Sclera as a Surrogate Marker for Determining AGE-Modifications in Bruch's Membrane Using a Raman Spectroscopy-Based Index of Aging

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PURPOSE. Raman spectroscopy is an effective probe of advanced glycation end products (AGEs) in Bruch's membrane. However, because it is the outermost layer of the retina, this extracellular matrix is difficult to analyze in vivo with current technology. The sclera shares many compositional characteristics with Bruch's membrane, but it is much easier to access for in vivo Raman analysis. This study investigated whether sclera could act as a surrogate tissue for Raman-based investigation of pathogenic AGEs in Bruch's membrane.

METHODS. Human sclera and Bruch's membrane were dissected from postmortem eyes ($n = 67$) across a wide age range (33–92 years) and were probed by Raman spectroscopy. The biochemical composition, AGEs, and their age-related trends were determined from data reduction of the Raman spectra and compared for the two tissues.

RESULTS. Raman microscopy demonstrated that Bruch's membrane and sclera are composed of a similar range of biomolecules but with distinct relative quantities, such as in the heme/collagen and the elastin/collagen ratios. Both tissues accumulated AGEs, and these correlated with chronological age ($R^2 = 0.824$ and $R^2 = 0.717$ for sclera and Bruch's membrane, respectively). The sclera accumulated AGE adducts at a lower rate than Bruch's membrane, and the models of overall age-related changes exhibited a lower rate (one-fourth that of Bruch's membrane) but a significant increase with age ($P < 0.05$).

CONCLUSIONS. The results suggest that the sclera is a viable surrogate marker for estimating AGE accumulation in Bruch's membrane and for reliably predicting chronological age. These findings also suggest that sclera could be a useful target tissue for future patient-based, Raman spectroscopy studies. (*Invest Ophthalmol Vis Sci.* 2011;52:1593–1598) DOI:10.1167/iovs.10.6554

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Aging of the Bruch's membrane is associated with modification of its constituent proteins by reactive intermediates from metabolism and lipid peroxidation.^{1,2} So-called advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) are established pathogenic agents in ocular and nonocular tissues during age-related diseases and diabetic complications.¹ Using a Raman spectroscopy-based approach, it has been demonstrated that various AGE and ALE adducts accumulate in Bruch's membrane during normal human aging,^{3–6} and this makes them potentially useful biomarkers for age-related disease risk. The Raman approach is ideally suited to the investigation of biological samples because it is effectively noninvasive (using visible or near-infrared radiation) and nondestructive and is capable of yielding complex structural information in a single measurement.^{7,8} In the context of Bruch's membrane and in addition to the information on AGE and ALEs, the Raman signal is able to simultaneously provide information on a broad range of biochemical molecules, including lipids, collagens, elastin, heme, cytochrome c, and α -crystallin.³

In the human eye, Bruch's membrane is only 2- to 6- μ m thick, and it is sandwiched between two deeply pigmented tissues. This necessitates highly confocal optics coupled to a highly sensitive Raman spectra-capturing device to isolate the Bruch's membrane signal from the surrounding environment. Such an approach is not realistic in vivo because the eye is effectively an f2.1–14.1 optical system given the variation of the iris from 8 to 2 mm; therefore, confocality cannot be achieved without considerable loss of throughput. Raman is an inherently weak effect (approximately 1/1,000,000 incident photons are Raman shifted), and collection efficiency is critical. Raman spectroscopy has previously been used for measurement of macular carotenoids and risk for age-related macular degeneration (AMD) in the living eye.⁹ That work was able to exploit a phenomenon called resonance Raman scattering, in which the use of a Raman excitation wavelength that coincides with absorption bands of the scattering species increases the efficiency of the Raman scattering by several orders of magnitude.

Raman has been used to probe the biochemical structure of the retina ex vivo^{10,11} and to probe age-related accumulation of AGEs and ALEs in human postmortem Bruch's membrane.^{3,4} Unfortunately, detection of such biomarkers is not possible using resonance Raman spectroscopy because of the lack of absorption bands at accessible wavelengths in AGE and ALE species. Higher laser input power is required to collect usable information. The presence of light-sensitive photoreceptors and highly absorbent (thereby heat-inducing) pigmented layers close to Bruch's membrane severely limits the input laser power that can be used in the living eye (approximately 1 mW for 0.25-second exposure at 785 nm¹²). Thus, it seems unlikely in the foreseeable future that age-related changes in

Bruch's membrane will be able to be analyzed *in vivo* using this approach.

