Exploring the possibility of early cataract diagnostics based on tryptophan fluorescence

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A novel route for early cataract diagnostics is investigated based on the excitation of tryptophan fluorescence (TF) at the red edge of its absorption band at 317 nm. This allows penetration through the cornea and aqueous humour to provide excitation of the ocular lens. The steepness of the red edge gives the potential of depth control of the lens excitation. Such wavelength selection targets the population of tryptophan residues, side chains of which are exposed to the polar aqueous environment. The TF emissions around 350 nm of a series of UV-irradiated as well as control lenses were observed. TF spectra of the UV cases were red-shifted and the intensity decreased with the radiation dose. In contrast, intensity of non-tryptophan emission with maximum at 435 nm exhibited an increase suggesting photochemical conversion of the tryptophan population to 435 nm emitting molecules. We demonstrate that the ratio of intensities at 435 nm to that around 350 nm can be used as a measure of early structural changes caused by UV irradiation in the lens by comparison with images from a conventional slit-lamp, which can only detect defects of optical wavelength size. Such diagnostics at a molecular level could aid research on cataract risk investigation and possible pharmacological research as well as assisting surgical lens replacement decisions.

Keywords: cataract; diagnostics; tryptophan fluorescence

1. INTRODUCTION

Cataract is the leading cause of blindness worldwide affecting some 17 million people and causing 1.3 million cataract operations annually in the United States alone [1,2]. The two most common effects caused by post-translational modification of ocular lens proteins are cataract and presbyopia that give rise to impaired vision, usually starting when individuals reach middle age and beyond [3]. The modifications are usually caused by either age-related metabolic changes, concomitant diseases or external risk factors such as significant UV dosage [4]. The clinical observations of loss in accommodation and lens opacification are likely to represent the end-stage of a more prolonged process of inexorable modifications to lens proteins progressing subclinically.

Currently, methods of cataract detection are based on measurements of lens opacity by Rayleigh light scattering. This is achieved by using a conventional slit-lamp-based technique, or other more complex methods such as Scheimpflug photography and optical coherent tomography (OCT) [5,6]. This can enhance anatomical localization of cataract probing, but would not provide the protein-level detail offered by TF owing to the limitation of these scattering techniques in that the sizes of the structural defects must be comparable with the wavelength of light used, i.e. ca 400–600 nm. Therefore, these methods do not reveal structural changes on a molecular level. A method such as TF could be of importance for identifying risk factors and to act as a research tool in the development of any pharmacological approaches for cataract treatment. It could help us to establish the point at which the irreversible crystallin protein change has occurred triggering the need for surgical intervention. In addition, clinical applications of this method would help in the diagnosis of early stages of metabolic disorders, e.g. diabetes, the preventative treatment of which could delay the development of chronic diseases.

TF has been widely used for monitoring protein changes in biophysical research to detect protein folding, conformation and aggregation by virtue of shifts in the emission spectrum in different polar microenvironments.

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When a tryptophan side chain is located in a non-polar microenvironment its emission spectrum has a maximum at 308 nm. In folded multi-tryptophan proteins, spectra usually exhibit maxima between 325 and 335 nm, while a denatured protein exhibits a spectral shift to 355–360 nm. Thus, changes in the concentration of the relative fraction of tryptophan residues situated in polar environment should correlate with structural changes in the lens.

While TF is a promising technique, in vivo applications have, so far, not exploited the useful transparency of the cornea and aqueous humour in the 310–320 nm spectral range [11], where TF can still be excited. These wavelengths could give a choice of penetration depth and hence have the potential for three-dimensional mapping of the lens structure.

In this paper, we present preliminary evidence of this novel application of red-edge TF as a potentially useful non-invasive method for monitoring very early changes in the lens structure at the molecular level, which could not be detected by any light scattering-based methods.

2. MATERIAL AND METHODS

Porcine eyes were obtained from the local abattoir shortly after death. The eyes were dissected to extract the lenses taking care to avoid mechanical damage to the lens capsule. Measurements were carried out with freshly isolated lenses on the same day. Spectral measurements were taken using an Edinburgh Instruments FLS920 double-grating spectrometer in a temperature controlled 1 cm quartz cuvette in PBS (Dulbecco’s phosphate-buffered saline, modified (Sigma Aldrich, D8537)) at 22°C (this temperature retained uniform tissue viability and proved to give consistent results over approx. 16 h without visible change in observed lenses).

The experimental geometry between excitation and emission was 90° with the samples aligned at 45°. Fluorescence spectra of tryptophan emission were measured in the 330–550 nm spectral range with excitation at 317 nm, while a denatured protein exhibits a spectral shift to 355–360 nm. Thus, changes in the concentration of the relative fraction of tryptophan residues situated in polar environment should correlate with structural changes in the lens.

