Alterations in the Biomolecular Signatures of Developing Chick Corneas as Determined by Biospectroscopy and Multivariate Analysis

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PURPOSE. Biospectroscopy tools are increasingly being recognized as novel approaches toward interrogating complex biological structures in a nondestructive fashion. This study was conducted to apply these tools to interrogate alterations in the molecular signatures of developing chick corneas during the onset and development of transparency.

METHODS. Embryonic chick corneas (n = 46) were obtained at 2-day intervals from embryonic day (E)10 to E18 of incubation and investigated with attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy and Raman microspectroscopy. Resultant spectra were analyzed for variance by using principal component analysis and linear discriminant analysis (PCA-LDA).

RESULTS. Mean spectra after ATR-FTIR spectroscopy or Raman microspectroscopy derived from corneas at each developmental stage showed some overlap; however, in PCA-LDA scores plots, a clear segregation of spectra was evident, and two-category discrimination indicated that significant molecular alterations occur during tissue morphogenesis. Notable by both techniques was the increasing intensity of DNA signal (1080 cm\(^{-1}\)) from E10 onward. Major segregating biomarkers identified by ATR-FTIR spectroscopy between E10 and E18 were in the DNA/RNA (1126 cm\(^{-1}\)), glycogen (1045 cm\(^{-1}\)), protein (1470 cm\(^{-1}\)), and amide II (1512 cm\(^{-1}\) and 1524 cm\(^{-1}\)) spectral regions. Raman spectroscopy also identified major distinguishing vibrational modes that included proteins, amino acids (tyrosine, proline, phenylalanine, and valine), and secondary structures of proteins (amide I and amide II).

CONCLUSIONS. The developing chick cornea undergoes significant changes in its biomolecular composition in the E10 to E18 developmental period, with the major changes occurring over the spectral regions associated with DNA/RNA, proteins, glycogen, and secondary protein structures. (Invest Ophthalmol Vis Sci. 2012;53:1162-1168) DOI:10.1167/iovs.11-9262

The chicken embryo cornea, which develops into a fully functioning transparent tissue in the week leading up to hatching at day 21 of incubation, is an excellent model for the study of matrix morphogenesis and corneal structure-function relationships.\(^1\)\(^{-1}\) Pioneering work documented fundamental anatomic and biochemical aspects of chick corneal development.\(^3\)\(^{-1}\) Later investigators added more detail about the roles of various collagen types that are influential in corneal development.\(^1\)\(^{-1}\); the mechanisms by which cells deposit an organized fibrillar matrix;\(^1\)\(^{80}\);\(^{-24}\) the tissue’s changing gene expression profile;\(^35\); and the compositional alterations in proteoglycans, their sulfation status, and their relationship with collagen.\(^26\)\(^{-33}\) To further clarify the developmental events in chick corneal morphogenesis, we undertook a series of investigations using infrared (IR) spectroscopy or Raman microspectroscopy to elucidate the biomolecular changes in the cornea as it grows and becomes transparent between embryonic day (E)10 and E18.

Attenuated total reflection Fourier-transform IR (ATR-FTIR) spectroscopy is based on the concept that biomolecules absorb in the mid-IR region (\(\lambda = 2.5-25\) \(\mu\)m) through the vibration of chemical bonds. The vibration is detected, and derived from this is a detailed biomolecular fingerprint of the cells or tissue being interrogated in the form of an IR spectrum that relates to chemical structure.\(^34\),\(^35\) Raman microspectroscopic techniques, on the other hand, work on the principle that chemical bonds are excited to a virtual state through excitation of photons that subsequently relax to a different vibrational state. This relaxation causes inelastic photon-scattering and a frequency shift,\(^36\) and acquired spectra provide data with a high spatial resolution and a wide vibrational profile, with minimal impact of aqueous.\(^34\),\(^37\) ATR-FTIR spectroscopy and Raman microspectroscopy, although fundamentally different, provide complementary information about the vibrational modes of molecules within tissues and have recently been used for the characterization of cell cycle events, disease diagnosis, and cancer diagnosis.\(^34\)\(-39\) ATR-FTIR spectroscopy and Raman microspectroscopy experiments generate complex biochemical datasets containing hundreds and thousands of spectra, and analysis is best achieved by multivariate approaches such as principal component analysis (PCA) and/or linear discriminant analysis (LDA). This allows for data reduction to facilitate the identification of wavenumber-related biomarkers, such as glycoprotein content, conformational protein changes and phosphorylation, and structural alterations in DNA/RNA.\(^39\) In the present

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study, we used ATR-FTIR spectroscopy and Raman microspectroscopy to obtain biomolecular signatures from developing chick corneas in the E10 to E18 developmental period when the tissue is undergoing embryonic growth and transition into a mature transparent tissue.

