GLYCOSAMINOGLYCAN CONTRIBUTION TO
THE STRUCTURE-MECHANICAL PROPERTIES
OF THE POSTERIOR SCLERA

by

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Abstract

The sclera is the white outer shell and main load-bearing tissue of the eye. It resists the intraocular pressure, therefore maintaining the shape of the eye, and protects the more fragile intraocular structures, such as the retina, from external forces. Alterations to the scleral mechanical properties can lead to the initiation and development of ocular pathologies such as myopia, often characterized by an axial elongation of the globe, or glaucoma, where the transmission of the visual information to the brain is impaired by a detrimental mechanical environment at the optic nerve head, at the back of the eye. While studies reported alterations in the mechanical properties of the posterior sclera of glaucomatous and myopic eyes, others measured alterations in the tissue microstructure, including the glycosaminoglycan (GAG) content. The contribution of collagen and elastin to the mechanical behavior of connective tissues is well known and consistent among tissues. However, the mechanical role of GAGs is tissue-dependent and has never been studied in the sclera. Therefore, this work aims to investigate the contributions of GAGs to the structure and mechanical properties of the posterior sclera, as well as their possible mechanical role in glaucoma...
ABSTRACT

and myopia.

This work developed experimental and numerical approaches to measure the structural and mechanical effects of sulphated glycosaminoglycans (s-GAGs) digestion in the posterior sclera. A setup was first developed to compare the two-dimensional (2D) and three-dimensional (3D) versions of digital image correlation (DIC) for a membrane under inflation. Although 2D-DIC can be useful to evaluate the profile behavior of materials inflated under experimental conditions that discourage the use of a stereovision system, only 3D-DIC can capture the 3D heterogeneous anisotropic mechanical behavior of the sclera, and was therefore used in this work. A protocol for s-GAG digestion was then developed and the inflation response of posterior scleral shells from pig and human eyes was measured before and after s-GAG degradation using 3D-DIC displacement tracking. Structural parameters such as the scleral thickness and hydration were also measured. A methodology was then developed to evaluate the error and uncertainty in strains due to the 3D-DIC displacement error and uncertainty for posterior scleral shells under inflation. An inverse finite element method (FEM) was finally applied to specimen-specific meshes of the porcine scleral shells to determine the effect of s-GAG degradation on the properties of the matrix and collagen components of the posterior sclera. It was concluded from this work that despite their low content compared to other structural components, s-GAG play measurable roles in the structure and mechanical properties of the posterior sclera, mainly through their effects on hydration and their interactions with the collagen
ABSTRACT

fibrils. Additionally, s-GAGs could be involved in the altered scleral mechanical properties measured in glaucoma and myopia.
ABSTRACT

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ACKNOWLEDGMENTS

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Dedication

This thesis is dedicated to my Mum, Christiane, for her support and encouragement throughout this journey.
Contents

Abstract ii

Acknowledgments vi

List of Tables xviii

List of Figures xxi

1 Introduction 1

1.1 The eye as part of the visual system 1

1.2 The sclera 3

1.2.1 Anatomy and function 3

1.2.2 Microstructure of the scleral connective tissue 5

1.2.2.1 Collagen 5

1.2.2.2 Elastin 7

1.2.2.3 Proteoglycans and glycosaminoglycans 8
CONTENTS

1.2.3 Mechanical Properties ........................................ 12
1.2.4 Mechanical testing ............................................. 14
1.2.5 Constitutive models ............................................. 17

1.3 The sclera in glaucoma and myopia ............................. 21
1.3.1 Glaucoma .................................................... 21
1.3.2 Myopia ...................................................... 22
1.3.3 Alterations to the scleral structure and microstructure .......... 24
1.3.4 Alterations to the scleral mechanical properties ................. 26

1.4 Objectives and significance of this thesis ......................... 26

2 Investigating the accuracy and uncertainty of digital image correlation ........................................ 33

2.1 Introduction ..................................................... 34
2.2 Methods ....................................................... 40
2.2.1 Specimen preparation ....................................... 40
2.2.2 Inflation testing ............................................. 41
2.2.3 Imaging ................................................... 43
2.2.4 Data analysis .............................................. 44
2.2.4.1 3D data analysis ....................................... 44
## CONTENTS

2.2.4.2  2D data analysis ........................................... 45  
2.2.5    3D-DIC static performance ................................. 46  
2.2.6    3D-DIC performance with inflation ......................... 47  
2.3    Results .......................................................... 48  
  2.3.1  3D regional variations ....................................... 48  
  2.3.2  Static noise .................................................. 50  
  2.3.3  Displacements ................................................ 50  
  2.3.4  Meridional strain ............................................ 54  
  2.3.5  3D-DIC static performance ................................. 55  
  2.3.6  3D-DIC performance with inflation ......................... 55  
2.4    Discussion ..................................................... 55  
2.5    Conclusion ..................................................... 61  

3  Alterations in the posterior sclera following glycosaminoglycan degra-
dation: a porcine experimental study  ................................... 63  
  3.1  Introduction ..................................................... 64  
  3.2  Methods .......................................................... 68  
    3.2.1  Experimental design and glycosaminoglycan removal ........ 68  
    3.2.2  Specimen preparation ...................................... 70  
    3.2.3  Hydration measurement .................................... 71  
    3.2.4  Glycosaminoglycan staining ................................ 73  
    3.2.5  Glycosaminoglycan quantification .......................... 73  

xii
## CONTENTS

3.2.6 Mechanical testing ........................................... 74
3.2.7 Thickness measurement ......................................... 75
3.2.8 Digital image correlation ...................................... 76
3.2.9 Strain calculation .............................................. 77
3.2.10 Hoop stress calculation ....................................... 80
3.2.11 Mechanical data analysis ..................................... 80
3.2.12 Statistical analysis ........................................... 82

3.3 Results .......................................................... 83

3.3.1 Glycosaminoglycan content, hydration and thickness .......... 83
  3.3.1.1 Glycosaminoglycan staining ................................ 83
  3.3.1.2 Glycosaminoglycan quantification ......................... 84
  3.3.1.3 Hydration .................................................. 85
  3.3.1.4 Thickness .................................................. 86

3.3.2 Mechanical behavior ........................................... 86
  3.3.2.1 Comparing the experimental groups ....................... 86
  3.3.2.2 Comparing the circumferential and meridional directions .................................................. 93
  3.3.2.3 Group/quadrant interaction ................................ 94
  3.3.2.4 Comparing the quadrants ................................ 94

3.4 Discussion ...................................................... 97

3.5 Conclusion ....................................................... 108
CONTENTS

4 Alterations in the posterior sclera after glycosaminoglycan degradation: a human experimental study 109

4.1 Introduction ................................................. 110

4.2 Methods .................................................. 114

4.2.1 Specimens and glycosaminoglycan degradation ........ 114

4.2.2 Specimen preparation .................................. 115

4.2.3 Glycosaminoglycan quantification ....................... 117

4.2.4 Mechanical testing ...................................... 117

4.2.5 Thickness measurement .................................. 119

4.2.6 Digital image correlation .............................. 120

4.2.7 Strain calculation ...................................... 120

4.2.8 Hoop stress calculation ............................... 122

4.2.9 Mechanical data analysis ............................. 124

4.2.10 Statistical analysis ................................ 125

4.3 Results .................................................... 125

4.3.1 Glycosaminoglycan degradation ....................... 125

4.3.2 Thickness data ....................................... 126

4.3.3 Mechanical behavior .................................. 126

4.3.3.1 Comparing the buffer-treated and enzyme-treated groups ........ 126
## CONTENTS

4.3.3.2 Comparing the circumferential and meridional directions .................................. 129

4.4 Discussion ............................................................................................................. 132

4.5 Conclusion ........................................................................................................... 137

5 The effects of glycosaminoglycan degradation on the scleral matrix and collagen: a numerical approach ................................................................. 138

5.1 Introduction ......................................................................................................... 139

5.2 Methods .............................................................................................................. 142

5.2.1 Experiments ................................................................................................. 142

5.2.2 Anisotropic hyperelastic constitutive model ................................................... 143

5.2.3 Finite element model .................................................................................... 146

5.2.3.1 Geometry ............................................................................................... 146

5.2.3.2 Boundary conditions ............................................................................... 147

5.2.3.3 Model validation ..................................................................................... 148

5.2.3.4 Convergence study .................................................................................. 149

5.2.3.5 Sensitivity study ..................................................................................... 149

5.2.4 Optimization ................................................................................................. 151

5.2.5 Statistical analysis .......................................................................................... 153

5.3 Results ................................................................................................................ 154

5.4 Discussion .......................................................................................................... 155

5.5 Conclusion .......................................................................................................... 159
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Number of specimens used for the measurement of GAGs, hydration and changes in thickness and mechanical behavior. The * denotes that 5 of the 8 specimens used to measure the thickness change were used for mechanical testing. Similarly, the # denotes that 7 of the 9 specimens used to measure the thickness change were subjected to mechanical testing.</td>
</tr>
<tr>
<td>3.2</td>
<td>s-GAG content [µg/mg dry tissue weight] in the 4 quadrants and 3 experimental groups. Significant interaction between quadrant and group (p &lt; 0.0001).</td>
</tr>
<tr>
<td>3.3</td>
<td>Hydration ratio [mg/mg] in the 4 quadrants and 3 experimental groups. Non-significant interaction between quadrant and group (p=0.17).</td>
</tr>
<tr>
<td>3.4</td>
<td>Thickness [mm] in the 4 quadrants and 3 experimental conditions. Non-significant interaction between quadrant and group (p=0.43).</td>
</tr>
<tr>
<td>3.5</td>
<td>Comparison of the mechanical outcomes, averaged over all quadrants, between the control and buffer-treated groups, in the circumferential and meridional directions. Paired Student’s t-tests were used for statistical analysis.</td>
</tr>
<tr>
<td>3.6</td>
<td>Comparison of the mechanical outcomes, averaged over all quadrants, between the buffer-treated and enzyme-treated groups, in the circumferential and meridional directions. Paired Student’s t-tests were used for statistical analysis.</td>
</tr>
<tr>
<td>3.7</td>
<td>Comparison of the mechanical outcomes, averaged over all quadrants, between the control and enzyme-treated groups, in the circumferential and meridional directions. Unpaired Student’s t-tests were used for statistical analysis.</td>
</tr>
<tr>
<td>3.8</td>
<td>Comparison of the mechanical outcomes, averaged over all quadrants for 2 specimens, between the buffer-treated and enzyme-treated groups with protease inhibitors, in the circumferential and meridional directions. NA = not applicable, as no statistical analysis could be performed on 2 specimens.</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9</td>
<td>Comparison of the absolute values of the shear strains, averaged over all quadrants and pressure levels of the loading regime, between the control and buffer-treated groups, and buffer-treated and enzyme-treated groups. Paired Student's t-tests were used for statistical analysis.</td>
<td>91</td>
</tr>
<tr>
<td>3.10</td>
<td>p-values for the comparison of the mechanical outcomes, averaged over all quadrants, between the circumferential and meridional directions, in the control, buffer-treated and enzyme-treated groups. Paired Student's t-tests were used for statistical analysis.</td>
<td>94</td>
</tr>
<tr>
<td>3.11</td>
<td>p-values for the interaction between group and quadrant for all mechanical outcomes. Linear mixed models were used for statistical analysis.</td>
<td>95</td>
</tr>
<tr>
<td>4.1</td>
<td>Number of specimens used for s-GAG quantification, hydration measurement, mechanical testing and thickness measurements. 1/1 indicates that 1 eye was used from 1 donor, 9/5 indicates that 9 eyes were used from 5 donors, * denotes that both eyes were from the same pair and # denotes that the same eyes were used for both experiments.</td>
<td>115</td>
</tr>
<tr>
<td>4.2</td>
<td>s-GAG content [µg/mg dry tissue weight] in each quadrant of a single eye, for samples in the buffer-treated and enzyme-treated groups. No statistical analysis.</td>
<td>126</td>
</tr>
<tr>
<td>4.3</td>
<td>Thickness [mm] averaged over all specimens and quadrants in the buffer-treated and enzyme-treated groups. Repeated measures ANOVA used for the statistical analysis.</td>
<td>126</td>
</tr>
<tr>
<td>4.4</td>
<td>Comparison of the mechanical outcomes, averaged over all quadrants, between the buffer-treated and enzyme-treated groups, in the circumferential and meridional directions. Repeated measures ANOVA used for the statistical analysis.</td>
<td>128</td>
</tr>
<tr>
<td>5.1</td>
<td>Table showing 1) 2 sets of test parameters, determined from the fit of the average stress-strain curve obtained experimentally for the control and enzyme-treated group, in the circumferential direction for the IN quadrant, and 2) the corresponding optimized parameters returned by the optimization algorithm. NO = non-optimized parameter.</td>
<td>151</td>
</tr>
<tr>
<td>5.2</td>
<td>Table showing the 4 optimized model parameters and collagen fiber stiffness, averaged over all specimens in the control and buffer-treated groups of protocol 1. Paired t-tests were used for statistical analysis.</td>
<td>154</td>
</tr>
<tr>
<td>5.3</td>
<td>Table showing the 4 optimized model parameters and collagen fiber stiffness, averaged over all specimens in the buffer-treated and enzyme-treated groups of protocol 2. Paired t-tests were used for statistical analysis.</td>
<td>155</td>
</tr>
</tbody>
</table>
LIST OF TABLES

