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Zeta potential of tear samples: A tool to explore the effects of wear of contact lenses

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ABSTRACT

Purpose: The aim was to develop a method to assess the electrostatic properties of human tear samples, and to evaluate their modifications induced by the wear of contact lenses (CLs).
Method: The barrier method was developed for the measurement of the isoelectric point (IEP) on relatively small quantities. The method was applied to compare three groups: tears (T_{NW}) of non-wearers, tears (T_{W_etaf}) of regular wearers of etafilcon A CLs, and tears (T_{W_omaf}) of regular wearers of omafilcon A CLs. Zeta potential (ζ) as a function of pH was measured by a Zetasizer Nano ZS90 (Malvern Instruments) on 40%-diluted samples, obtained by mixing 57 μL of tears of different subjects of the same group with 85 μL of HCl aqueous solution. IEP was deduced as the pH at which ζ is zero, i.e. the net electric charge on tear constituents being neutralized.
Results: Within an error of about 0.05, IEPs were found to be 2.90 (T_{NW}), 2.80 (T_{W_omaf}), and 3.16 (T_{W_etaf}). On average, a lower H^+ concentration is needed to neutralize the surface charge of the tear components of etafilcon A wearers, compared to both T_{NW} and T_{W_omaf}.
Conclusion: IEP measurements on tear samples of wearers of different types of CLs are proposed in order to enhance the knowledge on the modifications of the profile of charged species in tears. The T_{W_etaf} results, compared to those of the other groups, are compatible with an increase, due to the wear of etafilcon A CLs, of the relative concentration of high-IEP proteins.

1. Introduction

The tear film is a transparent layer covering the cornea, the bulbar conjunctiva, the fornices, and the inner eyelids up to the Marx line. In the first approximation, the tear film is typically described by three layers [1]: (i) a superficial oily layer (thickness of the order of one hundred nanometres); (ii) a middle aqueous layer (thickness approximately 5–10 μm; (iii) a semi-solid mucin layer close to the cornea (thickness of the order of tens of nanometres). The main constituents of the oily layer are hydrocarbons, wax esters, cholesterol esters, triglycerides, diglycerides, monoglycerides, free fatty acids, free cholesterol, and phospholipids [2]. The aqueous layer contains water, electrolytes, metabolites, proteins with anti-bacterial functions (antibodies, lipocalin, lactoferrin, lysozyme, lacritin, immunoglobulin), vitamins (mainly A), and growth factors [3]. The mucin layer contains hydrophilic proteins of high molecular weight, which are heavily glycosylated [3,4]. In general, the main component of tears is represented by proteins [3–6]. Tears maintain the healthy functioning of the eye defending the eye from external irritants and infections, by supplying oxygen and nutrients. They prevent dryness by keeping the eye moist, thus creating a smooth surface on the eye which allows for clear vision. Under normal physiological conditions, tear volume is approximately 6 ± 2 μL [7]; pH is slightly alkaline, varying between 7.4 and 7.8 [8]; during the day, the normal osmolarity of the tears is about 290 mOsm/kg [9].

Many external factors can influence the characteristics of tears. For example, one of the major factors is contact lens (CL) wear. The most commonly used CLs consist of hydrogel or silicone-hydrogel material. Once a CL is placed on the eye, the three-layer structure of the pre-corneal tear film is divided into a pre-lens (PreLTF) and a post-lens (PostLTF) tear film [10,11]. The use of CLs can cause changes in the structure, in the composition, in the physical and

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chemical properties, and in the behaviour of the normal PreLTF affecting the tolerability of CLs, and the biocompatibility with the ocular surface [12–17]. For instance, the stability of the tear film can be altered, causing symptoms of dryness and secondary dryness [18–21], often associated with tear osmolarity changes. The change of osmolarity, however, seems to be controversial. Tear-film osmolarity may increase if the rate of evaporation increases. For example, an increase of osmolarity of about 10% was reported by Iskeleli et al. [22]. Sarac et al. [23] reported an increase of a few percent after four hours of wear, even though they found osmolarity to decrease towards the initial value, after eight hours. Even if referred to the same initial period of adaptation to CLs, other authors reported a decrease of osmolarity due to excessive lacrimation [24–30]. The initial decrease is then typically followed by a shift towards hyperosmolality at the equilibrium [25–27,31]. The variation is controversial also when considering a longer period. Over time, after weeks or months of CL daily wear, some authors reported a return to the osmolarity measured before wear [26,31,32]; other studies reported higher values [22,27].