MATERIALS AND METHODS

Tissue Source
Fertilized white leghorn chicken eggs were obtained from a commercial hatchery (Henry Steward and Co., Lincolnshire, UK), left to settle for approximately 24 hours, and placed in an incubator at 37.5°C (Octagon-100; Brinsea Products Ltd., Sandford, UK). After the required period (i.e., at embryonic day [E]10, E12, E14, E16, and E18), the corneas (n = 48) were excised at the limbus and dried under light compression between paraffin film-covered glass microscope slides. All work was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all corneas were interrogated by both spectroscopic approaches.

ATR-FTIR Spectroscopy and Raman Microspectroscopy
Dried and flattened corneas were placed on 1-cm² low-E-reflective glass slides (Kevley Technologies, Chesterland, OH) for analysis. IR spectra for ATR-FTIR spectroscopy were obtained with an FTIR spectrometer (model 1600; PerkinElmer, Waltham, MA) applied before LDA (thus, PCA-LDA) to reduce computational complexity and increase the recognition accuracy in different classes.40,41 PCA can be applied before LDA (thus, PCA-LDA) to reduce computational complexity and increase the recognition accuracy in different classes.40,41 Principal component analysis (PCA) is a data separation technique that maximizes interclass variance. LDA is a data separation technique that maximizes interclass variance and increase the recognition accuracy in different classes.40,41

RESULTS
All corneas were interrogated by ATR-FTIR spectroscopy followed by Raman microspectroscopy, and typical representative spectra are shown (Fig 1A, ATR-FTIR spectroscopy; Fig. 1B, Raman microspectroscopy). Initial PCA on the average ATR-FTIR spectra on each embryonic day (E10 [n = 6], E12 [n = 9], E14 [n = 9], E16 [n = 10], and E18 [n = 12]) indicated a degree of overlap between the developmental days, but with a notable difference in the increasing of intensity of DNA (1080 cm⁻¹; symmetric phosphate, νₗ₉,PO₄²⁻) from E10 onward (data not shown). The average Raman spectra (E10 [n = 4], E12 [n = 5], E14 [n = 9], E16 [n = 10], and E18 [n = 11]) confirmed the overlap observed after ATR-FTIR spectroscopy, and again highlighted the between-class discrimination noted at 1080 cm⁻¹.

Owing to the overlap of data and subtlety of the spectral variations between developmental days, exploratory multivariate analysis (i.e., PCA-LDA) was performed. In this more powerful discriminatory approach, each spectrum becomes a point at which the best segregation and associated wavenumber are identified. By this analysis, a clear cluster segregation of the chick corneas at different developmental days was observed, as seen in 2-D and 3-D PCA-LDA scores plots (Figs. 2A, 2C). In particular, the PCA-LDA data indicated that there was less overlap of spectral points between developmental stages with greater intervals (e.g., E10 vs. E18). Consistent with the ATR-FTIR spectroscopy observations, 2-D and 3-D PCA-LDA score plots of the Raman data reveal clustering and segregation of data points from corneas at different developmental days (Figs. 2B, 2D), although these clusters were less tight than those derived after ATR-FTIR spectroscopy, especially at E10 and E14. The discriminating wavenumbers responsible for the segregation ATR-FTIR and Raman spectra with progressive corneal development as revealed by PCA-LDA are shown in the derived spectrometric data with the same software (R2009a, MatLab; The MathWorks) and a graphic user interface toolkit for spectroscopy (http://biophotonics.lancs.ac.uk/software).