5.4 Table showing the 4 optimized model parameters and collagen fiber stiffness, averaged over all specimens in the control and enzyme-treated groups of protocols 1 and 2, respectively. Unpaired t-tests were used for statistical analysis .............................................................. 155

6.1 Summary of the main structural and mechanical changes measured in the posterior porcine sclera after s-GAG degradation and the current best hypothesis for those changes ......................................................... 168

6.2 Summary of the main structural and mechanical changes measured in the posterior human sclera after s-GAG degradation and the current best hypothesis for those changes ......................................................... 168

6.3 Summary of the main changes in the mechanical behavior and corresponding matrix/collagen properties observed in the posterior porcine sclera after s-GAG degradation and the current best hypothesis for those changes ......................................................... 168

A.1 PID parameters used to equilibrate the porcine specimens at baseline pressure 0.28 kPa for 30 min and hold them at baseline pressure while taking the thickness measurements ............................................. 177

A.2 PID parameters used to load the porcine specimens from baseline pressure 0.28 kPa to 6 kPa at 0.13 kPa/s, unload them to 0.28 kPa at the same rate and hold them at baseline pressure for 30 min for recovery ......................................................... 178

A.3 PID parameters used to load the porcine specimens from baseline pressure 0.28 kPa to 6 kPa at 1.03 kPa/s, hold them at 6 kPa for 20 min, unload them to 0.28 kPa at the same rate and hold them at baseline pressure for 40 min for recovery ......................................................... 178

B.1 Table showing the 4 optimized model parameters and corresponding cost function for the control porcine specimens from protocol 1 .............................................................. 192

B.2 Table showing the 4 optimized model parameters and corresponding cost function for the buffer-treated porcine specimens from protocol 1 .............................................................. 193

B.3 Table showing the 4 optimized model parameters and corresponding cost function for the buffer-treated porcine specimens from protocol 2 .............................................................. 193

B.4 Table showing the 4 optimized model parameters and corresponding cost function for the enzyme-treated porcine specimens from protocol 2 .............................................................. 193
List of Figures

1.1 Schematic of the human eye and path of the light rays. Adapted from: https://nei.nih.gov/photo/anatomy-of-eye (Courtesy: National Eye Institute, National Institutes of Health (NEI/NIH)). . . . . . . . 2

1.2 Scanning electron micrograph of the cross-section of a human optic nerve head (Courtesy: Dr. Harry A. Quigley). The optic nerve head diameter is on average 1.8 mm in humans. . . . . . . . . . . . . . . . 4

1.3 Histophotograph of the longitudinal section of a human optic nerve head. The scleral flange (white arrows) connects the peripapillary sclera (black arrow) to the lamina cribrosa (yellow arrows), and forms the anterior boundary of the cerebrospinal fluid space (black star). Reproduced from Vurgese et al. with permission from Vurgese et al. 5

1.4 (a) Electron micrograph of the rabbit sclera showing collagen lamellae cl with different orientations, as well as fibroblasts f and elastin fibers ef. Adapted from Young et al. with permission from Company of Biologists Ltd. (b) Electron micrograph of the human sclera showing cross-striated collagen fibrils cf and GAGs in 3 different orientations, 1: radiating from the fibrils, 2: around the fibrils, 3: along the fibrils. Adapted from Young et al. with permission from Company of Biologists Ltd. 7

1.5 (a) Schematic of the 4 main proteoglycans of the human sclera and their associated glycosaminoglycans. CS/DS: chondroitin sulfate/dermatan sulfate, KS: keratan sulfate, N-glycan: N-linked oligosaccharide, Cys: cystein residue, TyrSO4: tyrosine sulfate, LRR: leucine-rich repeat. Adapted from Riley’s review article with permission from Cambridge University Press. (b) The repeating disaccharide unit of a dermatan sulfate glycosaminoglycan. COO−: carboxyl group, SO3−: sulfate group. Reproduced from Fig. 19-36 in Alberts et al. with permission from Garland Science/Taylor & Francis Group LLC. 9
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>Schematic of the interactions between proteoglycans and collagen fibrils based on electron tomography results of the bovine cornea. The proteoglycan core proteins (red and yellow ovals) are attached to the collagen fibrils (blue). The glycosaminoglycan chains (orange and brown lines) are represented in 3 different orientations: running along the fibrils, extending from or encircling the fibrils to form anti-parallel interactions with each other. Black arrowheads point to anti-parallel interactions between keratan sulfates (orange lines), while black arrows indicate anti-parallel interactions between chondroitin sulfates or dermatan sulfates (brown lines), and white arrowheads indicate mixed anti-parallel interactions between chondroitin sulfate or dermatan sulfate with keratan sulfate. White arrows show proteoglycan filaments running along the collagen fibrils. Adapted from Lewis et al. with permission from Elsevier.</td>
</tr>
<tr>
<td>1.7</td>
<td>(a) Second harmonic generation image of crimped collagen fibrils from a porcine scleral sample. Scale bar = 10 µm. Reproduced from Han et al. with permission from OSA Publishing. (b) Strain-stress curve for a typical collagenous tissue showing the collagen fibril recruitment as a function of the deformation. Reproduced from Freed and Doehring with permission from ASME.</td>
</tr>
<tr>
<td>1.8</td>
<td>(a) Schematic showing the morphological differences between normal and glaucomatous optic nerve heads, including the excavated shape of the optic nerve head, thinning of the lamina cribrosa and its more posterior insertion into the sclera and larger cup-to-disc ratio. Adapted from Quigley’s review article with permission from the Massachusetts Medical Society. (b) Schematic showing the influence of the scleral mechanical properties on the deformation mode of the lamina cribrosa. Reproduced from Sigal et al. with permission from Elsevier.</td>
</tr>
<tr>
<td>1.9</td>
<td>Schematic of a myopic eye showing the increased axial length and resulting focus of light rays from distant objects in front of the retina. Adapted from: <a href="https://nei.nih.gov/photo/refractive-errors">https://nei.nih.gov/photo/refractive-errors</a> (Courtesy: National Eye Institute, National Institutes of Health (NEI/NIH)).</td>
</tr>
<tr>
<td>1.10</td>
<td>(a) Average pressure versus strain curve, in the meridional direction, for the posterior sclera of normal, undamaged glaucoma and damaged glaucoma human eyes tested under inflation. Reproduced from Coudrillier et al. with permission from ARVO. (b) Percent extension versus time curve for the posterior sclera of myopic, contralateral control and normal tree shrew eyes tested under uniaxial tension. Reproduced from Phillips et al. with permission from ARVO.</td>
</tr>
<tr>
<td>2.1</td>
<td>Transilluminated latex membrane showing variations in light intensity.</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

2.2 (a) Top view and (b) side view of the inflation experimental setup, showing the latex membrane, inflation chamber, pressure transducer, stereo-vision system (blue) and mono-vision system (red). (c) Top view and (d) side view of the inflated latex membrane. The blue axes define the 4 quadrants used to analyze the 3D-DIC data and the red axis defines the direction of the profile-edge imaged by the mono-vision system. 42

2.3 3D-DIC (a) horizontal displacement, (b) vertical displacement and (c) meridional strain averaged over each quadrant of the membrane and over the entire membrane, at each ξ position. 49

2.4 Probability distributions for the static noise for 2D-DIC and 3D-DIC (a) horizontal displacement, (b) vertical displacement and (c) meridional strain, using a 0.0005 bin size. 51

2.5 (a) Horizontal displacement and (b) vertical displacement for the 2D meridians and the 3D meridians from quadrants 1 and 3. (c) Absolute difference between the 3D-DIC and 2D-DIC displacement components, averaged over the 2 meridians or 60 meridians considered, at each ξ position. 53

2.6 (a) Meridional strain for the 2D meridians and the 3D meridians from quadrants 1 and 3. (b) Probability distributions for the 2D-DIC and 3D-DIC meridional strain, calculated using a 0.0020 strain bin size. 54

2.7 Maps of absolute circumferential (a) and meridional (b) strains, averaged over a 3 mm wide annulus, for a sphere subjected to a 195 ± 13.4 µm uniform inflation. SD = standard deviation. 56

3.1 (a) Scleral shell mounted on an acrylic holder. (b) 3D-DIC image of a speckled scleral shell. Side (c) and top (d) views of the mechanical testing setup. 72

3.2 Pressure-controlled inflation protocol for the posterior scleral specimens. 75

3.3 Schematic of the posterior sclera mounted on a holder showing the ONH and the locations of the 8 mm punches for hydration measurement, 3x4 mm rectangles for s-GAG quantification, thickness measurements and strain averaging regions. 76

3.4 Schematic of the mechanical outcomes extracted from the load-unload stress-strain and ramp-hold stress-time curves. 81

3.5 Images of GAG staining for 3 specimens, each belonging to a different experimental group, and 2 different MgCl2 concentrations. Scale bars = 100 µm. Unpaired Student’s t-tests were used for statistical analysis. 84

3.6 Average stress-strain (a) and strain-time (b) behaviors for the control, buffer-treated and enzyme-treated groups, for the IN quadrant, in the circumferential direction. The curves for the buffer-treated group represent the average responses of the specimens from protocol 1. 92
LIST OF FIGURES

3.7 Schematic of the spatial variations for all mechanical outcomes, in the circumferential and meridional directions, assessed using linear mixed models. For the mechanical outcomes and directions having a significant interaction between group and quadrant, these variations were presented for each group. Each segment linking 2 quadrants indicates a significant difference between them. The plus and minus signs indicate the quadrants with the highest and lowest parameter value, respectively.