The sclera, a 400- to 1000- μm -thick tissue on the ocular surface can be readily addressed by a Raman probe and is predominantly an extracellular collagenous matrix.¹³ Because it is opaque, it scatters light efficiently, despite the presence of the very highly pigmented lamina fusca (within the sclera) and choroid (immediately underneath). Consequently, it could be anticipated that no significant amounts of laser light are able to penetrate the sclera to the photoreceptors, and laser power restrictions are not as stringent as for the retina. There is also the added advantage that confocality is not required, whereas its location at the ocular surface allows more choice of collection optics, enabling use of high numerical aperture microscope objectives.

The sclera itself is known to undergo significant age-related biomechanical change,^{14,15} including thinning, stiffening, and changes in permeability. At the biochemical level, a number of distinct age-related trends have been observed, including aggregation of lumican by aggrecan,¹⁶ a shift in the composition of proteoglycans from biglycan and decorin to aggrecan,¹⁷ accumulation of AGEs^{18,19} (which induce altered permeability²⁰), suggesting it has potential as a viable surrogate tissue for monitoring ocular age-related risk and diagnostic screening. In the current investigation, we have compared the Raman spectral data obtained from the sclera with those obtained from the Bruch's membrane in a cohort of human eyes across a range of chronological ages. The primary aim has been to determine whether AGE/ALE adducts could be readily recognized and quantified in the sclera and to what extent these correlated with age and with the levels already observed in Bruch's membrane.

METHODS

Human globes ($n = 67$, without corneas) from donors of various ages were obtained in saline solution from Bristol Eye Bank. Full ethical approval was obtained from Research Ethics Committees, and all methods used in the research were carried out in accordance with the tenets of the Declaration of Helsinki for research involving human tissue. The samples were drained of saline and stored at -80°C until dissection. The Bruch's membrane/choroid complex was dissected from the eyecup, and the sclera was placed on an extra white glass slide (Menzel-Glaser), air dried, and stored at -20°C until measure-

ment (within 1 month of dissection). The scleral tissue used in this study was from the same groups of donors as used in our previous Bruch's membrane study.³ Sclera from donors of different ages were divided into decades (30–49, $n = 6$; 50–59, $n = 8$; 60–69, $n = 11$; 70–79, $n = 12$; 80–89, $n = 16$; 90–92, $n = 10$).

Raman spectroscopy of both dissected Bruch's membrane and sclera was performed on a high-resolution Raman spectrometer (LabRam HR 800; Horiba Jobin-Yvon) using 633-nm excitation, at the same power level (10 mW) so as to allow direct comparison of the data obtained from the two tissues. A 300 L/mm diffraction grating was used throughout, giving a spectral resolution of 12 cm^{-1} . The samples were flat mounted, and spectra were recorded for 10 seconds per point from selected areas (8×8 grid, 64 points in $256\text{ }\mu\text{m}^2$), with three areas measured per sample. The Raman data were processed by first removing the non-Raman background using a linear combination of backgrounds identified in principal component analysis (PCA) and normalized about the heme-amide I region, as previously described.^{21,22} The multivariate models previously developed for Bruch's membrane were applied to the sclera data to obtain predicted results for the same range of parameters.⁴ All preprocessing and prediction of data from previously generated PCA models were carried out in computing software (MatLab; MathWorks, Cambridge, UK). An additional PCA model was generated directly from the sclera data (mean centered, not scaled to unit variance) using data analysis software (Unscrambler; Camo, Oslo, Norway). Prediction from existing partial least squares (PLS) regression models was carried out in data analysis software (Unscrambler; Camo) and the spectral residuals calculated. These spectral residuals measure how much of the spectral signal is not accounted for by the model. If the residual is high, then some of the signal cannot be explained by the model, rendering it unsuitable. If the residual is low, the signal is well summarized by the model and therefore relevant. The regression between age and each constituent was fitted using linear, quadratic, and exponential lines of best fit. The quadratic and exponential fits did not perform significantly better than the linear fit and so were ignored, based on Ockham's razor of simplicity.

For identification purposes, Raman spectra were also recorded from pure samples of oleic acid methyl ester, palmitic acid methyl ester, collagen III, and elastin purchased from Sigma-Aldrich (St. Louis, MO).