As far as proteins are concerned, an increase of lysozyme and lactoferrin concentrations was reported in CL wearers compared to non-wearers [33–36]. An excess of albumin was also detected during CL wear, due to changes of the blood-tear barrier [37]. Furthermore, lacrimal proteins can undergo structural modification such as unfolding [38]; this process also causes the loss of their biological functions. A study showed that CLs made of etafilcon A (IV FDA group) can modify the surface properties of the PreLTF, since the distribution of tear film rupture sites during breakup was found to differ from that of the normal eye [39]. CLs belonging to IV group FDA (etafilcon A) present more protein deposits and generate more changes in tear film than CLs belonging to the other three FDA groups [40]. This phenomenon is attributed to the ionicity of the materials of IV FDA group, and to their relatively high equilibrium water content. In this frame, analyses of photon correlation spectroscopy (PCS) on human tears have also been recently reported [41,42]. PCS detects intensity fluctuations of scattered light by a sample and provides information on the mean hydrodynamic diameter of components and on the so-called polydispersity index. Results obtained on tears of CL wearers and non-wearers were compared and a statistically-significant difference was found, the resulting mean hydrodynamic diameter being significantly higher for CL wearers [41,42]. The difference was attributed to changes in the interactions between tear constituents due to CL wear. However, the obtained hydrodynamic diameter is not attributable to the size of a specific component, due to the relatively high polydispersity of the sample, therefore the technique can only be adopted for comparative purposes.

In order to improve the knowledge on tear modifications due to CL wear, the measure of Zeta potential (ζ) of tear samples is here proposed. ζ is a measure of the magnitude of the electrostatic repulsion between particles in a suspension [44]. ζ varies with the pH of the surrounding environment and an isoelectric point (IEP) can be defined as the pH at which ζ is zero. IEP provides information on the net electric charge on particles, through the measure of the H^+ concentration of the surrounding environment which is required to neutralize it. The aim of this work was to explore the feasibility and the potential of IEP measurements to assess electrostatic properties of tear constituents, and to compare the effects of wear of CLs of differing materials. For example, etafilcon A and omafilcon A are here compared. The results obtained on tear samples of wearers of these two materials, and tears of non-wearers are reported and discussed.

2. Materials and methods

2.1. Tear collection and sample preparation

Tears of 35 subjects were divided into three groups: tears (T_{NW}) of non-wearers of CLs (15 subjects, age interval: 18–30 years), tears (T_{W_etaf}) of regular wearers of daily disposable etafilcon A CLs (1 Day Acuvue Moist, Johnson & Johnson, Jacksonville, FL, USA) belonging to IV FDA group (10 subjects, age interval: 19–26 years), and tears (T_{W_omaf}) of regular wearers of daily disposable omafilcon A CLs (Proclear One day, Cooper Vision, Victor, NY, USA) belonging to II FDA group (10 subjects, age interval: 21–32 years). Subject inclusion criteria were the absence of ocular pathologies, the absence of any medical therapy, and to be aged between 18 and 32 years.

After obtaining the informed consent from a subject, the procedure used for the collection of tears was to place a glass capillary of 5 μL, parallel to the lower meniscus tear [44]. The capillary was filled by capillarity in a few minutes. For CL wearers, tears were collected with CLs in-situ. For each subject, about 10 μL of tears were collected (about 5 μL from the right eye and about 5 μL from the left eye). Over a period of four months, tears were extracted from the same subject from six to eight times, at least two weeks apart from one sample to another. Each subject provided a total volume in four months of between 60 and 80 μL. The total tear volumes available for the measurements over the period of four months were about 900 μL of T_{NW} tears, about 700 μL of T_{W_etaf} tears, and about 700 μL of T_{W_omaf} tears.

Based on the adopted protocol of analysis, each independent ζ measurement required 57 μL of tears, so that tears of six or seven different subjects of the same group (either T_{NW}, or T_{W_etaf}, or T_{W_omaf}) were mixed to obtain at least a volume of about 60 μL. Therefore, for each group (T_{NW}, T_{W_etaf}, T_{W_omaf}) it was possible to acquire at least ten independent values of ζ at ten different pH values.