3.8 Model of the effect of (a) buffer-treatment from a control state and (b) enzyme-treatment from a buffer-treated state, on the scleral hydration and thickness. The collagen fibrils are represented in grey, the s-GAGs in blue and the water molecules in red and white.

3.9 Model of the arrangement of the GAG-associated water molecules (a) during fibril uncrimping at low pressures and (b) during fibril stretching at high pressures, in buffer-treated sclera. (c) Model of the increased initial fibril crimp after enzyme-treatment from a buffer-treated state. (d) Model of the displacement of the free water molecules during fibril stretching in enzyme-treated sclera. The collagen fibrils are represented in grey, the s-GAGs in blue and the water molecules in red and white.

4.1 (a) Side view and (b) top view of the experimental setup showing the inflation chamber, pressure transducer, MTS, hydration chamber, as well as the imaging and lighting systems.

4.2 Schematic of the human posterior sclera protruding from the holder showing the ONH as well as the locations of the samples used for the s-GAG quantification and hydration, the locations of the thickness measurements, and the regions where the strains were averaged. The letter B indicates the samples incubated in buffer alone, while the letter E indicates samples treated with the enzyme. For the s-GAG quantification and hydration measurement, samples from the same eye were subjected to each condition, while for the mechanical testing the specimens were subsequently treated with the buffer alone and then with the enzyme solution.
4.3 (a) Image of a left human scleral shell mounted on a holder and speckled for 3D-DIC tracking, showing the eye anatomical quadrants and directions. (b) Schematic showing how the local basis was defined for each Delaunay triangle. For each triangle, the vector $v_{MN}$ was defined from the centroid of the triangle M to the center of the ONH base N. This vector was then projected onto the plane of the triangle and normalized to find the meridional direction $e_\phi$. The circumferential direction $e_\theta$ was defined as the cross product $e_\phi \times e_n$, where $e_n$ is the outward normal unit vector to the plane of the triangle. $(n_{AB}^{sph}, n_{BC}^{sph}, n_{CA}^{sph})$ represent the unit orientation vectors for the sides of the triangle in the local spherical coordinate system of the triangle.

4.4 Stress-strain curves in the circumferential direction for all human specimens, averaged over the 4 anatomical quadrants, after buffer-treatment and after enzyme-treatment. The outliers were locally treated with the Matlab function smooth.

4.5 Stress-strain curves in the meridional direction for all human specimens, averaged over the 4 anatomical quadrants, after buffer-treatment and after enzyme-treatment. The outliers were locally treated with the Matlab function smooth.

4.6 Model of the effect of enzyme-treatment on the scleral hydration, thickness and collagen fibril structure in the posterior human sclera. The collagen fibrils are represented in grey, the s-GAGs in blue, the collagen non-enzymatic crosslinks in black and the water molecules in red and white.

5.1 (a) Top view and (b) side view of a representative mesh.

5.2 Boundary conditions on the cross-section of a representative mesh.

5.3 Comparison of the stress-stretch curves for the analytical model and FEM for a cube under equibiaxial stretch and various degree of anisotropy of the collagen fibers.

5.4 Plot showing the percent difference between the average $L_2$-norm of the displacements on the top surface nodes of various meshes and that of a reference mesh, as a function of the smallest element volume. The element density of the reference mesh corresponded to the 3D-DIC resolution in the meridional direction and 4 times the 3D-DIC resolution in the circumferential direction.

5.5 Difference in the top surface displacements with and without a +30% change in each of the model parameter, calculated at each node and averaged over the top surface for (a) the control group and (b) the enzyme-treated group.
## LIST OF FIGURES

| A.1 | Two-dimensional and three-dimensional views of the scleral holder designs (a) 2a and (b) 2b with dimensions. | 176 |
| A.2 | Schematic of the loading regime used to equilibrate the porcine specimens and measure their thickness at baseline pressure. | 177 |
| A.3 | Schematic of the loading regime for a load-unload cycle followed by a recovery period for the porcine specimens. | 178 |
| A.4 | Schematic of the loading regime for a creep test followed by a recovery period for the porcine specimens. | 178 |
| A.5 | (a) Image and (b) schematic of the ultrasound apparatus for the scleral thickness measurement, showing the ultrasound transducer, pulser-receiver, oscilloscope and computer, as well as their connections. The T/R connector is on the front panel of the pulser-receiver, while the SYNC OUT and RF OUT connectors are on the pulser-receiver back panel. (c) Electrical trace from the ultrasound transducer showing the peak-to-peak time difference between the echo signals from the outer and inner scleral surfaces. | 182 |
| B.1 | Maps comparing the experimental and FEM displacement components [mm] at maximum pressure for a representative control porcine specimen from protocol 1. | 195 |
| B.2 | Maps of the experimental and FEM displacement components [mm] at maximum pressure for a representative buffer-treated porcine specimen from protocol 1. | 196 |
| B.3 | Maps of the experimental and FEM displacement components [mm] at maximum pressure for a representative enzyme-treated porcine specimen from protocol 2. | 197 |
Chapter 1

Introduction

The introduction describes the role of the eye in the visual system, the anatomy and function of the sclera, as well as its microstructure and mechanical behavior. This chapter summarizes the various mechanical tests and mathematical models commonly used to evaluate the scleral mechanical behavior and properties. Finally, it highlights the importance of the sclera in ocular pathologies such as glaucoma and myopia, and concludes with the objectives of this thesis.

1.1 The eye as part of the visual system

The eye is the organ of sight. It transforms the light signals from an object into electrical signals. These signals are then transmitted to the brain, where they are interpreted to form an image. For an object to be clearly seen, the light must travel
CHAPTER 1. INTRODUCTION

Figure 1.1: Schematic of the human eye and path of the light rays. Adapted from: https://nei.nih.gov/photo/anatomy-of-eye (Courtesy: National Eye Institute, National Institutes of Health (NEI/NIH)).

through the eye and focus onto the retina. Light is focused on the retinal surface by the cornea and the lens (Fig. 1.1). To accommodate for the object distance, the ocular lens adjusts its curvature, bending the light rays to a precise length. Failure to focus light waves onto the retinal surface results in blurred vision. The light information is first encoded into electrical signals by specialized photoreceptor cells in the retina: cells sensitive to light intensity (the rods) and cells sensitive to colors (the cones). The signal is then transmitted to the retinal ganglion cells (RGCs), whose axons exit the eye through the optic nerve head (ONH) to transmit the electrical signal to the brain (Fig. 1.1).
1.2 The sclera

1.2.1 Anatomy and function

The average diameters of the human eye are 22–25 mm (anterior-posterior) × 24 mm (nasal-temporal) × 24 mm (superior-inferior) and the sclera, which is the white outer shell of the eye, comprises 80% of the eyewall. The sclera is connected anteriorly to the cornea at the limbus and posteriorly to the dura mater at the ONH (Fig. 1.1). The anterior sclera is located between the limbus and the equator of the eye, while the posterior sclera is located behind the equator and includes the peripapillary sclera, a 2 mm wide region immediately adjacent to the ONH (Figs. 1.2 and 1.3). At the ONH, which is on average 1.8 mm in diameter in humans, the sclera surrounds the lamina cribrosa (LC). The LC is a porous structure of interconnected collagen and elastin beams (Fig. 1.2). The collagen and elastin beam network occupies only 50% of the LC area, while the pores, ranging from 25 to 250 µm in diameter in humans, occupy the remainder area. The pores are filled with the RGC axon bundles that exit the eye to transmit the visual information to the brain. The thickness of the human sclera varies throughout the eye. It decreases from 0.6 mm at the limbus to 0.5 mm at the equator, where it is the thinnest, and increases to 1 mm near the ONH, where it is the thickest. Adjacent to the LC, the scleral thickness shows a complex variation, thinning dramatically from 0.9 mm in the peripapillary sclera to 0.4 mm in the scleral flange that connects the peripapillary sclera to the
CHAPTER 1. INTRODUCTION

Figure 1.2: Scanning electron micrograph of the cross-section of a human optic nerve head (Courtesy: Dr. Harry A. Quigley). The optic nerve head diameter is on average 1.8 mm in humans.

Moreover, the human superior and temporal quadrants are thicker than the inferior and nasal quadrants at the posterior pole differing by about 0.2 mm in the peripapillary sclera.

As the main load-bearing structure of the eye, the sclera resists the intraocular pressure (IOP) and external forces. It maintains the shape of the eye and protects the more delicate intraocular structures, such as the retina and choroid (Fig. 1.1). The sclera also anchors the muscles responsible for eye movements. The IOP is determined by the production of aqueous humor by the ciliary body and the outflow capacity of the trabecular meshwork as well as the release of water from the endothelial layer of the cornea (Fig. 1.1). The IOP is typically around 10-21 mmHg in humans and shows diurnal variations.
1.2.2 Microstructure of the scleral connective tissue

The extra-cellular matrix (ECM) of the sclera is composed of 3 main structural components: collagen fibrils, elastin fibers, proteoglycans (PGs) and their associated glycosaminoglycans (GAGs) (Fig. 1.4(a) and (b)). The ECM components are produced by the scleral fibroblasts.[18]

1.2.2.1 Collagen

All collagen types are formed of collagen molecules, each one made of a triple helix of polypeptide chains. Collagen represents 80% of the dry weight of the sclera.[19] Eight
different types of collagen are found in the human sclera, which are divided into 4 families based on their supramolecular arrangement: fibril-forming collagens (types I, III, V), fibril-associated collagens (type XII), network-forming collagens (types IV, VI, VIII) and cell-associated transmembrane collagens (type XIII). Type I collagen is the most abundant in the sclera. It is characterized by cross-striated fibrils with a D-band periodicity of 67 nm (Fig. 1.4(b)), that are stabilized by covalent intramolecular and intermolecular crosslinks. The collagen fibril size distribution in humans is similar in the anterior and posterior sclera, but is smaller in the peripapillary region. The fibrils of the outer human sclera (closer to the ocular cavity) are on average larger in diameter (58-160 nm) than those of the inner sclera (50-76 nm) (closer to the choroid). The fibrils are organized into lamellae, each one containing parallel fibrils aligned in one direction (Fig. 1.4(a)). The lamellae are stacked and interwoven but always lie roughly parallel to the scleral surface, with various in-plane orientations. At the limbus and in the outer peripapillary region the lamellae are circumferentially oriented, whereas they are radially arranged in the inner peripapillary region. Elsewhere, the lamellae form reticular patterns in the outer sclera and rhombic patterns in the inner sclera, and are more interwoven. The lamellae of the outer human sclera are on average thinner (0.5-2 μm) and narrower (1-5 μm) than those of the inner sclera (0.5-6 μm thin, 1-50 μm wide). The scleral fibroblasts are mostly located between the collagen lamellae.
CHAPTER 1. INTRODUCTION

![Figure 1.4:](a) Electron micrograph of the rabbit sclera showing collagen lamellae cl with different orientations, as well as fibroblasts f and elastin fibers ef. Adapted from Young et al. with permission from Company of Biologists Ltd. (b) Electron micrograph of the human sclera showing cross-striated collagen fibrils cf and GAGs in 3 different orientations, 1: radiating from the fibrils, 2: around the fibrils, 3: along the fibrils. Adapted from Young et al. with permission from Company of Biologists Ltd.

