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Research paper

ToF-SIMS analysis of ocular tissues reveals biochemical differentiation and drug distribution

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ABSTRACT

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) was used to obtain mass spectra from three ocular tissues, the lens, the vitreous and the retina. All three tissues were extracted from control ovine eyes and ovine eyes treated with model drug. To identify variations in surface biochemistry of each ocular tissue, principal component analysis (PCA) was applied to ToF-SIMS data. Interesting physiological differences in Na^+ and K^+ distribution were shown across the three tissue types, with other elements including Ca^{2+} and Fe^{2+} distribution also detected. In addition to the identification of small molecules and smaller molecular fragments, larger molecules such as phosphocholine were also detected. The ToF-SIMS data were also used to identify the presence of a model drug compound (amitriptyline – chosen as a generic drug structure) within all three ocular tissues, with model drug detected predominantly across the vitreous tissue samples. This study demonstrates that PCA can be successfully applied to ToF-SIMS data from different ocular tissues and highlights the potential of coupling multivariate statistics with surface analytical techniques to gain a greater understanding of the biochemical composition of tissues and the distribution of pharmaceutically active small molecules within these tissues.

1. Introduction

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is an extremely surface sensitive spectrometric technique that can provide detailed chemical information of a wide variety of systems, including biological tissue [1,2], drug formulations [3] and polymers [4]. ToF-SIMS has previously been successfully applied to ocular tissues; however, studies have been limited and mainly focused on physiological composition of retina tissue from a specific animal model [5–10]. Work by Amemiya et al. demonstrated the presence of vitamin A and E in cross sections of rat retina as well as differences in counts obtained in response to light stimulus [5]. Using the same model, the group also showed differences in retina expression of fatty acids (maleic, palmitic, oleic, stearic, arachidonic and docosahexaenoic acid) with the highest counts obtained for maleic acid [6]. The ability of ToF-SIMS to spatially map sample cross sections was not exploited in this retinal work as in each case work focussed on total ion counts measured for the whole-tissue cross section. Total ion count is often not an ideal method of direct comparison between samples due to matrix effects where the ion count achieved can vary depending on the chemical environment of the sample [11]. Following on from this work, a study by Kim et al. moved onto demonstrate spatial distribution in ocular tissue by mapping the distribution of Na^+, K^+, Mg^{2+} and Ca^{2+} in the mouse retina [8]. Very limited work has been carried out on lens tissue using the technique of ToF-SIMS with only one study reporting use of this technique. Kinoshita et al. used the rat model to demonstrate that concentration differences of Ca^{2+}, Fe^{2+}, Mg^{2+} and Na^+ existed in 4-month-old rats when compared with 15-month-old rats [12].

Similarly to the lens, limited studies have been performed on the vitreous tissue of the eye. The structure of the vitreous, mainly water, makes it a difficult tissue to prepare and analyse, perhaps explaining the previous lack of use of the ToF-SIMS technique in this tissue. Only one previous study, by Kishikawa et al., has been performed on vitreous tissue, looking at the distribution of various elements, fatty acids and vitamins A and E in patients with diabetic retinopathy and vitreoretinopathy [9]. The vitreous in this case was subjected to a washing stage to facilitate salt removal and was treated as a mass of tissue, analysing the outer layer of the tissue mass rather than a cross section through the tissue. Although ToF-SIMS has been used to consider organic substance distribution within some ocular tissues, ocular tissues have not previously been investigated as a collective to reveal any important physiological differences between tissues. In addition to this, the potential of ToF-SIMS to map drug distribution within ocular tissues has not previously been explored. Interest in mapping drug distribution

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within ocular tissues has increased recently, especially when treating certain posterior eye disease states, such as diabetic retinopathy [13] and age-related macular degeneration [14], where effective treatment is currently limited. The process of generating ocular drug distribution data can be time-consuming and requires the eye to be separated into its various ocular tissues. Typically, following separation, ocular tissues are homogenised before the drug is extracted from the tissue, providing no spatial awareness of the drug location within the tissue. The ability to detect drug concentrations directly from a cross section of ocular tissue using ToF-SIMS without the need for extraction method could provide useful distribution information.

Analysis of ToF-SIMS mass spectrum data can be seriously challenging with a simple sample, such as a homopolymer, producing thousands of secondary ion mass peaks [15]. This challenge is increased in magnitude for biological tissue due to the biochemical complexity and composition (from small molecules to kiloDalton weight proteins and polysaccharides) of tissue. It is now commonplace with analytical chemistry to utilise data mining techniques to fully explore large data sets. In the field of ToF-SIMS, both principle component analysis (PCA) [4,16] and partial least squares regression (PLS) [17] are extensively used and have established rules for data pre-processing and processing [16]. PCA is used to establish variance with a data set and determine where the direction of this variance originates. Through the use of PCA, chemically distinct regions in ToF-SIMS data can be identified and key peaks associated with image features selected, in both biological and non-biological samples [18,19]. Prior to carrying out MVA, it is important to select an appropriate means of scaling, as the method selected is widely known to have a major influence on extracting the most useful information from the data set. The use of various data scaling methods has been shown to aid the ability to identify chemical information governing differences within data sets; however, there appears to be no clear guidance on which method is most effective [20]. As a pre-processing step, normalisation is often carried out first before the selected scaling method is applied and PCA carried out. Various types of pre-processing include mean centring, root mean scaling, autoscaling, filter scaling and shift variance scaling. In this study, we show for the first time how PCA can be successfully applied to both positive ToF-SIMS data in order to highlight similarities and differences within the physiological chemistries of the lens, vitreous and retinal ocular tissues. We also present the first use of the ToF-SIMS technique to identify the presence of a model drug compound within these ocular tissues.