RESULTS

A range of Raman spectra was collected from both Bruch's membrane and sclera samples (Fig. 1). A number of differences

FIGURE 1. Mean Raman spectra of Bruch's membrane and sclera compared with spectra of collagen type 3, elastin, and heme. All spectra were recorded at 633 nm. Spectral intensities were normalized by the mean intensity of the spectra. Spectra were offset for clarity.

TABLE 1. Variation in Biochemical Signals Identified in the Raman Signals of Sclera

	Tissue	Age
Heme	Bruch's membrane*	NS ($R^2 = 0.41$)
MUFA	Sclera†	Increase ($R^2 = 0.62$)
Cholesterol	Bruch's membrane†	NS ($R^2 = 0.46$)
HHE	NS	NS ($R^2 = 0.00$)
CML/CEL	Bruch's membrane†	Increase ($R^2 = 0.74$)
GO	Bruch's membrane†	NS ($R^2 = 0.11$)
GH-1	Bruch's membrane†	Increase ($R^2 = 0.57$)
DHP-lys	Bruch's membrane†	NS ($R^2 = 0.26$)
Oxidized PUFA	Bruch's membrane†	NS ($R^2 = 0.44$)
Collagen 1	NS	NS ($R^2 = 0.42$)
Collagen 3	Bruch's membrane†	NS ($R^2 = 0.43$)
Collagen 4	Sclera	Increase ($R^2 = 0.88$)
Elastin	Bruch's membrane*	Increase ($R^2 = 0.65$)
Alpha crystallin	Sclera†	NS ($R^2 = 0.42$)

NS, not significant.

* $P < 0.01$.† $P < 0.05$, significant increase.

are immediately apparent within the signals of these two tissues. There was a higher intensity of features in Bruch's membrane-derived spectra at 1260 and 1590 cm^{-1} , and this contrasted with the sclera spectra, which had higher intensity features at 860 and 940 cm^{-1} (Fig. 1). Both spectra were dominated by the same three signals: collagen, elastin, and heme, as shown by comparison with the reference "finger-print" spectra for these three molecules (Fig. 1). The Raman spectra recorded for Bruch's membrane exhibited a much higher contribution from heme (4 $\times$ greater heme/collagen ratio) and elastin (5 $\times$ elastin/collagen ratio) than the sclera spectra. Table 1 summarizes the variation in the levels of the different molecular constituents identified from the Raman signals recorded for sclera. Although all the constituents identified within Bruch's membrane were also present in the sclera, the actual levels and their variations with age showed significant differences between the two tissues.

PCA of the sclera data revealed two additional signals—a carotenoid and a fatty acid—neither of which was observed in Bruch's membrane (Figs. 2a, 2f). The fatty acid-based lipid signal observed in Bruch's membrane (Fig. 2b) demonstrated a much stronger contribution from unsaturated fatty acid (Fig. 2d) than fatty acid-based lipid signals from the sclera (Fig. 2a). The bandwidth of the Raman features broaden as intermolecular packing disorder increases, and the triplet of bands from 1060 to 1120 cm^{-1} are excellent indicators of physical state in fatty acid-based lipids, with the two extreme bands arising from solid fat and the central band from disordered (liquid) fat.²³ Spectral differences between the sclera fat and the Bruch's membrane fat confirm that the degree of unsaturation has a clear effect on the solid fat content of these lipids at room temperature. The position of the carotenoid bands is dependent on the total length of the conjugated chain, with the band positions occurring very close to those observed in beta carotene, suggesting that the carotenoid present in sclera has a similar length of conjugated chain. Unsaturated bonds in fatty acids are responsible for the band at 1660 cm^{-1} .¹⁶ The height of this band in the fatty acid-based lipid Raman spectrum from Bruch's membrane was comparable to that obtained for mono-unsaturated oleic acid and adipose tissue,^{24,25} whereas the sclera spectral profile exhibited a greater degree of saturation.

Figure 3 displays a signal extracted from the sclera, the seventh constituent extracted by the PCA model from the Bruch's membrane. The equivalent signal from the Bruch's membrane is broadly similar with one notable exception, indicated by an asterisk. The band that is missing in the sclera is

that of carboxymethyl-lysine (CML), whereas the spectra of CEP and G-H1 can account for the signal from constituent 7 of the sclera data. The primary CML contribution in both sclera and Bruch's membrane occurred in constituent 5. Amino adipic acid had been a minor contributor to the Bruch's membrane signal but was not deemed to be detectable in this signal.