Different aqueous HCl solutions were prepared with variable pH ranging from 4.6 ± 0.1 to 2.2 ± 0.1. For each HCl solution, a volume of 57 ± 2 μL of human tears (of either T_{NW}, or T_{W_etaf}, or T_{W_omaf}) was added to 85 ± 2 μL of the HCl aqueous solution. In this way, different diluted tear samples of volume 142 ± 4 μL were obtained with variable pH, with the concentration of tears equal to $40\%^{V}/_{V}$. The pH of the diluted samples was deduced as $-log\frac{85\ 10^{-pH_{85}}}{142}$, where pH_{85} is the pH of the 85 μL of HCl aqueous solution. These 40%-diluted tear samples of 142 μL were used to fill the lower part of a capillary cell (Fig. 1). The upper part (650 μL) was filled with the corresponding aqueous HCl solution.

Some analyses were also carried out on solutions of albumin. Bovine serum albumin (BSA, Sigma Aldrich, molecular weight ~66 kDa) was dissolved in saline solution (aqueous NaCl $0.9\%^{W}/_{V}$, Alcon) with a BSA concentration 1 mg/mL. Saline solution was used because the sample of interest for this work is represented by tears with their intrinsic concentration of salts. Different aqueous HCl solutions were prepared with variable pH ranging from 6.4 ± 0.1 to 2.8 ± 0.1. For each HCl solution, a volume of 57 ± 2 μL of BSA solution was added to a volume of 85 ± 2 μL of the HCl aqueous solution, following the same procedure as for the preparation of tear samples. In this way, different 40%-diluted BSA samples of volume 142 ± 4 μL were obtained with variable pH. The pH of the diluted samples was deduced as $-log\frac{85\ 10^{-pH_{85}}}{142}$, where pH_{85} is the pH of the 85 μL of HCl aqueous solution. These samples of 142 μL were used to fill the lower part of the capillary cell (Fig. 1). The upper part (650 μL) was filled with the corresponding aqueous HCl solution.

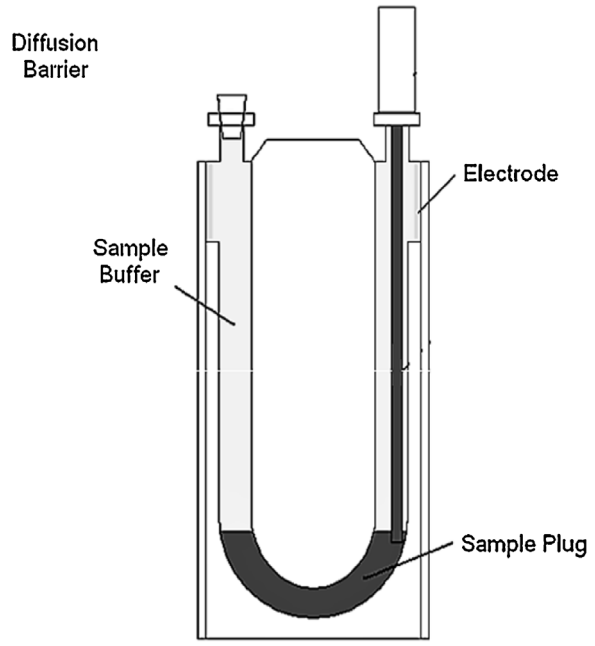

Fig. 1. Capillary cell used for ζ measurements.