2. Materials and methods

2.1. Ocular tissue preparation

A batch of ovine eyes was obtained from a local abattoir within one hour of slaughter. On arrival, the eyes were warmed to body temperature at 37 °C and excess extraorbital tissue and eyelids were removed. Eyes were then cannulated using one of the long ciliary arteries which typically wrap around the optic nerve. A small volume of perfusion fluid was slowly pumped through the eye, whilst the vortex veins were inspected for exit of the perfusion fluid out of eye. Once flow had been established, the eye was introduced into the perfusion system and perfused with physiological media (pH 7.4) based on a method previously used by Koeberle et al. [21]. Eyes were maintained at 37 °C throughout the experiment with a perfusion flow rate of 1 ml/min. Details of the composition of the perfusion fluid administered into the eyes via the ciliary artery are presented in Table 1, all components were purchased from Sigma–Aldrich (Dorset, UK). Once arterial perfusion pressure was maintained, an intravitreal injection of model drug amitriptyline (Fig. 1A) was administered to both eyes. Amitriptyline was identified as a suitable representation of a routinely administered small, generic, biologically active drug molecule. Amitriptyline was selected due to similarities in its basicity, molecular weight and partition coefficient to small drug molecules typically used in the treatment of ocular disease. Intravitreal injections of model drug amitriptyline hydrochloride (Sigma–Aldrich Dorset, UK) were prepared at two concentrations in 100 μl of deionised water. A low dose and a high dose injection were prepared, containing 250 μg and 2000 μg of drug, respectively. The injection was performed using a 1 ml syringe and 23G needle, inserted 1 mm into the vitreous approximately 3–4 mm from the limbus in the posterior direction. Non-perfused eyes, with and without drug administration, were used as controls. To non-perfused eyes with drug administration, intravitreal injection was performed using the same method as perfused eyes. Following exposure for 2 h, the perfusion procedure was ended and perfused eyes were removed from the perfusion kit. All eyes were immediately frozen in liquid nitrogen, and the lens, retina and vitreous were removed. The anterior eye was then cut away in a circular manner and the lens removed. In order to achieve cross sections of the vitreous and the retina, the eye was sliced at four positions from the anterior to the posterior section. The vitreous was removed and the sclera was pressed back for cryosectioning. All tissues were stored at −80 °C prior to sectioning. The lens, vitreous and retina from each eye were mounted onto a cryostat chuck using Shandon M-1 embedding matrix (Thermo-Scientific, Chesire); 20-μm-thick sections were cut through the centre of the tissue on a Leica CM1850 Cryostat (Leica Microsystems, Milton Keynes, UK). Tissue sections were mounted directly onto 1 × 1 cm silicon wafers which were previously cleaned with isopropyl alcohol.

Table 1
Perfusion media composition.

Chemical	Concentration
Tissue culture medium	1000 ml
Sodium bicarbonate	2.2 g/l
Atropine sulphate	0.005 g/l
EDTA	0.2922 g/l
Penicillin G	100k U/l
Streptomycin	75.6kU/l
Gentamycin	0.08 g/l
Insulin bovine	50 U/l
Bovine holo-transferrin	0.0025 g/l
Sodium selenite	2.4 μg/l

2.2. ToF-SIMS analysis

Time-of-flight secondary ion mass spectrometry was performed on an Ion-TOF ToF-SIMS IV instrument (IONTOF, GmbH, Munster, Germany) using a Bi^{3+} cluster source and a single-stage reflectron analyzer. Samples were mounted on a standard sample stage rather than a cold/cryostage as samples were taken directly from frozen conditions and then exposed to ultra high vacuum removing thawing issues. The standard sample stage also allowed greater sample dimension flexibility. A primary ion energy of 25 kV along with a pulsed target current of approximately 1 pA and post-acceleration energy of 10 kV were employed throughout the analysis. The primary ion dose density was maintained at less than 10^{12} ions per cm^2 throughout to ensure static conditions. Spectra were acquired in both positive and negative mode by rastering the stage under the primary ion beam over a sample area of 500 × 500 μm. Low energy electrons (20 eV) were delivered to the sample surface to compensate for positive primary ion beam induced surface charging, a common insulating effect of biological surfaces. Data