Although all the constituents detected in Bruch's membrane were also found in the sclera, as already noted, the concentration levels were generally different, with the consequence that *t*-tests of the measured proportions show a significant difference between the tissues in most instances (Table 1). For both tissues, AGEs showed an age-related increase, exemplified by the important AGE adducts, carboxymethyl-lysine/carboxyethyl-lysine (CML/CEL) and N δ -(5-hydro-4-imidazolone-2-yl)-ornithine (GH-1) (Figs. 4a, 4b). By contrast, there was no change observed in the sclera with the AGE/ALE precursors glyoxal (GO) and 4-hydroxyhexenal (HHE) (Figs. 4c, 4d). An aggregate of AGE signals demonstrated the similarities between sclera and Bruch's membrane in terms of accumulation with chrono-

FIGURE 2. Raman spectra of fatty acid lipids from (a) sclera, (b) Bruch's membrane, and (c) lung surfactant compared with Raman signals from pure fatty acid methyl esters (d), oleic (18:1; unsaturated, liquid), (e) palmitic (16:0; saturated, solid), and (f) scleral carotenoid compared with (g) beta carotene. All spectra were recorded at 633 nm at 21°C. Spectral intensities were normalized by the mean intensity of the spectra. Spectra were offset for clarity. Bandwidth of the features was dependent on the physical state—sharp in solids, broad in liquids. Bruch's membrane fat has a low solid fat content with significant unsaturation, whereas scleral fat has a high solid fat content and low saturation.

FIGURE 3. Comparing Raman spectra of (a) scleral biochemical constituent 7 with (b) the equivalent signal extracted from the Bruch's membrane. Also included are (c) G-H1 and (d) CEP. Scleral constituent 7 can be wholly accounted for by a combination of signals from AGE/ALEs, but, unlike the Bruch's membrane, CML (prominent peak indicated by *asterisk*) was not detected in this signal.

logical age, with aggregate R^2 scores of 0.824 and 0.717, respectively (Fig. 5).

To test whether the sclera exhibited essentially the same age-related changes as Bruch's membrane, the Raman spectral model of aging in this matrix was applied.⁴ In the case of sclera

FIGURE 4. Comparison of the trends in Raman predicted AGE/ALE levels with age for Bruch's membrane (*diamond*) and sclera (*square*). (a) CML/CEL. (b) GH-1. (c) GO. (d) HHE. These predicted scores were calculated by applying a PCA model developed using Bruch's membrane⁴ to a test set of Bruch's membrane data³ and sclera data.

FIGURE 5. Aggregate PC score of all identified AGE/ALEs against donor chronological age in (a) sclera and (b) Bruch's membrane. This aggregate score was calculated by summing the PC scores for each of the individual AGE/ALE. The individual scores were calculated by applying a PCA model developed using Bruch's membrane⁴ to a test set of Bruch's membrane data³ and sclera data.

data, there was a clear linear increase with age, although this occurred at a reduced rate compared with Bruch's membrane (Fig. 6). To determine how well a Bruch's membrane-derived model fits the sclera data, residuals were calculated. It was found that the scleral residual was not significantly different

FIGURE 6. Raman-PLS regression for the predicted index of aging for Bruch's membrane data (*diamond*) and sclera data (*square*). This predicted age was calculated by applying a model of aging developed using Bruch's membrane⁴ to a test set of Bruch's membrane data³ and sclera data.

from that obtained for Bruch's membrane data. In contrast, applying the same Bruch's membrane model to a series of spectra from an unrelated cell line subjected to AGE accumulation in vitro gave a residual 200 times higher.

DISCUSSION

Although the sclera is rich in extracellular matrix proteins, it cannot be assumed that age-related changes reflect what is happening in other ocular tissues. Bruch's membrane, for example, is subject to a unique metabolic environment with high oxygenation, flux of proteins, and metabolites and oxidative products. Indeed spectral analysis of the individual biochemical constituents (Table 1) confirms that the sclera is not directly analogous to Bruch's membrane. It is already known that the protein composition of the tissues differs, depending on the proportion of elastin present; the sclera contains only approximately 2% elastin,²⁶ but one of the five layers of the Bruch's membrane is composed almost entirely of elastin.²⁷ Although the precise levels differ, the range of constituents found in sclera and the Bruch's membrane is similar. Where there are differences in the constituents, such as in fat and carotenoid composition, they induce very minor spectral changes.

