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Protocols

Photon correlation spectroscopy applied to tear analysis

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ABSTRACT

This study aims to deepen the knowledge on tear film properties by the development of a protocol for analyses of Photon Correlation Spectroscopy (PCS) on human tears and by the comparison between PCS results obtained on tears of contact lens wearers and non-wearers. Tears (5 μL) were collected by a glass capillary. The analyses provide the hydrodynamic diameter of tear components by analyzing intensity fluctuations in time of scattered light. PCS appears a promising technique for studying tear features and for shedding light on specific eye conditions, such as on the clinical effects of CL wear. In fact, statistical difference ($p < 0.001$) was found between the measured mean hydrodynamic diameter of tear components of wearers and non-wearers, the resulting value significantly higher for CL wearers. The scenario does not substantially change after (25 ± 5) min from the CL removal. The difference is attributed to changes in the interactions between tear constituents due to CL wear. In order to get deeper insights on the influence of CL wear on aggregation and structure of tear components, a preliminary Electron Spin Resonance (ESR) investigation was performed, monitoring Fe^{3+} species. ESR spectra on tears of both CL wearers and non-wearers showed the presence of intense signals, probably associated to iron (III) centers in proteins such as lactoferrin, and a weaker resonance attributable to Fe^{3+} species interacting with S–S bridges of lysozyme. Differences in ESR spectra between CL wearers and non-wearers were detected and tentatively ascribed to changes in coordination or in local environment of Fe^{3+} centers connected to aggregation phenomena induced by CL wear, which promote their interaction with other neighboring iron species.

1. Introduction

The tear film is a highly specialized structure that performs several functions such as optical, mechanical, and defensive functions. Its volume is typically (6 ± 2) μL [1]. The tear production was estimated to be 1–2 μL/min and increases up to 4 μL/min after stimulation of the ocular surface [2]. Under normal physiological conditions, the thickness of tear film is a few micrometers [3]. After blinking the thickness is reduced by about 20% after 5 s and it is reduced by 50% after 30 s [4]. The average refractive index is 1.337 [5]. The pH is slightly alkaline, with an average value between 7.4 and 7.5 [6]. The osmolarity, under normal conditions, is expected to be lower than 320 mOsm/kg [7]. The properties of the tear film change during the day [8,9]; some of these variations can depend on the method used to extract tears from the eye [10,11]. From the biochemical point of view, the main components of tear film are lipids, proteins, mucins and electrolytes [12].

The application of a contact lens (CL) can change the structure, the composition, the physical and chemical properties and the behavior of the normal tear film. Many studies have been reported in the literature concerning the properties of new and worn CLs [13–20]. As far as the tear properties of CL wearers are concerned, Bahgat showed that tear break up times and tear production are affected by CL wear [21]. Guillon et al. [22] demonstrated that wearing CLs produces a greater evaporation than that experienced by non-wearers. This behavior may be associated to an intrinsic change of the tear film, the effect being partially present also 24 h after CL wear. Another variation observed in the tear film was an increase of the ocular surface temperature immediately following CL wear, the temperature being significantly higher compared to non-wearers (37.1 ± 1.7 °C versus 35.0 ± 1.1 °C), predominantly in the continuous wear group (38.6 ± 1.0 °C) [23]. Similar trends were also observed in a study reported many years ago by Martin et al. [24]. Remarkably, Reim and Schrage [25] showed that wearing CLs causes an increase of glucose and lactate levels in the cornea

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and in tears, while ATP and glycogen in the cornea were found to decrease. The presence of CLs can also affect the properties of lacrimal proteins, which can undergo structural modifications such as unfolding, as recently discussed by Mann and Tighe [26]. This process also causes the loss of their biological functions and the formation of protein aggregates. The same authors also discussed the possible variation both in type and concentration of the tear film components, the immobilization of lipid on the CL surface and their possible oxidative degradation, the deposition on the CL and denaturation of proteins, the stimulation of cascade processes leading either to the generation of additional proteins and peptides or to an increase in fraction of components. In general, there are significant gaps in the understanding to what extent CLs induce tear changes and, in turn, to what extent these changes are responsible for discomfort and other negative effects.

Prompted by this background and aiming to better understand tear properties and the consequences of CL wear, we have developed a new methodological tool to reveal tear film changes between CL wearers and non-wearers based on Photon Correlation Spectroscopy (PCS), also known as Dynamic Light Scattering (DLS). Azharuddin et al. [27] recently reported the results of DLS analyses on tears collected by Schirmer's strips, extracted and centrifugated. They found differences between patients with dry eye and healthy subjects and attributed these differences to a higher abundance of aggregated proteins in dry eye patients. Apart from these preliminary outcomes, many aspects deserve to be investigated. In this work, a novel method for tear analyses by PCS has been established. The method was applied for measuring the tear properties of CLs wearers and non-wearers. In detail, the hydrodynamic diameter of the main tear components was determined and a comprehensive comparison between the results obtained for CLs wearers and non-wearers was achieved. In order to get deeper insights on the influence of CLs wear on the aggregation and structure of tear constituents, in particular proteins, a preliminary Electron Spin Resonance (ESR) investigation was performed, to our knowledge for the first time, on both tears of CL wearers and non-wearers, monitoring the Fe^{3+} species. The results, besides revealing the presence of different iron centers associated to proteins and enzymes, supported the PCS data and highlighted the validity of ESR for characterizing metal binding sites in tears.

