figure 10 Rheogram of 100DHS.

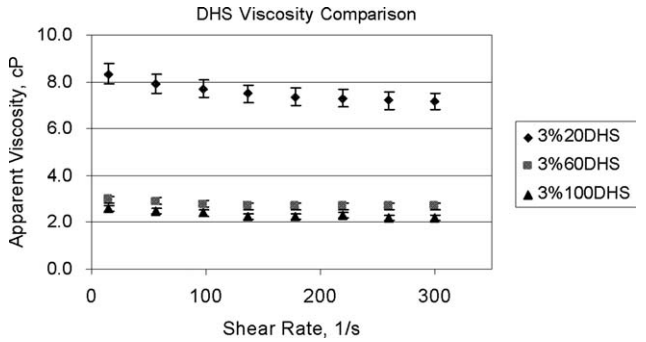

Figure 11 Viscosity comparison of DHS solution at the same concentration.

create polymers with relatively high viscosity under low shear rate and relatively low viscosity under high shear rate. Figure 8, 9 and 10 demonstrate the rheograms of 20, 60 and 100DHS solutions at varied concentrations.

The viscosities of all solutions were concentration dependent, and shear-thinning behaviors were observed for most formulations. Figure 11 shows the rheograms of 3% 20DHS, 60DHS, and 100DHS solutions. Among them, 20DHS showed the highest viscosity, followed by 60DHS and 100DHS, respectively. This difference is most likely due to the different molecular weights of the solutes.

The relationship between apparent viscosity and shear rate was studied for all samples using the Ostwald model.

$$\eta = K\dot{\gamma}^{n-1}$$

where: η: apparent viscosity, cP $\dot{\gamma}$: shear rate, 1/s, K: consistency index, cPs^{n-1}, n: flow index, dimentionless.

When $n > 1$, $n = 1$, and $n < 1$ the flow patterns denote shear-thickening, Newtonian and shear-thinning patterns, respectively. n, as well as K, were estimated by linear regression according to the Ostwald equation and are listed in Table III.

TABLE III
Regression of Steady Shear Flow Data

Polymer	K (Pa s)	n	R^2
1% 20DHS	0.0025	0.97	0.99
2% 20DHS	0.0048	0.96	0.99
3% 20DHS	0.0096	0.95	0.98
4% 60DHS	0.0029	1	0.97
8.5% 60DHS	0.0092	0.99	0.98
17% 60DHS	0.065	0.93	0.99
4% 100DHS	0.0019	1	0.99
10% 100DHS	0.0073	0.99	0.97
20% 100DHS	0.041	0.96	0.98

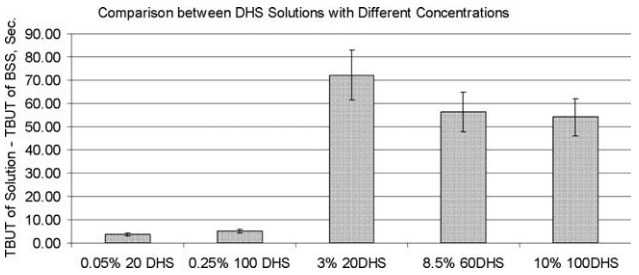

Figure 12 TBUT comparison of DHS solutions with different concentrations.

The Oswald model is appropriate here since all of the correlation coefficients (R^2) are higher than 0.97, indicating a good fit to the data. In accordance with the viscosity data shown in above figures, the consistency index also increases with an increase in the concentration. Shear-thinning behavior was observed for most solutions. Because of the equipment limitation, we were only able to measure the viscosity under shear rate no less than 15 L/s. It is likely that increasing the shear rate range to low values of the shear rate would generate lower values of the flow behavior index. The viscosity drops more quickly with a dilute solution than that of a solution with higher concentration. This explains why n decreases with all DHS solutions when concentration is increased.

TBUT

The normal tear film is continuous and maintained by blinking. If the eye is held open, the tear film will start to break apart. The eye will feel uncomfortable and the individual will blink to redistribute the tear film. In subjects with dry eyes the tear film is unstable. Therefore the tear breakup time in subjects who have dry eyes is shorter. However, an effective artificial tear can extend the tear breakup time.

Solutions of 20, 60, and 100DHS were tested at varied concentrations (Fig. 12) and compared with commercially available products (Fig. 13).

Solutions at higher concentrations had significantly longer TBUT than those at lower concentrations. This occurs for multiple reasons. High

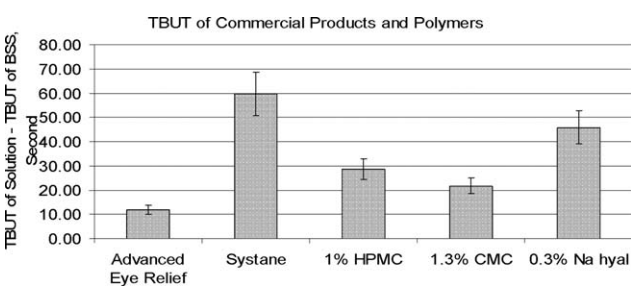

Figure 13 TBUT of commercial products and polymers.

TABLE IV
Viscosity Drop After Autoclaving

Sample	Viscosity drop%, shear rate = 300 1/s
3% 20DHS	41.6
8.5% 60DHS	28.4
10% 100DHS	17.5
1.3% CMC	20.7

concentration solutions, when compared to lower concentration solutions, have higher viscosities, shorte relaxation times, and stronger mucoadhesion. Therefore a higher concentration solution resists the shear caused by blinking and is retained on the ocular surface.