Computational Analysis
PCA allows for the reduction of the number of variables in the spectral dataset, whose principal components (PCs) can capture more than 95% of the variance present in the original dataset.40 LDA is a data separation technique that maximizes interclass variance. PCA can be applied before LDA (thus, PCA-LDA) to reduce computational complexity and increase the recognition accuracy in different classes.40,41

Initially, using OPUS software, ATR-FTIR spectra were individually cut to the biochemical cell fingerprint region (1800–900 cm⁻¹). Subsequently, spectra were baseline corrected and normalized to amide I (1650 cm⁻¹).

Raw Raman spectra were subtracted for cosmic rays or atypical peaks using the spectrometer system software (Wire 3.1; Renishaw). In particular, some residual paraffin peaks caused by the specimen environment were evident; thus, the following Raman spectral regions were removed before analysis: 1470 to 1405, 1305 to 1285, 1145 to 1115, 1070 to 1050, and 760 to 690 cm⁻¹. Data from every three spectra were then averaged and cut between the 1750 and 500-cm⁻¹ (1536 data points) spectral range. Subtraction of background fluorescence was achieved by baseline correction using the polynomial technique followed by vector normalization (MatLab; The MathWorks, Natick, MA). Multivariate analysis (i.e., PCA-LDA) was applied to the

**FIGURE 1.** Average spectra of all spectral acquisitions from developing chick corneas at different embryonic days (E10 [red], E12 [blue], E14 [green], E16 [magenta], and E18 [yellow]) after (A) ATR-FTIR spectroscopy and (B) Raman microspectroscopy.
cluster vector plots in Figure 3, and the main biomarkers in each category are listed in Table 1. These cluster vector plots indicate the discriminating variables in the corresponding scores plots proportional to peak intensity.

Two-category discriminant analysis of ATR-FTIR spectra from chick corneas at different stages of embryonic development was used to determine the wavenumbers responsible for between-category spectral variance. PCA-LDA scores plots show an obvious discrimination between E10 versus E18 with little overlap (Fig. 4A). The E12 versus E18 comparison reveals clustering and segregation of data, with distinguishing wavenumbers observed at 1543 (amide II), 1709 (amide I), 1126 (DNA/RNA), and 1053 (glycogen) cm\(^{-1}\) (Figs. 4C, 4D). A relatively good segregation was notable between E14 and E18, with the main distinguishing wavenumbers being 1524 (amide II), 1466 (proteins), 1512 (amide II), 1524 (amide II), 1732 (lipid), and 1612 (amide I) cm\(^{-1}\) (Figs. 4E, 4F). Owing to progressive corneal development, it is not surprising that the segregation between E16 and E18 was not as marked as that in the other between-category comparisons. Nevertheless, some segregation was found, and the wavenumbers most responsible were 1466 (proteins), 1512 (amide II), 1524 (amide II), 1732 (lipid), and 1612 (amide I) cm\(^{-1}\) (Figs. 4G, 4H).

Scores plots of the Raman microspectroscopy data when investigated by PCA-LDA confirmed between-category discrimination based on developmental day and revealed that, consistent with progressive developmental change, the segregation was more notable the larger the interval between developmental time points. No overlap was apparent in the E10 versus E18 comparison in which the following wavenumbers were responsible for segregation: 1395 (proteins), 1384 (proteins), 855 (tyrosine and proline), 1673 (amide I), and 1679 (amide I) cm\(^{-1}\) (Figs. 5A, 5B). There was also a clear discrimination between E12 and E18, with 855 (tyrosine and proline), 1681 (amide I), 919 (proline and valine), 935 (proline and valine), and 1656 (amide I) contributing to the segregation (Figs. 5C, 5D). The degree of segregation was less evident when the E14 and E18 spectral points were compared, for which the main distinguishing wavenumbers were found to be 856 (tyrosine and proline), 937 (proline and valine), 814 (proteins), 1635 (amide I), and 1002 (phenylalanine) cm\(^{-1}\) (Figs. 5E, 5F). Unlike the clear segregation seen in comparisons between E18 and E10/E12, notable overlap was seen for the E16 versus E18 between-category comparison, with the wavenumbers 1002 (phenylalanine), 1685 (amide I), 1688 (amide I), 1035 (phenylalanine), and 1657 (amide I) cm\(^{-1}\) responsible for the segregation that was observed (Figs. 5G, 5H).

