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Review

From nano to macro: Studying the hierarchical structure of the corneal extracellular matrix

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ABSTRACT

In this review, we discuss current methods for studying ocular extracellular matrix (ECM) assembly from the 'nano' to the 'macro' levels of hierarchical organization. Since collagen is the major structural protein in the eye, providing mechanical strength and controlling ocular shape, the methods presented focus on understanding the molecular assembly of collagen at the nanometre level using X-ray scattering through to the millimetre to centimetre level using non-linear optical (NLO) imaging of second harmonic generated (SHG) signals. Three-dimensional analysis of ECM structure is also discussed, including electron tomography, serial block face scanning electron microscopy (SBF-SEM) and digital image reconstruction. Techniques to detect non-collagenous structural components of the ECM are also presented, and these include immunoelectron microscopy and staining with cationic dyes. Together, these various approaches are providing new insights into the structural blueprint of the ocular ECM, and in particular that of the cornea, which impacts upon our current understanding of the control of corneal shape, pathogenic mechanisms underlying ectatic disorders of the cornea and the potential for corneal tissue engineering.

1. Introduction

In the vertebrate eye, the extracellular matrix (ECM) plays a fundamental role in defining tissue form and function. The ocular connective tissues serve both as a mechanically tough and protective outer layer and at the same time define the shape and transparency of the cornea necessary to form a refractive lens for focussing light back to the retina. In general, the properties of the ocular ECM are thought to be controlled by the unique spatial organization of the tissue components, which, as is the case in other connective tissues such as tendon and ligament, are predominantly proteins, glycoproteins and glycosaminoglycans/proteoglycans. Collagen is the principal structural element of connective tissues and while the molecular and cellular events involved in collagen fibrillogenesis are well known (Zhang et al., 2005), there is a major gap in our understanding of how the ECM and its different components are structurally organized and assembled to facilitate the functional demands of such diverse tissues.

On the cellular and molecular level, small diameter collagen fibrils in connective tissues are formed by triple helical chains of collagen peptides synthesized within the cell and then secreted and self-assembled within the extracellular space. As shown for developing tendon, short (10–30 μm) collagen fibril segments are assembled by fibroblasts within specialized extracellular compartments. These segments then grow both linearly and laterally, increasing fibril thickness and length (Birk and Trelstad, 1984). Collagen fibril segment growth has been shown using knockout mice to be related, in part, to expression of leucine-rich repeat proteoglycans and glycoproteins that influence both linear and lateral fibril fusion (Chakravarti et al., 1998, 2000; Danielson et al., 1997; Svensson et al., 1999). Elongating fibril segments also coalesce in the developing matrix to form larger fibers, which may branch and anastomose; a process that has been suggested to be controlled by cellular contacts and exertion of cytoskeletal forces within the boundaries of the specialized extracellular compartments formed by tendon fibroblasts during development.

A similar developmental program has been proposed for the cornea involving the intracellular synthesis, modification and packaging of procollagen, followed by directed fibril assembly within corneal fibroblast-organized extracellular compartments

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(Birk and Trelstad, 1984). More recently, filipodial extensions from keratocytes, termed keratopodia, have been identified in developing chick cornea also suggesting the cellular directed assembly of collagen fibrils and fibril bundles during development (Young et al., 2014). While cornea and tendon show distinct developmental similarities when considering collagen fibril formation, the tissues differ dramatically in both form and function with one showing parallel alignment of collagen fibers supporting uniaxial mechanical load, while the other shows a predominantly orthogonal, interwoven arrangement supporting formation of a 3-dimensional refractive lens. How these connective tissues are constructed from the same general materials to give very different structural and functional properties is unknown, and as noted by Trelstad and Birk in 1984, the story of "the weaving of the body fabric from the warp and woof of the matrix has yet to be told (Trelstad and Birk, 1984)."

As suggested by Kokott in his studies of eye structure (Kokott, 1938), insights into the mechanisms controlling corneal shape and function may be obtained by developing a blueprint of the cornea's architecture. Over the past 20–30 years, new technologies have become available that have furthered our understanding of hierarchical structures the cornea from the smallest (nano) to the largest (macro) scale. The purpose of this article is to review some of the influential imaging technologies which have been applied to the study of the ocular ECM, and describe advances in our knowledge to which they have contributed. Application and continued refinement of these (and, of course, other) technologies will lead to a better understanding of the mechanisms controlling corneal shape and function, the discovery of pathogenic mechanisms leading to refractive error and ectatic disorders, as well as help direct novel strategies for engineering more biomimetic corneal constructs.