Effect of autoclaving

The purpose of this test was to study the effect of autoclaving (121°C, 20 min) on the polymer molecular weight. In this test the viscosity was measured as an indicator of the molecular weight change. DHS solutions were tested at pH 7. The viscosity of these samples was measured with shear rate set at 300 1/s by a Haake rheometer. The viscosity measurement was repeated twice before and after autoclaving and the results are shown as follows.

Besides fixed shear rate viscosity change was recorded as above (Table IV), we also measured the rheological property change before and after autoclaving. The rheograms of tested solutions are shown as follows (Figs. 14, 15, 16).

A drop in the viscosity was observed with all DHS solutions, with 20DHS having the largest drop indicating that hydrolysis occurred under the high temperature and pressure conditions. However, this hydrolysis should not affect the performance of ophthalmic solutions too much, as long as autoclaving does not impact their rheological properties too much. We also observed that the viscosity drop takes place in CMC solutions, which is one of the most commonly used polymers in ophthalmic solutions.

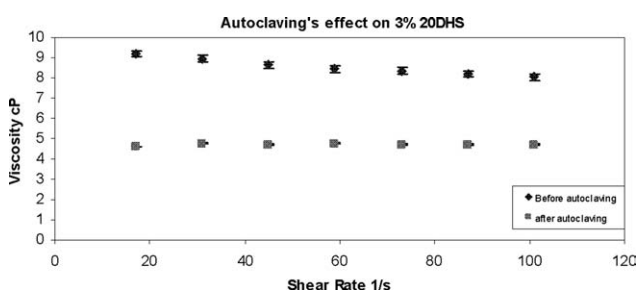

Figure 14 Rheological property comparison of 20DHS before and after autoclaving.

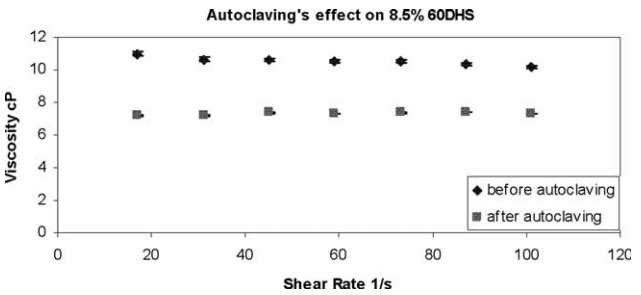

Figure 15 Rheological property comparison of 60DHS before and after autoclaving.

Ocular toxicity

All of the polymers demonstrated greater than 80% viability after 256 min of exposure (Fig. 17). Greater than 80% viability corresponds to a Draize eye irritation score of 0–15, which is considered nonirritating.[10] The positive control demonstrated 2% viability. Significant differences were noted between the viability of the negative control when compared to the positive control ($P = 1\times10^{-11}$), 5% 20DHS ($P = 0.007$), and 10% 20DHS ($P = 0.0006$).

CONCLUSIONS

In summary, we synthesized a series of starch based polymers whose modifications were characterized utilizing FTIR, NMR, and chemical methods. The degree of modification was controllable by varying reaction conditions. The products were water soluble. Solutions of these polymers demonstrated the essential characteristics of ocular lubricants. There was strengthened adhesion with mucin, which resulted in prolonged ocular retention times, when in contact with mucin; the polymers demonstrated excellent rheological properties of a tear substitute, which aids in ocular retention and stabilitization of the tear film. They also showed certain stability during autoclaving and low ocular toxicity. *In vivo* tear

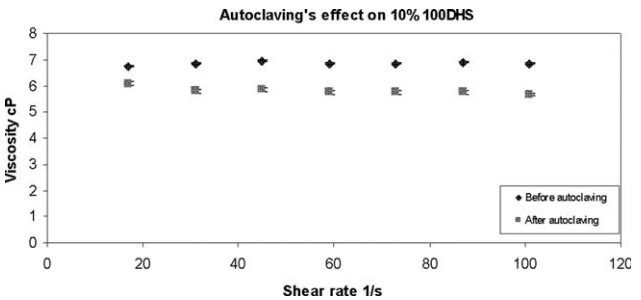

Figure 16 Rheological property comparison of 100DHS before and after Autoclaving.

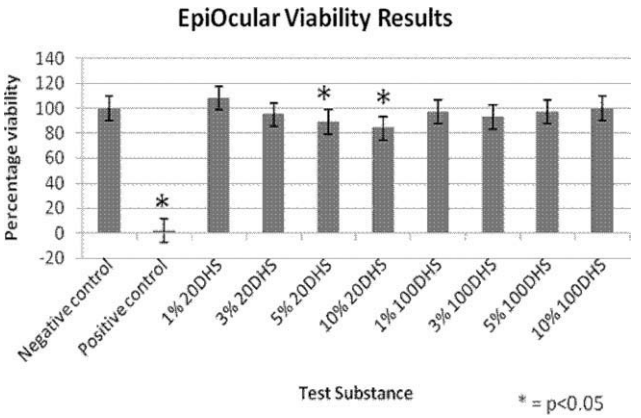

Figure 17 EpiOcular results of 20DHS and 100DHS.

break-up time tests provided promising results suggesting the need for further investigation of potential commercial applications as ocular lubricants. All polymers showed promising potential as ocular lubricants, especially 20DHS. It had the longest TBUT comparing with solutions at the same viscosity.

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