Changes in the lens structure were induced by UV irradiation from a FLS920 single-grating spectrometer using 310 nm light with 20 nm bandwidth over time periods between 20 min and 12 h using a 450 W Xenon arc lamp as the source providing approximately 7 mW at the sample. This irradiation model was employed to mimic cortical cataract for which ambient UV is a known epidemiological risk factor in humans. The emission spectra were corrected for spectral sensitivity of the emission tract.

The spectra were excited at 317 nm wavelength. Numbers near the curves show irradiation time in minutes.

Figure 1 shows the normalized emission spectra of non-irradiated and irradiated porcine lenses over different periods of time. The spectra exhibit a bi-modal shape with two bands at around 350 nm, attributed to TF and 345 nm to a non-tryptophan emission. Maxima of the non-tryptophan to tryptophan emission bands both integrated over 338 to 365 nm and intensity decreased along with the increase in the irradiation time suggesting a conformational change of the crystallin proteins. In contrast, intensity of the 345 nm band increased in the course of irradiation. Ratios $F$ of the non-tryptophan to tryptophan emission bands both integrated over 25 nm of bandwidth as a function of the lens UV irradiation time.

3. RESULTS

Figure 1 shows the normalized emission spectra of non-irradiated and irradiated porcine lenses over different periods of time. The spectra exhibit a bi-modal shape with two bands at around 350 nm, attributed to TF and 345 nm to a non-tryptophan emission. Maxima of the non-tryptophan to tryptophan emission bands both integrated over 338 to 365 nm and intensity decreased along with the increase in the irradiation time suggesting a conformational change of the crystallin proteins. In contrast, intensity of the 345 nm band increased in the course of irradiation. Ratios $F$ of the non-tryptophan to tryptophan emission bands both integrated over 25 nm of bandwidth as a function of the irradiation time are plotted in figure 2. It is noteworthy that slit-lamp comparative observations (figure 3) showed no structural defects in the lenses irradiated for less than 240 min. The structural changes (as seen by fluorescence) were independent of the elapsed time between termination of irradiation and slit-lamp
imagery suggesting that the defects were produced during the irradiation. The slit-lamp could detect only large-scale structural defects whereas the TF method shows structural changes at a significantly earlier stage.

Figure 4 shows that TF excited by a 320 nm LED gave an image showing high-contrast visualization of the UV damaged region in the lens.

The above findings were confirmed by repeated experiments on five samples over approximately 2 days. This was supported by later measurements on some 60 pigs’ lenses and 40 complete eyes over extended timescales and wavelengths, where the effects of irradiation were consistent. The irradiation effects on the pigs’ lenses were consistent in that all samples contained TF around 350 nm, but with variability, and also steadily increasing 450 nm emission with irradiation dose. The spectral profiles while varying nonetheless give information on the photochemical effects at the early stages of UV irradiation.

Figure 5 illustrates the possibility of exciting lens fluorescence at tryptophan wavelength through the
cornea and aqueous humour with data from Ambach et al. [11]. Spectra requiring more detailed future analysis were obtained from these complete pigs’ eyes.

The above early results inspired similar measurements on four human donor lenses obtained from the Bristol eye bank—figures 6 and 7. Some detail of history is given with slit-lamp images. Figure 7 shows that two of these cases gave consistent spectra with the pigs’ lens results. However, the optical precision (approx. 1 mm square area) was insufficient at this stage to draw conclusions regarding in vivo application but TF and non-TF spectra were observed. The results on human lenses were consistent with the above, with the reservation that the optical system did not have adequate spatial resolution, but nonetheless showed that the human lens could be excited in the TF region. The penetration depth of this excitation wavelength is yet to be determined. By contrast, the pigs’ lens results showed that the depth of excitation could be varied by altering the excitation wavelength. The older human lenses showed more emission at the 450 nm range and the 27552A lens showed marked nuclear sclerotic changes as the predominant clinical characteristic whereas the remaining three lenses showed cortical or peripheral wedge opacification as the main feature suggesting that the TF profile does discriminate between the two anatomic markers. Also 27557B shows central white rather than sclerotic opacification and the remaining two show a similar clinical and TF profile.

4. DISCUSSION
The major components of the lens are highly stable and water-soluble crystallin proteins. Once deposed, the proteins remain in the lens and create a structure providing unique transparency and accommodation properties [12]. In spite of the high stability, molecular changes accumulate in the proteins over time caused by chemical, photochemical, environmental factors or by ageing and concomitant diseases, e.g. diabetes [13,14]. The changes lead to protein misfolding, denaturation and aggregation and hence structural defects [15]. Currently, cataract diagnostics based on light scattering only detect defects comparable in size to the wavelength so that detection of the impact of photochemical change is hardly possible at the stage when a cataract is currently diagnosed. Thus, surgical treatment is the only currently possible method.