2. Materials and methods

2.1. Tear collection

Tears of 63 subjects were analyzed, 24 wearers of CLs (14 females and 10 males, age interval: 21–34 years) and 39 non-wearers (23 females and 16 males, age interval: 20–45 years). CL wearers wore CLs of different materials. The hydrogel materials belong to II FDA group (nelfilcon A, omafilcon A, hilafilcon B) and IV FDA group (etafilcon A, methafilcon A). Silicone-hydrogels are comfilcon A, lotrafilcon B, narafilcon A, galyfilcon A, balafilcon A, delefilcon A, senofilcon A.

The procedure used for the collection of tears was to place, parallel to the lower meniscus tear, a glass capillary of 5 μL. The capillary was filled by capillarity in a few minutes. For CL wearers, tears were collected with CLs in-situ and after (25 ± 5) min from their removal. The measurements were performed on samples of 5 μL diluted with 45 mg (45 μL) of deionized water (tear concentration $(10 \pm 2)\%^{V}/_{W}$). The diluted tears were placed in 50 μL cells. On each tear sample, three PCS acquisitions were carried out three times (nine acquisitions). For each triple acquisition, the Malvern software provided an average result and a quality report obtained by a number of quality tests on the three measurements. The data reported in the paper were taken from one triple acquisition, but similar data were found in either two out of three or three out of three triple acquisitions. During the development of the PCS protocol, analyses were also performed on solutions with tear concentration $(5 \pm 1)\%^{V}/_{W}$ and $(3 \pm 2)\%^{V}/_{W}$. PCS results were found to be independent on tear concentration. Lower tear concentrations could not be used because there was not enough scattered light to make measurements. Tear concentrations higher than $(20 \pm 2)\%^{V}/_{W}$ were not taken into consideration due to the scarce repeatability of PCS results.

2.2. Photon correlation spectroscopy analyses

The hydrodynamic diameter (d_H) of the main components of tear content was determined using a DLS Malvern Zetasizer ZS90 instrument. The Zetasizer system detects the Brownian motion of particles suspended in a solution by illuminating them with a laser and analyzing the intensity fluctuations of the scattered light as a function of time. The speed of movement is then used to determine d_H, which is the diameter of hard spheres that would diffuse light at the same speed as the particles being measured. The relationship between d_H and the particle speed is defined in the Stokes-Einstein equation $d_H = \frac{kT}{3\pi\eta D}$, where k is the Boltzmann's constant, T is the absolute temperature, η is the viscosity, and D is the translational diffusion coefficient. The result is a particle size distribution calculated from the intensity of scattered light. The size distribution is displayed as relative intensity of light scattered by particles versus particle diameter. The most frequently used number to define d_H is called z-average ($d_{H,avg}$). It is the intensity-weighted mean of the diameters calculated from the intensity particle size distribution. A second parameter is the polydispersity index (I_{pd}), which describes the width of the intensity distribution. We also deduced $d_{H,avg}$ from (i) the fitting of the main peak of the intensity distribution and (ii) the particle size corresponding to maximum intensity of scattered light. These results are omitted here because a similar scenario was found as deduced from z-average. Although the fundamental size distribution generated by PCS is an intensity distribution, this can be converted, using Mie theory, to a volume distribution. This volume distribution can also be further converted to a number distribution. Here, volume and number particle size distributions are not taken into consideration because generated from the primary size distribution, which is the intensity one. Volume and number distributions are typically only recommended for comparison between relative amounts of different particles.

The comparison of $d_{H,avg}$ for CL wearers and non-wearers was obtained keeping the same experimental conditions: the temperature was set to 25 °C, the selected stabilization time was 60 s, the viscosity and the refractive index of solvent were assumed to be those of water (n = 1.330, v = 0.8872 cP). The algorithm used by the software for calculating the size distribution was the "General Purpose – NNLS (Non-negative least squares)" [28].

2.3. Electron spin resonance investigation

The electron spin resonance (ESR) investigation was performed by a Bruker EMX spectrometer operating at the X-band frequency and equipped with an Oxford cryostat working in the temperature range of 4–298 K. The capillary containing the tears samples were charged in quartz glass tubes and the spectra were recorded at 298 K. The spectra of pure lactoferrin were recorded at 298 K on a solution of the protein in a phosphate buffer (0.02 M at pH = 7.6). Three samples of lactoferrin in phosphate buffer solution were analyzed and the results were substantially equivalent. The *g* values were calculated by standardization with 2,2-diphenyl-1-picrylhydrazyl (DPPH). Care was taken to always keep the most sensitive part of the ESR cavity (1 cm length) filled.