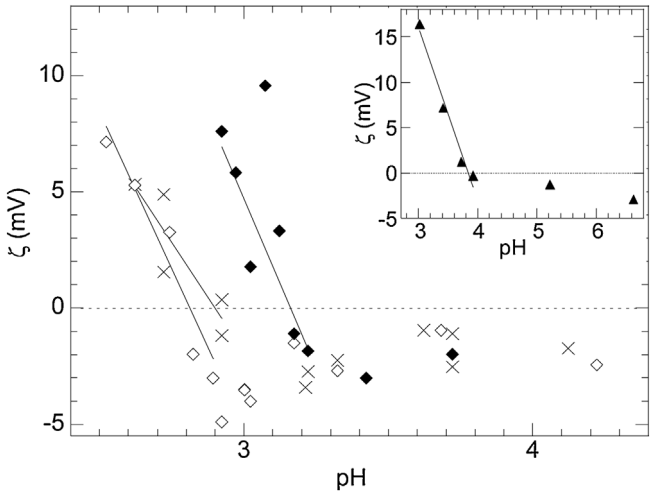

Fig. 2. ζ values measured following the procedure described in the text on 40%-diluted tear samples of the three groups (× for T_{NW}, ◇ for T_{W_omaf}, ◆ for T_{W_etaf}). Lines indicate the results of the linear regression of data corresponding to all the positive ζ data, and the first negative one (T_{NW}: y = - 18.97 x + 55.00, R = 0.911, IEP = 2.90; T_{W_omaf}: y = - 26.99 x + 75.91, R = 0.973, IEP = 2.80; T_{W_etaf}: y = - 29.06 x + 91.91, R = 0.918, IEP = 3.16).
Inset: ζ values (▲) of BSA samples and result of linear regression of the data corresponding to all the positive ζ data, and the first negative one (y = - 19.13 x + 73.50, R = 0.988, IEP = 3.84).

2.2. Zeta potential analyses

A Zetasizer Nano ZS90 (Malvern Instruments) was used for ζ analyses. During the measurements, the sample to be analysed fills a capillary micro-electrophoresis cell with electrodes at the ends (Fig. 1); a potential difference is applied which allows charged particles to move towards the opposite charge electrode. The Zetasizer system detects the Brownian motion of suspended particles by illuminating them with a laser. The incident laser beam passes through the centre of the sample cell, thereby detecting the scattered light. When an electric field is applied to the cell, any particle moving through the measurement volume causes the intensity of scattered light to fluctuate with a frequency proportional to the particle speed. The Zetasizer Nano software produces a frequency spectrum from which the electrophoretic mobility, and hence ζ is calculated. The equation with which ζ is calculated is $\zeta = \frac{U_E}{2}\frac{3}{\varepsilon}\frac{\eta}{f(ka)}$, where U_E is the electrophoretic mobility, η and ε are the viscosity and dielectric constant of the medium, respectively, and f(κa) is the so-called function of Henry, κa indicating the product between the inverse of Debye length and the particle radius. ζ varies with the pH of the surrounding environment, and an isoelectric point (IEP) can be defined as the pH at which ζ is zero. For the analyses, the temperature was set at 25 °C, the selected stabilization time being 60 s. The viscosity and the refractive index of solvent were assumed to be 0.888 cP and 1.330). The algorithm used by the software for calculating the size distribution was “Auto mode”.

3. Results

The values of ζ at different pH of the 40%-diluted tear samples of the groups T_{NW}, T_{W_etaf}, and T_{W_omaf} are reported in Fig. 2 in the pH range from 2.4 to 4.4. The data shows the typical ζ trend as a function of pH, with an approximately linear trend around the region where ζ = 0 [43]. Analyses at the higher pH up to 4.8 were performed (not shown here), where ζ was found to be between -2.5 and -0.5 mV for all three groups. At the lower pH, linear regression of data in Fig. 2 provided the equations reported in the figure caption. Starting from the lowest pH, the range of pH for linear regression was chosen including all the positive ζ data, and the first negative one. The intersections of the regression lines with the horizontal axis (y = 0) in Fig. 2 represent the IEPs which are found, within an error of about 0.05, to be equal to 2.90 for T_{NW}, 2.80 for T_{W_omaf}, and 3.16 for T_{W_etaf}.

For comparison purposes, analyses were also carried out on BSA solutions. The data is shown in the inset of Fig. 2. A similar trend of ζ was observed as a function of pH, as in the case of tear samples. Following the same approach as for the tear samples, linear regression of data at the lower pH allowed to extrapolate an IEP value of 3.84.

4. Discussion

ζ potential measurements have been sporadically proposed in CL research to study the charge behaviour of the surface of CL materials, and the bacterial cell surface potentially interacting with the CL [45,46]. In the present study, for the first time, this technique has been used to assess the electrostatic properties of human tear samples, and to evaluate their modifications induced by CL wear.