Although cataract surgery is generally recognized as being one of the safest operations, and there is a significant complication rate [16]. Furthermore, there is considerable interest in identification of the risk factors involved in cataractogenesis and possible protective strategies [17].

We note that the use of N-acetylcarnosine as treatment of cataract has been investigated with an...
attempt to provide quantitative assessment by image analysis, visual acuity and glare test [18]. The results presented have not been generally considered conclusive. Further development of non-surgical cataract treatment needs the application of non-invasive methods with resolution at the molecular scale, i.e. red-edge TF is likely to be a potentially useful clinical technique.

The eye lens exhibits a tryptophan emission in the 300–450 nm range and a non-tryptophan emission in the 400–550 nm range. The latter is also associated with the proteins [19,20] and may be of application as an indicator. Both of these emissions could be used for diagnostics of cataract.

We have demonstrated that in vivo applications of TF are a possibility by preliminary data on a complete pig’s eye that showed similar results for TF with transmission through the cornea and aqueous humour. It remains to be shown how practical this would be for human eyes but Ambach’s data and the possibility to monitor tryptophan and non-TF in human lenses are suggestive. In a future work, we aim to validate our findings through extended ex vivo and in vitro studies. To this end, we are going to quantify tryptophan and non-TF in human ocular lenses and compare it with data obtained by Scheimpflug and dynamic light scattering techniques. Given the clinical inter-individual variation in both cataract grading and progression rates, tryptophan and non-TF may provide deeper insight into the protein unfolding characteristics underlying this variation. Thus a research tool, hospital and optician use are envisioned.

The steepness of the red slope [21] allows selective excitation of the ‘red-shifted’ tryptophan residues, situated in the most polar environment. At the same time, a non-tryptophan emission of ocular lens proteins is also excited at this wavelength (figure 1). The opposite tendency in the changes of tryptophan and non-tryptophan emission intensities suggests that UV excitation triggers a photochemical conversion of tryptophan to a fluorescence product, which is probably 3-formylkynurenine according to Fukunaga et al. [22] or 3-hydroxykynurenine [23]. This led us to suggest the parameter $F$ determined as a ratio of intensity of the non-tryptophan and tryptophan emission bands, which correlates on one hand with concentration of a fraction of tryptophan residues located in polar environment and on the other hand with concentration of its photo chemical product. This $F$-parameter was found to be very sensitive to structural changes in porcine lenses induced by UV irradiation (figure 2). We could confidently detect changes already after 20 min of irradiation. The parameter doubled within the first 30 min and reached saturation after 240 min—the first time point when structural defects could be detected by the slit-lamp method.

The red-edge TF method operates below the daily safety threshold for UVA (ultra-violet A, ca 315–400 nm) exposure, which limits the total exposure to 1 J cm$^{-2}$ within 103 s [24]. Measurement of one fluorescence spectrum in a standard double-grating spectrometer with the use of 10 μW excitation focused into a 3 × 3 mm spot takes ca 30 s, which is equal to ca 3 mJ cm$^{-2}$. Optimization of the experiment by using UV high-throughput tunable interference filters [25,26] instead of grating monochromators and consequently the use of higher numerical aperture optics will further reduce the lens exposure in the course of spectral measurements by at least 10-fold and bring the method well below the safety limit. Additionally, the method might be used as a more efficient slit lamp since light scattering efficiency increases inversely as the fourth power of the wavelength. Compared with the visible light used in conventional slit lamps, the 320 nm light will scatter six-times more efficiently. The instrument could therefore ‘see’ scattering from the formation of pre-cataract aggregation of protein at a far earlier stage than is possible at present (figure 4).

5. CONCLUSION

The present results show that TF offers a sensitive method for monitoring very early changes in the lens structure that cannot be detected by the slit-lamp method. Exploiting this discovery should allow the development of a clinically useful tool sensitive enough to detect, diagnose and monitor lens change before significant damage, light-scattering, aggregation and visual impairment occurs. This could evaluate risk factors and potential future therapies including 2-photon bleaching of the human lens as pioneered by Kessel et al. [27] capable of slowing or reversing age-related changes in crystallin proteins. Thus a research tool, hospital and optician use are envisioned.

Funding for this project was provided by TTom and by the National Institute for Health Research (NIHR) Innovation for Innovation (i4i) programme and a Royal Society Industry Fellowship (D.M.G). The views expressed in this article are...
those of the authors and not necessarily those of the NHS, the NHIR or the Department of Health. Edinburgh Instruments provided use of two spectrometers and facilities of its R&D laboratory as well as access to the intellectual property described in International Patent Application No. PCT/GB2010/000332. The authors would also like to thank Dr Valerie Smith, Bristol Eye Hospital for the supply of the human lenses used in this study and Dr Line Kessel for fruitful discussion.

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