1.2.2.2 Elastin

Elastin fibers in the sclera are interwoven between collagen fibrils (Fig. 1.4(a)). They comprise 2% of the dry weight of the sclera. Elastin fibers consist of multiple elastin molecules crosslinked through covalent bonding. They are surrounded by longitudinal microfibrils, formed of glycoprotein such as fibrillin. The elastin core and microfibrils have been shown to interact directly with collagen and PGs or
CHAPTER 1. INTRODUCTION

through bridging molecules. Elastin fibers are circumferentially arranged within the 300 µm region around the ONH in humans, and show no preferred arrangement elsewhere. Distal to the peripapillary sclera, the elastin fibers are predominantly found in the inner sclera and their density decreases. The average diameter of the elastin fibers varies with age in humans, at 2-3 years old their diameter is 12 nm and it can increase to 20 nm at eighty years old.

1.2.2.3 Proteoglycans and glycosaminoglycans

PGs are composed of GAGs attached to a core protein through covalent bonding (Fig. 1.5(a)) and they form the ground substance in which the collagen fibrils and elastin fibers are embedded. GAGs are polysaccharide chains of various lengths consisting of repeating disaccharide units (Fig. 1.5(b)). PG core proteins make up 2% of the dry weight of the sclera, while GAGs represent 0.5 to 1%. The most abundant and most studied PGs in the human sclera are decorin, biglycan, aggrecan and lumican (Fig. 1.5(a)). The human sclera contains 6 main GAGs: chondroitin sulfate, dermanat sulfate, keratan sulfate, heparan sulfate, heparin and hyaluronic acid.

The most abundant PG in the human sclera is decorin, which represents 74% of the newly synthesized PGs, followed by biglycan (20%) and aggrecan (6%). Decorin (≈45 kDa core) has a single chondroitin sulfate or dermanat sulfate GAG side chain, while biglycan (≈45 kDa core) has 2 side chains of chondroitin sulfate and/or der-
CHAPTER 1. INTRODUCTION

Figure 1.5: (a) Schematic of the 4 main proteoglycans of the human sclera and their associated glycosaminoglycans. CS/DS: chondroitin sulfate/dermatan sulfate, KS: keratan sulfate, N-glycan: N-linked oligosaccharide, Cys: cystein residue, TyrSO₄: tyrosine sulfate, LRR: leucine-rich repeat. Adapted from Riley’s review article with permission from Cambridge University Press. (b) The repeating disaccharide unit of a dermatan sulfate glycosaminoglycan. COO⁻: carboxyl group, SO₃⁻: sulfate group. Reproduced from Fig. 19-36 in Alberts et al. with permission from Garland Science/Taylor & Francis Group LLC.

Aggrecan is the largest PG (≈350 kDa core) and contains up to 100 chondroitin sulfate and 30 keratan sulfate side chains. Decorin and biglycan, which contain multiple leucine-rich regions, are part of the small leucine-rich proteoglycans (SLRP) family (Fig. 1.5(a)). The human sclera also contains lumican (≈70 kDa core), another member of the SLRP family with short unsulfated lactosaminoglycan side chains. Other core proteins have also been identified in the human sclera such as PRELP (proline-arginine-rich end leucine-rich protein), fibromodulin, osteoglycin and chondroadherin. Among the GAGs, dermatan sulfate and hyaluronic acid...
are the most abundant in the sclera, followed by chondroitin sulfate/keratan sulfate, heparan sulfate and heparin (a highly sulfated version of heparan sulfate). Hyaluronic acid is unsulfated, not associated to a core protein and it provides binding sites for aggrecan in cartilage. However, the association between aggrecan and hyaluronic acid has not yet been demonstrated in the sclera. The PG/GAG content varies with location, the human posterior sclera being richer in aggrecan and chondroitin sulfate/dermatan sulfate side chains.

Being hydrophilic and structurally not very flexible, GAGs usually adopt a linear rather than globular conformation, filling the space between the collagen fibrils and the collagen-elastin network. As mentioned previously, PGs can bind elastin. Some PGs, such as lumican and decorin, can also bind collagen (including type I collagen) through their core protein. However, aggrecan’s core protein was observed between the collagen fibrils and the lamellae rather than in close association with the fibrils. Different PG cores have different binding sites on collagen fibrils.

Staining of the human sclera with Cuprolinic Blue showed GAGs, approximately 5 nm in diameter and 54 nm in length, in 3 different orientations: along the fibrils, around the fibrils and between adjacent fibrils. Other PGs, such as biglycan, also interact with collagen via their GAG side chains through electrostatic interactions, as shown in vitro. Through their interactions with collagen, PG core protein and side chains are believed to regulate collagen fibrillogenesis, including the fibril structure, diameter and rate of formation.
CHAPTER 1. INTRODUCTION

Figure 1.6: Schematic of the interactions between proteoglycans and collagen fibrils based on electron tomography results of the bovine cornea. The proteoglycan core proteins (red and yellow ovals) are attached to the collagen fibrils (blue). The glycosaminoglycan chains (orange and brown lines) are represented in 3 different orientations: running along the fibrils, extending from or encircling the fibrils to form anti-parallel interactions with each other. Black arrowheads point to anti-parallel interactions between keratan sulfates (orange lines), while black arrows indicate anti-parallel interactions between chondroitin sulfates or dermatan sulfates (brown lines), and white arrowheads indicate mixed anti-parallel interactions between chondroitin sulfate or dermatan sulfate with keratan sulfate. White arrows show proteoglycan filaments running along the collagen fibrils. Adapted from Lewis et al. with permission from Elsevier, based on Fig. 2 from Scott.

shown in vitro. In addition, mice deficient in decorin, fibromodulin and lumican show abnormalities in the structure and arrangement of their collagen fibrils.

GAGs are also responsible for the 70% water content of the sclera due to their negatively charged sulphated and carboxyl groups (Fig. 1.5(b)) that attract and retain water. GAGs also attract water through osmotic effects, due to the accumulation of counter-ions from the environment that balance their charges. However, the tissue swelling capability is restricted by the elasticity of the collagen network as well as by the size and architecture of the GAGs that either repulse each other or interact through anti-parallel interactions (Fig. 1.6), creating
more or less space for free water uptake. Therefore, GAGs, most of which are bound to the collagen fibrils through their core, are hypothesized to regulate the distance between the fibrils through hydration (swelling) GAG-GAG charge repulsion and GAG-GAG anti-parallel interactions. Most observations are based on studies of the cornea, which transparency depends on the regular arrangement of the collagen fibrils.

1.2.3 Mechanical Properties

The stress-strain response of the sclera is non-linear (Fig. 1.7) spatially-varying and anisotropic as measured for various species. It is also viscoelastic and shows hysteresis creep stress relaxation and rate dependence. The sclera is nearly incompressible and its mechanical properties are temperature and hydration dependent. The posterior sclera is more compliant than the anterior sclera, despite being thicker and having a higher collagen content. In addition, the peripapillary sclera deforms more than the rest of the posterior sclera.

The mechanical properties of the sclera are governed by the composition, dimensions and arrangement of its structural components. The collagen fibrils are mainly responsible for scleral stiffness and strength. They bear the load at moderate to elevated strain levels (linear region of the stress-strain curve) (Fig. 1.7(b)), as shown in various tissues using the targeted enzymatic degradation of structural com-
Initially crimped (Fig. 1.7(a)), the fibrils uncrimp and then stretch under mechanical loading, giving the tissue its characteristic non-linear “J-shaped” strain-stiffening stress response (Fig. 1.7(b)). The strain-stiffening behavior protects the tissues from being stretched beyond physiological levels. The presence of thicker fibrils in the outer sclera close to the eye cavity reinforces the tissue where the stress is higher. Similarly, the circumferential arrangement of fibrils around the limbus and ONH prevents the enlargement of the ONH and maintains the corneal curvature. The anisotropy of the scleral mechanical properties is dictated by the anisotropic organization of the fibrils. The tissue viscoelasticity can be attributed to fluid movements within the tissue, the reorientation of the collagen fibrils and the flow-independent viscoelasticity of the PGs. The sclera stiffens with age despite a decreased collagen content, due to an accumulation of collagen non-enzymatic crosslinks.

Elastin fibers are mainly responsible for the scleral elasticity and ability to resist deformation at low to normal levels of strain (toe region of the stress-strain curve) as shown in various tissues. They help the tissue restore its original length after mechanical unloading, provided there is enough time to recover and the microstructure of the collagen fibrils is not permanently altered. Elastin’s interaction with collagen also affects the stress-strain behavior in the toe region.

The mechanical role of GAGs varies among tissues and has never been studied in the sclera. GAGs digestion induced an increased creep rate in cartilage, a faster
relaxation in the aorta\textsuperscript{109} and a decrease in hysteresis in the aortic heart valve leaflet at low levels of stress\textsuperscript{113}. This suggests that GAGs might have a damping function in those tissues, by providing a viscous or frictional resistance to the sliding of the collagen fibrils. However, GAGs degradation did not alter the hysteresis\textsuperscript{113} and stress relaxation\textsuperscript{115} in ligaments. Other studies showed that GAG digestion increased the stiffness of mesenteric arteries\textsuperscript{116}, lungs\textsuperscript{117} as well as that of the aorta\textsuperscript{109} and ligaments\textsuperscript{108} at small levels of strain. However, the stiffness of the aorta was decreased at high levels of strain following GAG degradation\textsuperscript{119}. These findings indicate that GAGs might act as mechanical crosslinks or a lubricant between the collagen fibrils, reducing the friction between them and promoting fibril sliding rather than stretching to protect them from tearing\textsuperscript{118}. However, GAG degradation did not change the stiffness of cartilage\textsuperscript{112}, tendons\textsuperscript{119} and ligaments in a different study\textsuperscript{114}.

\subsection*{1.2.4 Mechanical testing}

The mechanical behavior of the sclera has been investigated using different experimental techniques, such as uniaxial\textsuperscript{80,82,81,86,87,89,90,93,94} and biaxial\textsuperscript{91,95} testing, inflation of whole globes\textsuperscript{93,97} and scleral shells\textsuperscript{81,85,89,92,96}, dynamic mechanical testing (DMA)\textsuperscript{122} and indentation with an air piston\textsuperscript{110} or atomic force microscopy\textsuperscript{123}. The testing method affected the results by several orders of magnitude\textsuperscript{124}. Uniaxial and biaxial testing are straightforward methods but they have important limitations, such as stress concentration and preconditioning effects. Preconditioning is charac-
CHAPTER 1. INTRODUCTION

Figure 1.7: (a) Second harmonic generation image of crimped collagen fibrils from a porcine scleral sample. Scale bar = 10 µm. Reproduced from Han et al. with permission from OSA Publishing. (b) Strain-stress curve for a typical collagenous tissue showing the collagen fibril recruitment as a function of the deformation. Reproduced from Freed and Doehring with permission from ASME.