Proteins and other biomolecules dispersed in a liquid generally display surface electrostatic charges, which determine an electric field responsible for the attraction of counter ions from the surrounding medium. The layer of surface charges and counter ions is defined as the “electrical double layer”. According to the Stern model, this electrical double layer is composed by two regions: an internal one, indicating the stationary layer in which the counter ions are strongly bound to the charged particle (Stern layer), and an external one, the diffused layer, where ions are driven away from the surface due to thermal fluctuations. ζ is the potential difference between this interfacial double layer at the so-called *slipping plane,* and a point away from it in the dispersion medium [43]. In aqueous media, the most common surface charge-determining ions for proteins are H^+ and OH^-. Hence, the pH value readily affects the surface charge, and consequently ζ, which becomes more positive and more negative in magnitude with acidic and basic pH, respectively [43]. Therefore, monitoring

ζ values at different pHs allows one to determine the isoelectric point (IEP), i.e. the pH at which ζ becomes zero. The measuring of the IEP provides information on the H^+ concentration, which is needed to neutralize the total surface charge of particles in suspension, thus allowing one to assess and compare the electrostatic properties of the different samples.

The IEP measure is here proposed to assess the electrostatic properties of tears, whose main component is represented by proteins [47–50]. A first comment concerns the results of the analyses on albumin (inset of Fig. 2), whose IEP is reported in the literature as being 5.2 [40]. In preliminary analyses, the capillary cell used for ζ measurements (Fig. 1) was completely filled with BSA dissolved in saline solution (BSA concentration 1 mg/mL), without any dilution. In these preliminary measurements, the most common method for ζ measurements was used (instead of the barrier method), the IEP of BSA measure being 4.9 ± 0.1 (results are not shown here), indicating reasonable agreement with data found in the literature [40]. However, the method for the analysis was not optimal, in view of the large volume of human tear samples needed to be analysed. Other limits to this approach, were the denaturation of proteins during the analysis and the damage of the cell. These effects were attributed to the contact between proteins and electrodes. Denaturation was evident due to the formation of white aggregates, attributable to the presence of unfolded proteins giving rise to flocculation. Moreover, electrodes appeared blackened after the analysis. Therefore, to avoid the contact between the electrodes and both saline solution and proteins, the barrier method was developed. As described in the method section, a volume of 57 ± 2 μL of BSA NaCl solution was added to a volume of 85 ± 2 μL of HCl aqueous solution (producing a $40\%^{V}/_{V}$ dilution of the BSA solution). The analyses with the barrier method were carried out on these 40%-diluted BSA samples, and the IEP was measured at 3.84. This decrease in the IEP of BSA samples could be due to the aqueous dilution, which can partially modify the intrinsic properties of albumin and its IEP [51].

A more varied composition, and greater complexity are expected for tears. In general, tear proteins are reported in the literature to have intrinsic IEP, varying from about 1 to 12 [40]. Examples of proteins that have received attention in CL research include lysozyme (IEP 11.4), lactoferrin (IEP 8.7), and albumin (IEP 5.2) [40]. The ζ curve of tears can be interpreted as the result from the average of all these constituents. The measured IEPs of tears (Fig. 2) are also relatively low compared to the IEPs of the main tear proteins. The explanation is reasonably the same as for BSA, namely the 40%-dilution with water of the sample, which was adopted to avoid flocculation of proteins and blackening of the electrodes.

Apart from the comparison with a reference sample, such as albumin, the crucial point of this work is the comparison between wearers and non-wearers of CLs. The results highlight clear IEP differences among T_{W_etaf} and the T_{NW} and T_{W_omaf} groups. In detail, the IEP of T_{W_etaf} occurs at a significantly higher pH, compared to the other two groups.