2. X-ray scattering

X-ray scattering is not, in the strictest sense, an imaging modality because tissue sub-structure is not visualised in real space. Nevertheless, very useful – and importantly, quantitative – structural information about the organisation of the ocular ECM can be obtained across a number of length scales. Also referred to as X-ray fibre diffraction, the applicability of this approach for ultrastructural investigations of the eye lies in the fact that when a beam of X-rays is shone through excised ocular tissue, diffraction patterns produced by X-rays are scattered by the main structural component of the ocular ECM, collagen. Most of the collagen in the eye exists in the form of fibrils, which are made up of approximately parallel arrays of long thin collagen molecules, arranged with quasi-hexagonal lateral packing (Hulmes and Miller, 1979). This fairly high degree of regularity in the spatial distribution of collagen molecules leads to the preferential constructive interference of scattered X-rays in certain directions. The upshot is the production of so-called diffraction maxima, the analysis of which allows researchers to gain conformational information about the collagen molecules which gave rise to the maxima in the first place. A comprehensive treatment of the theory behind X-ray fibre diffraction studies of the ocular ECM is beyond the scope of this article, and can be found elsewhere (Meek and Quantock, 2001; Meek and Boote, 2009). Suffice it to say that X-rays scattered at relatively wide-angles (i.e. in the region of 5° of arc) provide measurements of the spacing between collagen *molecules* within fibrils, which in the hydrated human cornea are separated by about 1.6 nm (Meek et al., 1991). X-ray scattering is not just limited to investigations at intermolecular distances because diffraction maxima from cornea are also found at smaller angles, which arise because of the regular spacing of uniform diameter collagen *fibrils* within lamellae, an arrangement which, as noted by Maurice (1957), is required for tissue transparency. In the hydrated human cornea the average centre-to-centre separation of collagen fibrils is about 65 nm (Meek et al., 1991). A schematic representation of wide-angle X-ray scattering (WAXS) and small-angle X-ray scattering (SAXS) from regularly spaced and uniform diameter collagen fibrils as would be found in the cornea is shown in Fig. 1. It is important to appreciate, at this point, that while WAXS patterns can be recorded for all collagen-rich ocular tissues, SAXS patterns are only produced by the cornea, because of insufficient structural homogeneity on the fibrillar scale in other ECMs such as the sclera.

X-ray scatter can be sub-categorised into *equatorial* (i.e. that which is scattered perpendicular to the collagen fibril axis) and *meridional* (i.e. X-rays scattered parallel to the fibrillar axis). As alluded to earlier, analysis of the equatorial portion of the SAXS pattern from cornea provides quantitative structural information about the diameter of the collagen fibrils, their spacing, and level of order, or regularity, in the mode of their packing. The meridional SAXS pattern, on the other hand, can be analysed to ascertain information about the electron density along the axis of the collagen fibrils. As well as generating robust quantitative information about collagen intermolecular arrangements, the WAXS signal can be further interrogated to yield a numerical measure of the orientation distribution of collagen fibrils as an average of the specimen thickness (Fig. 1) This can be achieved because collagen molecules are aligned near-axially within the fibrils that they make up, an approach described in more depth by Meek and Boote (2009). And a salient feature of X-ray scattering experiments is that they are typically carried out on hydrated, excised tissue which has not undergone any chemical fixation or other such treatment. Thus, structural data is acquired which is illustrative of the tissue close to its native state. Moreover, during data collection the X-ray beam is ordinarily passed through the whole thickness of the cornea (or the whole thickness of a dissected or partially ablated portion of cornea in some experiments), so the final values obtained are highly representative averages of the tissue as a whole.

The corneal stroma is the most widely studied ocular ECM by SAXS and WAXS. Early X-ray fibre diffraction analyses of cornea were obtained using lab-based X-ray generators, and it typically took several hours to obtain usable diffraction patterns (Goodfellow et al., 1978; Worthington and Inouye, 1985). Because a particular strength of the X-ray diffraction approach is that the tissue can be investigated in its native hydrated state this was clearly sub-optimal, so researchers led by Gerald Elliott and Keith Meek began to use the more intense X-ray beams produced by large synchrotron radiation sources to investigate cornea (Sayers et al., 1982). The first published study was in 1981 based on data collected at the DESY synchrotron in Germany (Meek et al., 1981). Since that time the vast majority of X-ray fibre diffraction studies of the eye have been conducted using synchrotron radiation, notably at Daresbury SRS and Diamond in the UK, ESRF in France, and SPring8 in Japan. Nowadays, synchrotron X-ray diffraction patterns can be recorded on sensitive detectors with sub-second exposure times, and collectively SAXS and WAXS have been readily exploited to allow the collection of numerical information on collagen architecture across the ocular ECM, making a number of noteworthy contributions to our current understanding of corneal and scleral development, homeostasis and disease.

2.1. X-ray studies of ocular development

Studies of corneal development in the chick cornea between embryonic days 13 and 18 have utilised WAXS and shown that the intermolecular spacing does not change over this developmental interval, despite the fact that the stroma undergoes a significant compaction and increase in transparency (Coulombre and Coulombre, 1958). As development progresses over the same