This phenomenon is partly due to the rearrangement of the collagen fibrils in the direction of stress and suggests that the tissue is not tested under physiological conditions. Preconditioning loading cycles are designed to obtain a unique and recoverable reference state of the tissue and load-unload curve. Moreover, the severed collagen fibrils at the tissue boundaries and the act of flattening the sclera when testing can introduce significant errors in the measured mechanical properties. While loads are typically measured using force transducers, the deformations are evaluated from the clamp extension, extensometer measurement or two-dimensional (2D) tracking of artificial markers. In
contrast, inflation testing mimics the *in-vivo* loading conditions of the eye. It allows the measurement of the pressure-induced 3D displacements of the scleral surface using image tracking techniques such as 3D digital image correlation (DIC)\textsuperscript{4,92} electronic speckle pattern interferometry (ESPI)\textsuperscript{89,96} or optical flow tracking of surface particles.\textsuperscript{85} Three-dimensional displacements within the scleral volume can also be measured using ultrasound speckle tracking.\textsuperscript{95} For the inflation of scleral shells, the boundary effects such as stress concentration are assumed to be negligible away from the clamp. In addition, tissue preconditioning was shown to be unnecessary due to the fully fixed boundary condition, providing the tissue was allowed time to recover and did not incur irreversible damage.\textsuperscript{125} For inflation tests, it is common to use Laplace’s approximation for thin-walled vessels to calculate the hoop stresses. For a thin-walled spherical eye or scleral shell, the hoop stresses in the circumferential ($\sigma_{\theta\theta}$) and meridional directions ($\sigma_{\phi\phi}$) can be derived directly from equilibrium as:

$$\sigma_{\theta\theta} = \sigma_{\phi\phi} = \frac{Pr}{2T}$$

with $P$ the pressure applied to the eye, $T$ its thickness and $r$ the radius of the sphere. For an ellipsoidal thin-walled eye or shell, the stresses are defined as:

$$\sigma_{\theta\theta} = \frac{Pr_2(2r_1 - r_2)}{2r_1T}, \sigma_{\phi\phi} = \frac{Pr_2}{2T}$$

where $r_1$ and $r_2$ are the radii of curvature of the ellipsoid in the meridional and
CHAPTER 1. INTRODUCTION

circumferential directions respectively.\textsuperscript{126} This approximation is typically considered valid if the thickness is small compared to the radius of the eye, i.e. \( r/T > 10 \).

Mechanical testing is usually limited to the macroscale characterization of the scleral mechanical behavior. However, simulations based on a constitutive model can be used to investigate scleral properties that are difficult to study or measure experimentally.

1.2.5 Constitutive models

Constitutive models are commonly used to characterize the scleral mechanical behavior. The model parameters can be extracted by directly fitting the model to the experimental stress-strain curves\textsuperscript{4,86,91,94,100,101} or by implementing an inverse finite element method (FEM)\textsuperscript{30,89,127} These parameters sometime represent material properties depending on the constitutive model used.

The mechanical behavior of the sclera is typically modeled as hyperelastic. The Cauchy stress for hyperelastic models is derived from a strain energy density function \( \Psi \) and computed as:

\[
\sigma = \frac{2}{J} F \frac{\partial \Psi(C)}{\partial C} F^T
\]  

(1.3)

where \( F = \frac{\partial \varphi(X)}{\partial X} \) is the deformation gradient, which maps a vector from the undeformed configuration into the deformed configuration through the deformation \( \varphi \), \( C = F^T F \) is the right Cauchy-Green strain tensor and \( J = \det(F) \) is the Jacobian of
CHAPTER 1. INTRODUCTION

the deformation gradient.

The simpler constitutive models used for the sclera are tissue-level phenomenological models, such as the one use by Eilaghi et al.\cite{91} The models have no physical interpretation and usually derive from the model initially developed by Fung.\cite{128} Fung’s model became popular, as it captured both the anisotropy and non-linearity of collagenous tissues by assuming an exponential form of the strain energy density. This model evolved over the years, resulting in many different versions.\cite{128}

Lanir then proposed a model based on Fung’s strain energy density, which incorporates aspects of the microstructure of collagenous tissues.\cite{129} and was used to describe the sclera.\cite{30,130} In this approach, the sclera is modeled as a distribution of collagen fibers, which correspond to the scleral collagen lamellae, embedded in a matrix of hydrated PGs. Assuming that both the matrix and collagen fibers are subjected to the same deformation gradient, the strain energy density can be defined as:

\[
\Psi(C) = \Psi_{\text{matrix}}(C) + \Psi_{\text{fibers}}(C)
\]  

(1.4)

The \(\Psi_{\text{matrix}}\) represents the matrix contribution to the strain energy density. The matrix is assumed to represent all non-fibrillar collagen components, including elastin fibers, non-fibrillar collagens, GAGs and water. It is usually considered isotropic and described by a Neo-Hookean model as:
\[ \Psi_{\text{matrix}}(C) = \frac{\mu}{2} (\bar{I}_1(C) - 3) + \Psi_{\text{vol}}(I_3(C)) \] (1.5)

where the first and second terms represent the deviatoric and volumetric part of the strain energy density of the matrix, respectively. \( \mu \) is the matrix shear modulus, 
\( \bar{I}_1 = I_3^{-\frac{1}{3}}I_1 \) is the deviatoric part of the first invariant \( I_1(C) = tr(C) \), and \( I_3(C) = \det(C) \) is the third invariant. \( \Psi_{\text{vol}} \) can assume different forms as shown in Doll and Schweizerhof \cite{131} and is typically used as a penalty to enforce incompressibility. \( \Psi_{\text{vol}} \) usually includes a logarithm to ensure that \( \Psi_{\text{vol}} \) goes to 0 for \( J = 1 \) (no volume change) and to \( \infty \) for \( J = 0 \) (zero volume is not acceptable). The \( \Psi_{\text{fibers}} \) represents the collagen fiber contribution to the strain energy density which is defined as:

\[ \Psi_{\text{fibers}}(C) = \int_{-\pi}^{\pi} \Psi_{\text{fiber}}(\lambda_f(\Theta)) P(\Theta) d\Theta \] (1.6)

where \( \lambda_f = \sqrt{a_0 C a_0} \) is the stretch of a collagen fiber with reference orientation \( a_0 = [\cos(\Theta), \sin(\Theta), 0] \), \( \Theta \) being the orientation of the collagen fibers in the plane of the tissue, \( P(\Theta) \) is the probability density distribution for the collagen fiber, and \( \Psi_{\text{fiber}} \) is the strain energy density for a single collagen fiber, which can be expressed as an exponential function. Coudrillier et al.\cite{30} used \( \Psi_{\text{fiber}}(\lambda_f) = \frac{a}{2} [\exp(\beta(\lambda_f^2 - 1)) - \beta\lambda_f^2] \) based on the work from Pinsky et al.\cite{132} for the cornea and sclera, while Girard et al.\cite{130} used \( \frac{\partial \Psi_{\text{fiber}}}{\partial \lambda_f} = \frac{C_4}{\lambda_f} [\exp(C_4(\lambda_f - 1)) - 1] \) based on the expression initially developed for ligaments by Weiss et al.\cite{133} In those studies,\cite{30,130,134} the probability density
distribution for the fibers was represented by a semi-circular von Mises distribution defined as follows:

\[
P(\Theta) = \frac{\exp(k\cos(2(\Theta - \Theta_p)))}{2\pi I_0(k)}
\]

(1.7)

where \(\Theta_p\) is the preferred collagen fiber orientation, \(k\) is the collagen fiber dispersion around \(\Theta_p\) and \(I_0(k) = \frac{1}{\pi} \int_0^\pi \exp(k\cos(\Theta))d\Theta\) is the modified Bessel function of the first kind of order zero. Coudrillier et al.\(^{30}\) also improved their model by including the collagen fiber orientation and dispersion determined from wide-angle X-ray scattering measurements.

Micro-mechanical models, which incorporate the collagen fiber crimp, have also been used to model the sclera.\(^{127}\) In Grytz et al.\(^{127}\) the sclera is modeled as a distribution of crimped collagen fibrils embedded in a ground matrix. Similar to other models, the isotropic matrix is described by a Neo-Hookean model, while the anisotropic fibrils are distributed using a von Mises probability distribution that has been integrated to give a generalized structure tensor. However, the model assumes a helical crimp conformation of the fibrils when unloaded and takes into account their unwinding depending on their orientation and stretch. Viscoelastic models have also been used to describe the stress relaxation behavior of the sclera.\(^{100,101}\)
CHAPTER 1. INTRODUCTION

1.3 The sclera in glaucoma and myopia

1.3.1 Glaucoma

Glaucoma is the second leading cause of blindness worldwide[^135] and is expected to affect 80 million people by 2020[^136]. It is characterized by a progressive and irreversible vision field loss, due to the malfunction[^137][^138] and eventual death[^139][^140] of the RGC axons (Figs. 1.1 and 1.8(a)). Insult to the RGC axons occur in the LC at the ONH[^141] (Figs. 1.2 and 1.8(a)). In early glaucoma, the LC and peripapillary sclera move posteriorly as the opening of posterior scleral canal increases. The LC thickens but there is no evidence of failure of the laminar beams yet, as summarized by Downs et al.[^142] As the disease progresses, the anterior laminar beams fail and the LC compresses. The LC insertion into the sclera moves posteriorly, leading to the characteristic “excavated” shape of the optic disk and enlargement of the cup-to-disc ratio[^143] (Fig. 1.8(a)).

An elevated IOP was initially believed to damage the RGC axons[^144] (the mechanical theory). In angle-closure glaucoma, the narrow angle between the cornea and iris physically blocks the trabecular meshwork, which is responsible for draining the aqueous humor outside the eye. In open-angle glaucoma, the aqueous humor is not evacuated fast enough by the trabecular meshwork. However, glaucoma is also observed in patients with normal levels of IOP[^145][^146]. Therefore, others suggested that an impaired blood supply at the ONH is responsible for the axonal damage[^144] (the
CHAPTER 1. INTRODUCTION

vascular theory). In 2005, Burgoyne et al.\textsuperscript{147} proposed a unifying theory that considered the ONH as a “biomechanical structure”, in which pathological levels of stress and strain could develop in the LC at any level of IOP. This mechanical insult could simultaneously damage the RGC axons, and lead to the activation of astrocytes and to a poor vascular nutrition. Numerical studies showed that the mechanical properties of the posterior sclera greatly influence the deformation of the more compliant LC\textsuperscript{90} for a given stress and might highly contribute to the development of glaucoma.\textsuperscript{148–150} A more compliant sclera would stretch the LC in response to an IOP elevation, while a stiffer sclera would make the LC bend backward\textsuperscript{151} (Fig. 1.8(b)). In patients with high-pressure glaucoma, medications such as eye drops can be effective in reducing the amount of aqueous humor produced in the eye, and laser therapy or surgery can prevent the aqueous humor built-up inside the anterior chamber.\textsuperscript{152} However, those treatments have drawbacks and they are not very efficient in patients with normal-pressure glaucoma.

1.3.2 Myopia

Myopia is a common refractive error anticipated to affect 35 million people by 2020 in the USA.\textsuperscript{153} It is often characterized by the abnormal axial elongation of the globe, which prevents the focus of images from distant objects on the retina and leads to nearsightness (Fig. 1.9). Among people affected by myopia, 12-15% suffer from high-myopia,\textsuperscript{154} which can eventually lead to blindness due to the increased risk for
retinal tissue degeneration\textsuperscript{155} and glaucoma\textsuperscript{156}. Such an axial elongation of the eye, especially pronounced at the posterior pole\textsuperscript{201}, could not occur without a change in the mechanical properties of the posterior sclera. Myopia can be corrected with corrective lenses or refractive surgery. However, neither option slows the eye elongation and its associated physiological changes.\textsuperscript{157}
CHAPTER 1. INTRODUCTION

Figure 1.9: Schematic of a myopic eye showing the increased axial length and resulting focus of light rays from distant objects in front of the retina. Adapted from: https://nei.nih.gov/photo/refractive-errors (Courtesy: National Eye Institute, National Institutes of Health (NEI/NIH)).