A first hypothesis to explain the measured IEP shift, is a decrease of tear electrolytes concentration due to the wear of etafilcon A CLs [52]. From a clinic point of view, a decrease of tear electrolytes could be due, for example, to an excessive lacrimation. The change of electrolyte concentration due to CL wear is controversial in the literature, as discussed in the introduction [21–32]. A possible reduction of the concentration of electrolytes was reported, but typically, alterations occur only in the initial period of adaptation to CL wear. Therefore, this hypothesis explaining the IEP shift is rather unlikely. Moreover, the following considerations suggest that the IEP shift observed for $T_{W\text{-}etaf}$ is too great to be entirely attributed to a possible change of electrolyte concentration, if present. Indeed, to check the reliability of this hypothesis, additional ζ measurements were performed on T_{NW} samples with different concentrations (results are not shown here). By following the usual procedure, samples were prepared either with T_{NW} tear concentration $8 \pm 1\%^{V}/_{V}$ or with T_{NW} tear concentration $40\%^{V}/_{V}$. The outcome was that the IEP occurred at significantly higher pH (3.34 ± 0.05) in the case of $8\%^{V}/_{V}$ samples than in the $40\%^{V}/_{V}$ samples (2.90 ± 0.05, Fig. 2), suggesting that a fivefold reduction of tear electrolytes concentration generates a IEP shift from about 2.9 to 3.3 pH units. According to these results, the IEP difference between T_{NW} and T_{W_etaf} (2.90 vs 3.16) would correspond to a reduction of electrolyte concentration of at least two/three times in tears of etafilcon A wearers, compared to non-wearers. These considerations suggest that the IEP shift observed for $T_{W\text{-}etaf}$ is too great to be entirely attributed to a decrease of electrolyte concentration.

Other possible phenomena are expected to play a role. One possible phenomenon is the aggregation of tear constituents, which is expected to generate the reduction of the protein surface exposed to the medium, and the depletion of the surface negative charge, so that the amount of ions H^+ to compensate it decreases, compared to a system with fewer aggregates. This hypothesis would support some recent results obtained with the PCS technique. Indeed, the mean hydrodynamic diameter of tear components was found to be higher in CL wearers [41], especially for the hydrogel CLs of the IV FDA group [42].

Another possible event could be a change in the profile of the tear constituents. Since the IEP of T_{W_etaf} occurs at the higher pH compared to non-wearers, a change of the tear protein profile is deduced to be in favour of species with the highest IEP. Biochemical changes of protein profile are mentioned in the literature [33–38,53]. For example, excess of albumin was also detected during CL wear, due to changes of the blood-tear barrier [34]. An increase of lysozyme and lactoferrin in CL wearers was reported by other authors [33–37]. The results of this work suggest that the change of the tear profile is in favour of species with the highest IEPs [40]. It is worth mentioning that ionic materials (such as etafilcon A) have a negatively charged surface and therefore, typically attract positively charged constituents of the tear film, i.e. the species with the highest IEPs [54–57]. However, the deposition of lysozyme and other positively charged proteins does not simply cause a reduction in their concentration in tears due to drainage to the CL. On the contrary, alterations in the production of the tears itself due to the presence of the CLs, cannot be excluded. The change of the protein profile in favour of high IEPs, could be a reaction to drainage towards the CL from which they are attracted, or a reaction to an adverse event associated with CL wear, such as irritation, corneal infiltrative events or bacterial adhesion to CLs [35]. Interestingly, no substantial evidence of differences is found when comparing T_{NW} and T_{W_omaf}. Omafilcon A CLs have a relatively low surface ionicity and reactivity [40,57]. It is worth mentioning the biomimetic nature of this CL that contains synthetic phosphorylcholine (PC) molecules. PC is the headgroup present on the outer surface of the human cell membranes, binding with the water molecules of tears. Omafilcon A CLs are expected tobring less changes to the lacrimal film, compared to CLs of the IV group. Some studies on omafilcon A CLs demonstrated less presence of lipids and protein deposits on the CL surface than etafilcon A CLs, together with less on-eye dehydration [55,57]. The present study shows that the H^+ concentration which is needed to neutralize the total surface charge of tear components, is similar in tears to those of non-wearers, and in tears of wearers of omafilcon A CLs, in contrast to the etafilcon A CLs. In this respect, ζ results confirm that tears of omafilcon A wearers are not affected by CL wear, in terms of tear electrostatic properties, in contrast to etafilcon A CLs. A potential limitation of this technique is linked to the amount of the sample required for the analysis (60 μL) that, at

the moment, forces pooling of tears from different people. For this reason, it is possible to look at changes in the tear IEP in groups of wearers of certain CL material, rather than changes in individual subjects. Notwithstanding this, the ζ potential measurement on tear film remains a potential intriguing and useful technique for future developments. ζ analyses could be extensively applied to compare the clinical effects of different types of CLs, and to develop more compatible CL materials and artificial tears.

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Declaration of Competing Interest

None.

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