The major structural constituent of both tissues is collagen. This accounted for most of the Raman signal in sclera and Bruch's membrane. The similarity in the range of constituents suggests that the models created from Bruch's membrane data could be directly transferable to the sclera. This was true for some individual AGE/ALE adducts (namely CML/CEL and G-H1), even though they were present at lower levels and most displayed weaker individual trends than had been observed for Bruch's membrane. As measured by Raman spectroscopy, sclera contains AGE levels approximately one-fourth the levels occurring in Bruch's membrane. Consequently, it appears that measuring individual AGE/ALE adducts in the sclera may not be a useful age index, with the possible exception of CML/CEL and G-H1 (which are highly abundant AGEs²⁸). Aggregated AGE data in sclera do, however, show a robust correlation with chronological age, and this reflects a similar outcome with Bruch's membrane.

The results in Figure 3 show that there is a subtle difference in the interactions of the various AGEs in the sclera compared with the Bruch's membrane. Both tissues feature CML in a separate principal component, indicating that most CML has no direct relationship with G-H1 and CEP. In the Bruch's membrane, the appearance of CML as part of the constituent 7 signal indicates a weak association between a small proportion of the CML and G-H1/CEP. The absence of CML in scleral constituent 7 suggests that there is no association between CML and G-H1/CEP in the sclera.

As indicated by the AGE results, some of the age-linked changes in molecular composition in Bruch's membrane occur at much lower concentrations in the sclera, which makes their individual determination less accurate. Therefore, using sclera as a surrogate for other ocular tissues necessitates the aggregation of individual results. This brings the advantage of serving to cancel out much of the error in the individual determinations, thus leading to a reliable index of retinal aging. In the present study, a previously generated model of age-related Raman spectral changes in Bruch's membrane was applied. This approach was chosen to test whether using a broader range of the biochemical composition within the sclera could provide a robust indicator of the extent of aging in this tissue.

This approach uses a PLS regression generated on the Bruch's membrane to calculate an inherently aggregated measure of age that used contributions from 28 constituents (14

factors, each describing the variation between two constituents).⁴ The model of aging showed a marked linear increase with age when applied to the sclera, suggesting that the same changes do indeed occur in the spectra of the sclera. The fact that the residuals after prediction were not significantly different between the sclera and Bruch's membrane data confirmed that the model is relevant for both tissues, unlike the negative control data for a cell line, which showed much higher residuals. The range of biochemicals in sclera and Bruch's membrane only showed slight differences spectrally. In contrast, a spectral dataset dominated by cellular proteins rather than collagens radically increases the residual by two orders of magnitude, despite the dominant signals having been due to the same class of biomolecule (protein).

Although we have concentrated on comparing the age-related changes in the sclera with those in the Bruch's membrane, the multiplexed Raman technique is capable of acting as a powerful tool for the study of age-related changes in the sclera and thereby for the study of stiffness and permeability of the sclera, which have important implications for pathology and for pharmaceutical drug delivery, respectively. In the sclera, the slope of the trend line is considerably lower (one-fourth that for Bruch's membrane), which suggests that these spectral changes occur at a much lower rate. This may be linked to the fact that Bruch's membrane and the sclera are separated by the choroid so that the sclera is buffered from the high metabolic rate occurring in the photoreceptors. Despite the changes occurring at a lower level in the sclera, they nevertheless do occur at detectable levels, opening up the possibility of predicting the events in the Bruch's membrane by means of remote measurement under less restrictive conditions in the sclera. The fact that the changes occur at a lower rate in the sclera than in the Bruch's membrane suggests that a larger data set would be required to achieve the same depth of insight into the sclera as was possible with the Bruch's membrane.³ However, it is anticipated that any further studies would necessitate increased sample numbers.

Further investigation of the usefulness of the sclera as a marker of Bruch's membrane status will clearly require substantial validation of the preliminary findings of this study. Ultimately, the aim will be to determine whether relatively early-stage biochemical changes observed in the sclera correlate with Bruch's membrane pathology and the risk for conditions such as age-related macular degeneration.

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