1.3.3 Alterations to the scleral structure and microstructure

Biomechanical studies of glaucoma have focused on microstructural changes in the peripapillary region. Scleral thinning occurs in the peripapillary sclera of experimental glaucoma monkey eyes\cite{158,159} but it was not observed in glaucomatous human eyes\cite{160} compared to controls. In another study, the change in thickness in the peripapillary region following fixation was higher in normal scleras than in glaucomatous scleras (mouse), suggesting a loss of non-fibrillar components in glaucoma\cite{161}. The collagen density is lower, but there is no significant change in the collagen fibril diameter or elastin density in the peripapillary sclera of human or experimental glaucoma monkey eyes\cite{24} However, in the peripapillary sclera of experimental glaucoma mouse
CHAPTER 1. INTRODUCTION

eyes, the collagen fibrils are thinner, the lamellae are thicker and increasingly oriented antero-posteriorly, but there is no change in the collagen density. In addition, the collagen fiber anisotropy is lower in the peripapillary sclera (human). The elastin fibers become curled in the LC (human) but there is so far no evidence of such a change in the posterior sclera. The content of some GAGs is increased in the iris (human) and LC (monkey, rat, human), but there is currently no data for the posterior sclera.

In myopia, most structural changes occur in the posterior sclera. The thickness of the posterior sclera decreases as well as its dry tissue weight (tree shrew), suggesting a loss rather than a redistribution of tissue. In the long-term, the frequency of smaller diameter fibrils increases (human, tree shrew), the fibril size gradient across the sclera is reduced (tree shrew) and the collagen lamellae become thinner and less interwoven (human) at the posterior pole. The collagen content of the posterior sclera decreases (human, tree shrew) due to a decreased synthesis, as well as an increased degradation (tree shrew). The GAG content also decreases in the posterior sclera (human, tree shrew) and is accompanied by a decreased synthesis (tree shrew, marmoset).
1.3.4 Alterations to the scleral mechanical properties

The peripapillary sclera of human glaucomatous eyes exhibits a lower creep rate and higher stiffness (Fig. 1.10(a)) than that of normal eyes in inflation tests. In experimental glaucomatous monkey eyes, the posterior sclera also shows a higher stiffness under inflation, and the peripapillary sclera exhibits a higher equilibrium modulus after stress relaxation, but no change in the time-dependent viscoelastic parameters compared to that of normal eyes in uniaxial strip tests. In comparison, the posterior sclera of human myopic eyes under uniaxial testing reaches a higher strain at maximum pressure than that of normal eyes. In experimental myopic tree shrew eyes, the posterior sclera also shows a greater strain at the peak pressure, as well as a higher creep rate (Fig. 1.10(b)) but a similar stiffness than those of normal eyes, in uniaxial strip tests.

1.4 Objectives and significance of this thesis

The mechanical properties of the posterior sclera have been shown to play an important role in the initiation and development of glaucoma and myopia. Alterations to the scleral mechanical properties could significantly influence one’s susceptibility
CHAPTER 1. INTRODUCTION

Figure 1.10: (a) Average pressure versus strain curve, in the meridional direction, for the posterior sclera of normal, undamaged glaucoma and damaged glaucoma human eyes tested under inflation. Reproduced from Coudrillier et al. with permission from ARVO\textsuperscript{[4]} (b) Percent extension versus time curve for the posterior sclera of myopic, contralateral control and normal tree shrew eyes tested under uniaxial tension. Reproduced from Phillips et al. with permission from ARVO\textsuperscript{[99]}

to the development of those diseases even at normal levels of IOP. Understanding how the structural components of the sclera contribute to its mechanical properties is crucial for understanding their role in the pathophysiology of glaucoma and myopia, and could lead to the development of new treatments in the long-term. Although the main role of collagen and elastin in the biomechanics of biological tissues is known, the role of GAGs varies greatly among tissues and has never been studied in the sclera. In addition, pathological changes in the scleral GAG content and mechanical properties have been observed independently in eyes with glaucoma and myopia, but the impact of GAG content variations in the mechanics of those diseases has not been determined.
CHAPTER 1. INTRODUCTION

Triggering specific microstructural changes that are beneficial against glaucoma or myopia could represent the next generation of treatments, thus avoiding the need for surgical intervention. Currently, most studies focus on collagen crosslinking, which was proven beneficial when applied to the cornea of patients with keratoconus, a disease where the cornea thins and elongates. Wang and Corpuz showed that in-vivo scleral collagen crosslinking in a guinea pig model of myopia using genipin stopped the eye elongation, although the long-term effects were not studied. However, Kimball et al. showed that in-vivo scleral collagen crosslinking in a mouse model of glaucoma using glyceraldehyde increased the damage of the RGC axons. Therefore, other microstructural changes, such as a change in the GAG content, might be of interest for the management of glaucoma or myopia.

The main goal of this thesis is to study the contribution of sulfated GAGs (s-GAGs) to the structure and mechanical properties of the posterior sclera using experimental and numerical approaches. Additionally, this work aims to investigate whether s-GAGs could be mechanically involved in glaucoma and myopia. This thesis is composed of 5 chapters beside the introduction, each containing the relevant literature review and discussion. Their content is summarized below.

One of the main experimental techniques used in this work beside s-GAG degradation is digital image correlation (DIC). DIC is commonly used in experimental mechanics to obtain the surface deformation of a specimen, and both its 2D and 3D versions present advantages and disadvantages under certain experimental conditions.
Chapter 1. INTRODUCTION

Chapter 2 directly compares 2D-DIC and 3D-DIC, experimentally, for a membrane subjected to inflation, as a model for the posterior scleral shells used in this work. An experimental setup was designed to simultaneously image a latex membrane from the top, using a stereovision imaging system, and in profile, using a single camera imaging system, during inflation. The horizontal (in the membrane plane) and vertical (out of the membrane plane) displacements, as well as meridional strain, from 2D-DIC and 3D-DIC were calculated for comparison. The results explored the differences, as well as advantages and disadvantages of using 2D-DIC versus 3D-DIC to characterize the mechanical response of materials under inflation. Since 3D-DIC is needed to analyze the three-dimensional anisotropic mechanical behavior of heterogeneous tissues such as the sclera, and was therefore extensively used in this work, chapter 2 also presents a methodology to evaluate the error and uncertainty in strains associated with the 3D-DIC displacement error and uncertainty for a specimen under inflation. This methodology combined the controlled translation of a spherical object to obtain the 3D-DIC displacement error and uncertainty, which were then applied to the numerical inflation of a synthetic sphere to calculate the corresponding error and uncertainty in strains.

The other main experimental method used in this work is s-GAG digestion. A protocol was first developed to experimentally digest s-GAGs in the posterior sclera of enucleated porcine eyes using chondroitinase ABC (ChABC), an enzyme that specifically degrades chondroitin and dermatan sulfates. Chapter 3 describes how s-GAG
CHAPTER 1. INTRODUCTION

digestion affects the structure and mechanical behavior of the posterior porcine sclera. The s-GAG content and hydration were measured in the 4 anatomical quadrants of the eyes divided into 3 experimental groups: no treatment (control), buffer-treated and enzyme-treated. Eyes from the control group were native controls, whereas eyes from the buffer-treated and enzyme-treated groups were incubated in buffer alone and buffer with ChABC, respectively, prior to the measurements. The inflation response and scleral thickness were also assessed in each anatomical quadrant of other eyes divided into 2 experimental groups: inflated as controls and re-inflated after buffer-treatment, or inflated after buffer-treatment and re-inflated after enzyme-treatment. The difference in the mechanical changes between the 2 groups was used to distinguish the effects of an increase in hydration from those of s-GAG degradation. The mechanical behavior was evaluated along the meridional and circumferential directions of the eyes, using pressure-controlled inflations and 3D-DIC to obtain the full-field surface displacements. The findings were interpreted for the contribution of s-GAGs to the structure and mechanical properties of the posterior porcine sclera and their role in the altered scleral mechanical behavior measured in glaucoma and myopia.

The experimental s-GAG degradation protocol of chapter 3 was then applied to human donor eyes. Chapter 4 evaluates the contribution of s-GAGs to the structure and mechanical behavior of the posterior human sclera, which is more relevant to the understanding of the mechanics of glaucoma and myopia. The thickness and inflation response were measured subsequently on the same eyes after buffer-treatment and af-
CHAPTER 1. INTRODUCTION

...ter enzyme-treatment. The experimental design described in chapter 3, including the equipment setup and data analysis, had to be improved to accurately measure smaller displacements because the posterior sclera is significantly stiffer in older humans than young pigs. The findings were analyzed for the role of s-GAGs in the structure and mechanical properties of the posterior sclera in humans and in the pathological scleral mechanical behavior measured in glaucoma and myopia.

In chapter 5 an inverse finite element method (FEM) was used to probe the effects of s-GAG digestion on the mechanical properties of the matrix and collagen components of the posterior porcine sclera. Three-dimensional (3D) specimen-specific FEM meshes for the scleral shells of chapter 3 were created and subjected to Cauchy tractions corresponding to the experimentally recorded inflation pressures. Parameters of an anisotropic distributed fiber constitutive model were optimized by minimizing the difference between the numerical displacements on the mesh top surface and the experimental surface displacements obtained from 3D-DIC in chapter 3. A surrogate-model accelerated random search algorithm was used to solve the inverse material characterization problem. The algorithm consisted of a genetic algorithm performed on a neural-network surrogate model obtained from the finite element model. The results were analyzed for differences in the matrix shear modulus, as well as the recruitment, stiffness and degree of anisotropy of the collagen fibers, which correspond to the scleral collagen lamellae, between the control, buffer-treated and enzyme-treated groups.
CHAPTER 1. INTRODUCTION

Finally, chapter 6 summarizes the key results, implications and main limitations of this work, and closes with future research perspectives.

The appendix contains further details about the experimental procedures and designs, as well as numerical results, relevant to this work.
Chapter 2

Investigating the accuracy and uncertainty of digital image correlation

This chapter compares the 2D and 3D versions of DIC for the same inflation experiment. A latex membrane was inflated vertically to 5.41 kPa (reference pressure), then to 7.87 kPa (deformed pressure). A two-camera stereo-vision system acquired top-down images of the membrane, while a single camera system simultaneously recorded images of the membrane in profile. 2D-DIC and 3D-DIC were used to calculate horizontal (in the membrane plane) and vertical (out of the membrane plane) displacements, and meridional strain, for comparison. This work is reprinted from *Optics and Lasers in Engineering*, Barbara J. Murienne and Thao D. Nguyen,
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

“A comparison of 2D and 3D digital image correlation for a membrane under inflation”, Vol. 77, Pages 92-99, 2016, with permission from Elsevier. This chapter also presents a methodology to evaluate how the error and uncertainty in the 3D-DIC displacements translate into a strain error and uncertainty for a specimen under inflation. The 3D-DIC displacement error and uncertainty were obtained from the rigid-body translation of a spherical object. They were then applied to the inflation of a numerical sphere to calculate the corresponding error and uncertainty in strains. This methodology was first described in The Journal of Biomechanical Engineering, Theresa K. Tonge, Barbara J. Murienne, Baptiste Coudrillier, Stephen Alexander, William Rothkopf and Thao D. Nguyen, “Minimal Preconditioning Effects Observed for Inflation Tests of Planar Tissues”, Vol. 135, Pages 114502-1 - 114502-14, 2013. However, the data presented here are reproduced from Acta Biomaterialia, Barbara J. Murienne, Joan L. Jefferys, Harry A. Quigley and Thao D. Nguyen, “The effects of glycosaminoglycan degradation on the mechanical behavior of the posterior porcine sclera”, Vol. 12, Pages 195-206, 2015, with permission from Elsevier.