**DISCUSSION**

In this study, ATR-FTIR spectroscopy and Raman microspectroscopy with multivariate analysis were used to characterize the changing biomolecular fingerprint of the embryonic chick cornea as it develops from E10 to E18 and becomes transparent. A major finding of both techniques was an enhanced peak at 1080 cm\(^{-1}\), specific to E10. This wavenumber corresponds to $v_{as}\text{PO}_2\}$, suggesting a major conformational difference in the genomic material at E10 compared with E12, E14, E16, and E18. Although the biological significance of this difference remains to be fully determined, it has been suggested that it may be associated with an altered methylation state.\(^{46,47}\) Previous measurements of the relative levels of...
mRNA in the developing chick cornea have shown an increase in the message for the keratan sulfate proteoglycan core proteins lumican, keratocan, and mimecan (osteoglycin) between E9 and E12, with a steady decrease thereafter. It is unlikely, therefore, that changes in keratan sulfate proteoglycan core proteins underlie the change in DNA conformation subsequent to E10, with the alterations in the genetic mechanisms underlying the biosynthesis of collagen, the major component of the cornea, likely to be important. In relation to the changes in DNA/RNA after E10, it is also noteworthy that recent investigations of developing chick corneas at E14 have indicated that the relative cellular volume of the stroma ascertained by volume scanning electron microscopy is in the region of 25%, higher than previous estimates. Parallel analyses of chick corneas at E10 and E18 have provided a similar overall volume estimate for the keratocytes in the stromal matrix (Young RD, and Quantock AJ, unpublished data, 2011). Although the cells tend to have flatter profiles. It is likely, therefore, based on the relatively high signal from H9263 at E10, that a greater proportion of the keratocyte cell body is occupied by the nucleus at this stage of embryogenesis compared with later developmental stages. As stated, it could be that methylation of the genome plays a significant role, with epigenetic mechanisms allowing for subsequent development stages.46

More subtle changes in the complex datasets of ATR-FTIR and Raman spectra may be extracted only by a multivariate

### Table 1. Top Five Ranked Tentative Peaks of Every Category Observed in the Cluster Vector Plots

<table>
<thead>
<tr>
<th>Embryonic Day</th>
<th>ATR-FTIR Spectroscopy</th>
<th>Raman Microspectroscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1038, 1126, 1470, 1620, 1524</td>
<td>853, 1035, 1679, 1684, 1675</td>
</tr>
<tr>
<td>12</td>
<td>1686, 1701, 1717, 1736, 1539</td>
<td>814, 919, 938, 1084, 1658</td>
</tr>
<tr>
<td>14</td>
<td>1015, 1701, 1593, 1616, 1736</td>
<td>814, 939, 919, 1084, 1655</td>
</tr>
<tr>
<td>16</td>
<td>1126, 1045, 1462, 1528, 1416</td>
<td>919, 938, 1250, 814, 1028</td>
</tr>
<tr>
<td>18</td>
<td>1701, 1736, 1531, 1601, 1123</td>
<td>856, 814, 1685, 1635, 919</td>
</tr>
</tbody>
</table>

Spectral ranges and their respective biological associations. ATR-FTIR spectroscopy: glycogen (1015, 1038, and 1045 cm⁻¹); DNA/RNA (1123, 1126, and 1219 cm⁻¹); proteins (1416, 1462, and 1470 cm⁻¹); amide II (1489, 1508, 1524, 1531, and 1539 cm⁻¹); amide I (1593, 1601, 1620, 1686, and 1701 cm⁻¹); and lipid (1717 and 1736 cm⁻¹). Raman microspectroscopy: proteins (813 and 814 cm⁻¹); proline, glucose, and lactic acid (919 and 921 cm⁻¹); proteins (938 and 939 cm⁻¹); phenylalanine (1035 cm⁻¹); proteins (1084 cm⁻¹); amide III (1250 cm⁻¹); and amide I (1635, 1658, 1679, and 1685 cm⁻¹).17,18