2.1 Introduction

DIC is a non-contact method used to calculate the 2D or 3D full-field surface displacement response of structures to mechanical loading. From the displacement field, the surface strain field can be calculated to characterize the local mechanical
behavior of the specimens. DIC has been utilized to characterize the mechanical properties of a wide range of materials, including biological materials.\textsuperscript{189} Zero-order or first-order shape functions are commonly used to describe the reference subset displacement/deformation in the deformed image during the matching process.\textsuperscript{189} Since first-order shape functions are more accurate but also more computationally expensive, most commercially available software use zero-order shape functions. Since there is no one-to-one pixel correspondence between the reference and deformed subsets, sub-pixel intensities in the deformed image are obtained using common interpolation schemes, such as bicubic interpolation, prior to matching.\textsuperscript{189} Alternatively, sub-pixel interpolation of the correlation coefficient can be performed.\textsuperscript{190,191} This process returns the new 2D positions of points on the specimen surface, from which 2D displacement vectors are calculated. This process can be repeated for all deformed images to obtain the 2D displacement of the specimen surface throughout the deformation. The main source of error in 2D-DIC is the
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

Image correlation error. This error is associated with the pattern matching process between different image frames and can be divided into 2 categories: a bias error and a random error. The bias error is responsible for the 2D-DIC error (i.e. accuracy), while the random error represents the 2D-DIC uncertainty (i.e. precision). The bias error is a function of the camera noise, image distortion, illumination conditions and noise, speckle pattern, how parallel the camera sensor and specimen are, matching process (subset size, shape function, correlation criterion) and interpolation method for sub-pixel resolution. The random error is mainly a function of the random noise from the camera and illumination conditions. It should be noted that the noise contributes to both the bias and random error. The use of 2D-DIC is often limited by the 3D nature of the material structure and deformation, and by its high sensitivity to out-of-plane motions, which generate significant errors in in-plane displacement and strain measurements. Therefore, 2D-DIC is optimal for deformation tracking of planar specimens showing a small out-of-plane contraction under planar deformation.

The 2D-DIC static error and uncertainty in displacements due to static noise can be evaluated by correlating multiple images of the specimen acquired under static conditions. From those, the corresponding baseline error and uncertainty in strains can easily be calculated. The 2D-DIC error and uncertainty in displacements and strains for a specimen under deformation can be estimated from the planar translation of the specimen and the numerical deformation of images of the specimen respectively.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

In comparison, 3D-DIC operates on a series of image pairs of a deforming object acquired by a two-camera stereo-vision system. The images from the reference pair are correlated to match image subsets between cameras, and the 3D reference surface geometry is reconstructed using the camera intrinsic and extrinsic parameters, and triangulation. The intrinsic parameters describe the characteristics of each camera-lens system, such as the location of the intersection between the optical axis and the sensor plane, the skew of the sensor plane, the focal length and the distortion coefficient. The extrinsic parameters describe the relative position of the camera-lens systems, including their distance and orientation. Changes in the 3D surface geometry throughout the deformation are then obtained through the correlation of the images in each subsequent deformed pair and triangulation. To locate corresponding points on the reference and deformed surfaces of the specimen, additional correlations between the reference and deformed image pairs are performed. This process returns the new 3D positions of points on the specimen surface from which 3D displacement vectors are calculated. 3D-DIC is associated with two main sources of error: an image correlation error and a 3D reconstruction error. The reconstruction error is a bias error associated with the imaging system calibration. It is mainly a function of the camera positioning (stereo-angle) and calibration (their orientation in space with respect to each other), and the objective focal length. 3D-DIC is recommended for non-planar specimens or planar specimens undergoing significant rotations and out-of-plane displacements.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

The 3D-DIC error and uncertainty in the reconstructed 3D positions have been evaluated experimentally by comparing the true and reconstructed shape of a cylinder. The 3D-DIC uncertainty in the reconstructed 3D positions has also been calculated theoretically for planar and curved objects. The 3D-DIC static error and uncertainty in the reconstructed 3D positions due to static noise have been measured experimentally by comparing the reconstructed 3D position of the same image subsets from multiple static image pairs for a planar object. Similarly, the 3D-DIC static error and uncertainty in the 3D displacements due to static noise have been evaluated experimentally for planar and curved objects, by correlating multiple image pairs of the object acquired under static conditions. The static error and uncertainty in displacements allow the calculation of the baseline error and uncertainty in strains.

The 3D-DIC error in the in-plane displacements with deformation has been evaluated by numerically applying a planar deformation to synthetic images of a planar target. Such numerically applied planar deformation can also be used to evaluate the 3D-DIC error and uncertainty in in-plane strains. Analogous experimental studies have compared the 3D-DIC in-plane displacements and strains to a physically applied in-plane rigid body translation as well as out-of-plane translation and rotation. Siebert et al. compared the in-plane displacements between 3D-DIC and an accelerometer on a vibrating specimen, and the strains between 3D-DIC and a strain gauge on a tensile specimen. However, to fully characterize the 3D-DIC per-
formance, it is also important to evaluate the error and uncertainty in out-of-plane displacements and strains with deformation. In fact, some studies on 3D-DIC reported a higher out-of-plane uncertainty than in-plane uncertainty on positions and displacements for static objects.\cite{195,200}

Measuring the 3D-DIC error and uncertainty in the out-of-plane displacements and strains with deformation requires either applying a known 3D deformation field or comparing to another well characterized and more accurate full-field measurement method. However, it is difficult to experimentally apply a known non-uniform 3D displacement field even for a simple structure, such as a membrane under inflation, because of irregularities and variations in the boundary conditions, geometry, and material properties. In addition, applying synthetic deformations to pairs of images to mimic a known 3D deformation is not feasible. Therefore, current studies have only examined the effect of rigid-body motion rather than deformation on the 3D-DIC out-of-plane displacements of curved objects,\cite{4,186} which prevents the calculation of the error and uncertainty in strains with 3D deformation.

The goal of this study was to compare the displacements and strain obtained from 2D-DIC and 3D-DIC for the same inflation test. Inflation testing is widely used to characterize the mechanical behavior of biological materials,\cite{188,182,186,188,4} as well as non-biological tubular materials\cite{204} and thin films.\cite{202,206} An experimental setup was developed to measure the deformation of a bulged latex membrane in response to pressure increase using DIC tracking on images acquired simultaneously by a single
camera and a two-camera stereovision system. The pressurization of the initially bulged membrane imposes a uniform strain condition away from the clamps. The 3D-DIC error and uncertainty in displacements and strains for a curved specimen under static and inflation conditions were also evaluated. The static performance of 3D-DIC was calculated from multiple images of a posterior scleral shell under static conditions. The performance of 3D-DIC with inflation was evaluated using a new methodology. Rigid-body displacements of a spherical object were used to assess the 3D-DIC displacement error and uncertainty, which were then applied to the inflation of a numerical sphere to calculate the error and uncertainty in strains.

2.2 Methods

This section describes the methods used to obtain the horizontal and vertical displacement components and the meridional strain from 2D-DIC and 3D-DIC.

2.2.1 Specimen preparation

A 0.2032 ± 0.0508 mm thick latex membrane (Abrasion-resistant natural latex rubber film, 85995K13, McMaster-Carr, Princeton, NJ) was glued flat to a custom-made acrylic holder with a 20.5 mm circular opening. The specimen was transilluminated using a lamp with a diameter much larger than that of the holder to ensure a uniform illumination of the membrane. Images of the transilluminated specimen
showed variations in light intensity across the membrane, which corresponded to thickness variations and a distinct material texture (Fig. 2.1). The membrane was speckled with black India ink (Figs. 2.2(c) and (d)) using an airbrush (ECL4500 HP-CS, Iwata Medea, Portland OR) to allow for DIC deformation tracking.

2.2.2 Inflation testing

The latex specimen was secured onto a custom inflation chamber through the holder. The pressure was increased by the controlled injection of water into the chamber using a custom MTS-driven syringe pump (MTS, Eden Prairie MN). The pressure in the chamber was measured using a high precision pressure transducer (TJE, Honeywell, Columbus OH). The membrane was first equilibrated at the baseline pressure 0.28 kPa for 30 min and then subjected to a pressure-controlled load test from the baseline pressure to 7.87 kPa at 0.13 kPa/s.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

Figure 2.2: (a) Top view and (b) side view of the inflation experimental setup, showing the latex membrane, inflation chamber, pressure transducer, stereo-vision system (blue) and mono-vision system (red). (c) Top view and (d) side view of the inflated latex membrane. The blue axes define the 4 quadrants used to analyze the 3D-DIC data and the red axis defines the direction of the profile-edge imaged by the mono-vision system.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

2.2.3 Imaging

The deforming membrane was imaged simultaneously by a stereo-vision system mounted above the specimen and a mono-vision system positioned to the side of the inflation chamber (Figs. 2.2(a) and (b)). The stereo-vision system consisted of two 2 Mpixels monochrome cameras (GRAS-20S4M-C, Point Grey, Richmond BC, Canada) fitted with 35 mm focal length objectives (Xenoplan 1.9/35mm-0901, Schneider Optics, Hauppauge NY), and oriented with a 24° stereo angle (Fig. 2.2(a)) that acquired top-down images of the membrane. A third camera with identical characteristics was positioned to capture images of the membrane in profile with the same 0.025 mm/pixel image resolution as the stereo-vision system (Fig. 2.2(b)). The three cameras were synchronized to simultaneously image the membrane using Vic-Snap 2009 (Correlated Solutions Inc., Columbia SC). The static error and uncertainty in the 2D-DIC and 3D-DIC displacement components and meridional strain due to static noise were calculated from the correlation of three sequential images taken at a near zero pressure, 0.28 kPa and after the specimen was left to equilibrate for 30 min, to minimize the effect of creep. The static error was defined as the mean of the static displacements and meridional strain measured, while the static uncertainty was defined as the standard deviation of the measurements from the mean. A finite pressure was used to measure the static error and uncertainty rather than a zero pressure to ensure the membrane was unwrinkled. A high reference pressure, 5.41 kPa, was used for the inflation test to start from a bulged, spherical cap, configuration. This allowed
a central region of uniform strain to develop from inflation to the higher 7.87 kPa pressure. During inflation from baseline to 7.87 kPa, images were acquired every 2 s.