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933464/) Two-category discriminant analysis using PCA-LDA after ATR-FTIR spectroscopy. (A) 1-D scores plot (E10 vs. E18); (B) cluster vector plot (E10 vs. E18); (C) 1-D scores plot (E12 vs. E18); (D) cluster vector plot (E12 vs. E18); (E) 1-D scores plot (E14 vs. E18); (F) cluster vector plot (E14 vs. E18); (G) 1-D scores plot (E16 vs. E18); and (H) cluster vector plot (E16 vs. E18).
approach, such as PCA-LDA. This highlighted good between-category segregation (i.e., developmental days) in scores plots. Moreover, scores plots derived from two-category discrimination between E18 and earlier developmental days revealed that comparisons over larger intervals result in less overlap. Nearness in multivariate distance implies pattern recognition, whereas the separation of sample clusters in the plots signifies structurally dissimilar groups. Both ATR-FTIR spectroscopy and Raman microspectroscopy disclosed the greatest degree of separation between E10 and E18 and the most similarity at E16 and E18. Thus, biospectroscopy can be indicative of alterations in the biomolecular signatures of developing chick corneas that are progressive and cumulative. Biospectroscopy tools appear to hold great promise toward lending new insights into molecular questions.

As the secondary chick cornea grows in ovo from E10 to E18, the presumptive keratocytes synthesize and deposit new and progressively more well-organized extracellular matrix. ATR-FTIR spectroscopy reveals how biomarkers at E10 are clearly different from those at later developmental days and, as well as changes in DNA/RNA previously discussed, these can be attributed, predominantly, to changes in glycoprotein, protein, and secondary structures of protein (amide I and II), consistent with new matrix deposition. Raman spectroscopy also indicates that the major distinguishing vibrational modes that differ between E10 and later stages include proteins, constituent amino acids (tyrosine, proline phenylalanine, valine), and secondary structures of proteins (amide I and II). Collagen is undoubtedly the major component of the newly deposited corneal matrix, and, as comprehensively reviewed by Linsenmayer et al.,¹ a multitude of alterations in the production and assembly of the various fibril- and non-fibril-forming collagens, their subtypes and potential interactions, occur throughout the development of the chick stroma.¹⁰⁻¹⁹ Previous studies of corneal morphogenesis have indicated a progressive doubling in the amount of hydroxyproline in the embryonic corneas between E12 and E14, between E14 and E16, and between E16 and E18,³³ suggestive of a fairly linear rate of growth and collagen deposition over these 2-day intervals (E10 corneas were not included in the previous analysis). The new spectroscopy data presented herein point to a post-E10 accelerated matrix deposition, with a presumed steady rate of synthesis thereafter. Collagen is the most abundant protein of animal origin and FTIR spectroscopy has been applied to investigate its molecular structure;⁴⁸ however, it was not possible in this study to assign specific spectral regions such as the amide I peak solely to collagen structure, given that entire corneas were analyzed. Future studies could shed new insights of collagen-specific alterations in corneal development by observing analysis-isolated constituents.

Within reason, one would surmise that these bioanalytical methods are relatively nondestructive in comparison with other conventional methodologies. ATR-FTIR spectroscopy requires the diamond to come into contact with the biological material,³⁵,⁴⁰,⁴¹ so some pressure effects will be exerted on the corneal samples. However, the sample is still retained if, for instance, one wishes to extract DNA/RNA from it or stain it. The 785-nm laser diode appears to be minimally destructive of biological samples.⁴⁹,⁵⁰ A promising observation was that these two biospectroscopy methodologies exhibited a good correlation between their respective findings.

In summary, this study demonstrates that ATR-FTIR spectroscopy and Raman microspectroscopy can be used to reveal significant spectral discrimination and changing biomolecular discrimination using PCA-LDA after Raman microspectroscopy. (A) 1-D scores plot (E10 vs. E18); (B) cluster vector plot (E10 vs. E18); (C) 1-D scores plot (E12 vs. E18); (D) cluster vector plot (E12 vs. E18); (E) 1-D scores plot (E14 vs. E18); (F) cluster vector plot (E14 vs. E18); (G) 1-D scores plot (E16 vs. E18); and (H) cluster vector plot (E16 vs. E18).
signatures in the developing chick cornea and that, by the use of multivariate analysis, the biomarkers responsible for the segregation of corneas in the E10 to E18 developmental period can be discriminated.

References