2.2.4 Data analysis

2.2.4.1 3D data analysis

The 3D-DIC analysis was performed on the image pairs obtained from the stereo-vision system using Vic-3D 2009 (Correlated Solutions, Columbia SC). A 45-pixel correlation window size and a 5-pixel step size were used, which provided reference position and displacement vectors on a 2D Cartesian grid with a 0.125 mm spacing. It was assumed that away from the boundaries, the reference bulged configuration can be described as a sphere, and an equation for a generalized sphere was fitted to the position vectors. The fit returned a radius $R_{3D} = 15.7$ mm for the spherical cap with a negligible average residual of 9.54e-5 mm. Using $R_{3D}$, a spherical grid spanning 360° in the circumferential $\theta$ direction with a 3° spacing and 14° in the meridional $\varphi$ direction with a 1° spacing was created, and the displacement vectors were interpolated onto that grid using the Matlab function spherefit.m (Levente Hunyadi, 2010). The transformation thus provided reference positions ($X$, $Y$, $Z$) and displacements ($U_x$, $U_y$, $U_z$) at 1° intervals along 120 meridians. The horizontal displacement component $U_\xi$ in the projected radial direction $\xi = R_{3D} \sin(\varphi)$ along a meridian with angle $\theta$ (Fig. 2.2(c)) was calculated at each grid point from the interpolated displacements...
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

as,

$$U_\xi = U_x \cos(\theta) + U_y \sin(\theta).$$

(2.1)

The interpolated $$U_z$$ provided the vertical displacement component in the vertical direction $$z$$. The meridional strain was evaluated from the stretch of the reference spherical grid. At each grid point $$n$$ along a meridian, the deformed positions were calculated as $$x_n = X_n + U_{xn}$$, $$y_n = Y_n + U_{yn}$$, $$z_n = Z_n + U_{zn}$$. The reference length $$L_{\varphi n}$$ and deformed length $$l_{\varphi n}$$ of the grid were evaluated as,

$$L_{\varphi n} = \sqrt{(X_{n+1} - X_{n-1})^2 + (Y_{n+1} - Y_{n-1})^2 + (Z_{n+1} - Z_{n-1})^2},$$

$$l_{\varphi n} = \sqrt{(x_{n+1} - x_{n-1})^2 + (y_{n+1} - y_{n-1})^2 + (z_{n+1} - z_{n-1})^2}. \quad (2.2)$$

The meridional stretch $$\lambda_{\varphi\varphi}$$ and Green-Lagrange strain $$E_{\varphi\varphi}$$ were evaluated at each grid point using the reference and deformed grid lengths as,

$$\lambda_{\varphi\varphi n} = \frac{l_{\varphi n}}{L_{\varphi n}}, \quad E_{\varphi\varphi n} = \frac{1}{2} (\lambda_{\varphi\varphi n}^2 - 1). \quad (2.3)$$

2.2.4.2 2D data analysis

The 2D-DIC analysis was performed on images obtained from the mono-vision system using Vic-2D 2009 (Correlated Solutions, Columbia SC). The same correlation window size and step size as for the 3D-DIC analysis were used. The horizontal and vertical displacement components ($$U_\xi, U_z$$) were extracted for a series of points on the
membrane boundary in the reference configuration, which corresponded to the $\theta_{2D}$ and $\theta_{2D}+180^\circ$ meridians in Fig. 2.2(c). The location $(X, Z)$ of those points coincided with the location of the 3D-DIC reference spherical grid points along the $\xi$ direction from the membrane center. The center of the membrane in profile was calculated by fitting a generalized circle to the position vectors on the membrane boundary using the Matlab function $\texttt{CircleFitByTaubin.m}$ (Nikolai Chrenov, 2009). The meridional strain was calculated from the 2D reference positions $(X, Z)$ and displacements $(U_\xi, U_z)$ using the 2D version of equations (2.2) and (2.3).

### 2.2.5 3D-DIC static performance

To estimate the static noise level responsible for the static error and uncertainty in the 3D-DIC measurements, a porcine scleral specimen, incubated in buffer for 18 hours at $37^\circ$C, was left to equilibrate at baseline pressure 0.28 kPa for 30 min (see chapter 3 for details), after which 10 consecutive pictures were rapidly taken at 0.5 s intervals. The displacements and corresponding strains were calculated in the circumferential and meridional directions, and averaged over a 3 mm wide annulus and over the 9 image sequence, using the first image as the reference. The mean and standard deviation across the annulus and image sequence provided a measure of the 3D-DIC static error and uncertainty in displacements and strains due to static noise.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

2.2.6 3D-DIC performance with inflation

To evaluate the error and uncertainty in the 3D-DIC displacements and corresponding strains with inflation, measurements of the 3D-DIC displacement error and uncertainty from another study were used. In this study, a piece of sclera glued onto a rigid spherical object was translated using a micromanipulator and a 3D-DIC measurement of $195 \pm 12 \, \mu m$ was reported for a $200 \, \mu m$ applied out-of-plane displacement. Those measurements were then applied to the inflation of a numerical sphere to calculate the error and uncertainty in strains. A discrete spherical shell with radius $13.3 \, \text{mm}$, corresponding to the mean radius over all specimens and tests, was created in Matlab and subjected to an inflation defined as a $195 \pm 13.4 \, \mu m$ Gaussian distribution across its surface. The distribution mean and standard deviation values accounted for the 3D-DIC error and uncertainty in the out-of-plane displacements, which were larger than for the in-plane displacements, and therefore provided an upper bound for the error and uncertainty in strains with inflation. A random noise of $1.4 \, \mu m$ was added to the uncertainty measured by Coudrillier et al, based on the mean confidence margin across the surface of one specimen, for the out-of-plane displacement at maximum pressure, obtained from Vic-3D (Correlated Solutions Inc., Columbia SC). The confidence margin represents a one standard deviation taking into account initial position and displacement uncertainties due to 3D-DIC dynamic noise. The corresponding strains were computed over a $3 \, \text{mm}$ wide annulus, in the circumferential and meridional directions. The mean and standard deviation across
this annulus represented a measure of the 3D-DIC error and uncertainty in strains with inflation due to the error and uncertainty in the displacement measurements.

2.3 Results

2.3.1 3D regional variations

To examine the spatial variations in the inflation response, the specimen was divided into 4 quadrants (Fig. 2.2(c)) and the 3D-DIC displacements and meridional strains were averaged over the 30 meridians of each quadrant, at each $\xi$ position. The 3-D-DIC displacements and meridional strains were also averaged at each $\xi$ position over all 120 meridians. The results are plotted as a function of the horizontal position $\xi$ in Fig. 2.3. Significant variations were observed in the 3D-DIC measurements along $\xi$ across the membrane. The maximum absolute difference along $\xi$ between quadrants was 0.016 mm between quadrants 2 and 4 for the horizontal displacement, 0.033 mm between quadrants 3 and 4 for the vertical displacement, and 0.033 between quadrants 2 and 4 for the meridional strain. The variation in displacements and strain along $\xi$ were smaller when the results were averaged over the entire membrane. Due to the large regional variations in the 3D-DIC measurements, which could be partly due to the membrane heterogeneity shown in Fig. 2.1, the 2D-DIC measurements were compared to the 3D-DIC measurements of quadrants 1 and 3 only, which contained the 2D meridians.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

Figure 2.3: 3D-DIC (a) horizontal displacement, (b) vertical displacement and (c) meridional strain averaged over each quadrant of the membrane and over the entire membrane, at each $\xi$ position.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

2.3.2 Static noise

The probability distributions of the static noise for 2D-DIC and 3D-DIC displacement components and strain were calculated using a 0.0005 bin size and are plotted in Fig. 2.4. Fifteen additional values were extracted along each 2D meridians to obtain a representative statistical distribution. The probability for each bin was calculated as the number of measurements in the bin divided by the total number of measurements. The static error was defined as the mean of each distribution and the static uncertainty was defined as the standard deviation from the mean. For the horizontal displacements, the absolute error was 13 times higher for 2D-DIC than for 3D-DIC, whereas the uncertainty in 2D-DIC was only twice the uncertainty in 3D-DIC. For the vertical displacements, the absolute error was 0.7 times smaller for 2D-DIC than for 3D-DIC, and the uncertainty in 2D-DIC was 0.3 times smaller than the uncertainty in 3D-DIC. For the meridional strain, the absolute error was 0.9 times smaller for 2D-DIC than 3D-DIC, whereas the uncertainty in 2D-DIC showed a value 5 times higher than the uncertainty in 3D-DIC.

2.3.3 Displacements

The 3D-DIC displacement measurements for pressurization from 5.41 kPa to 7.87 kPa are plotted in Figs. 2.5(a) and 2.5(b) along the horizontal direction $\xi$ for the 60 meridians of quadrants 1 and 3. The 2D-DIC displacement measurements for the
Figure 2.4: Probability distributions for the static noise for 2D-DIC and 3D-DIC (a) horizontal displacement, (b) vertical displacement and (c) meridional strain, using a 0.0005 bin size.
2 meridians of the membrane profile are also plotted for comparison. The variation in the horizontal and vertical displacements were generally higher for 3D-DIC than 2D-DIC, though the difference was significantly larger for the vertical displacement. The 3D-DIC vertical displacements were also generally larger than those measured by 2D-DIC. For 3D-DIC, the variation in the vertical displacement was larger than for the horizontal displacement, whereas 2D-DIC showed similar variations for both displacement components. To further compare the variation in 2D-DIC and 3D-DIC measurements, the range in the displacement components was calculated at each ξ position as the difference between the maximum and minimum displacement values among the 60 meridians or 2 meridians considered. Further averaging over all ξ positions gave an average range for the horizontal displacement of 0.0044 mm for 2D-DIC and 0.017 mm for 3D-DIC. The average range for the vertical displacement was 0.0035 mm for 2D-DIC and 0.052 mm for 3D-DIC. The absolute difference between the average 3D-DIC and 2D-DIC displacements was calculated as the absolute value of the difference between the 3D-DIC displacements averaged over the 60 meridians, and the 2D-DIC displacements averaged over the 2 meridians. The results are plotted for the horizontal and vertical displacements along ξ in Fig. 2.5(c). Further averaging over all ξ positions gave an average absolute difference of 0.0024 mm for the horizontal displacement and 0.014 mm for the vertical displacement.
Figure 2.5: (a) Horizontal displacement and (b) vertical displacement for the 2D meridians and the 3D meridians from quadrants 1 and 3. (c) Absolute difference between the 3D-DIC and 2D-DIC displacement components, averaged over the 2 meridians or 60 meridians considered, at each $\xi$ position.
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

2.3.4 Meridional strain

The meridional strain is plotted as a function of the horizontal position $\xi$ for the 2D meridians and 3D meridians from quadrants 1 and 3 in Fig. 2.6(a) for comparison between 2D-DIC and 3D-DIC. The variation in the 2D-DIC and 3D-DIC strain were comparable over the 4 mm region around the membrane apex. Assuming that the meridional strains were uniform within the central 4 mm region, the 2D-DIC and 3D-DIC strains were plotted as probability distributions using a 0.0020 bin size as shown in Fig. 2.6(b). The probability distributions for the meridional strain were similar for 2D-DIC and 3D-DIC. The means of the 2D-DIC and 3D-DIC distributions differed by 0.0029, while the standard deviations were nearly identical.

**Figure 2.6**: (a) Meridional strain for the 2D meridians and the 3D meridians from quadrants 1 and 3. (b) Probability distributions for the 2D-DIC and 3D-DIC meridional strain, calculated using a 0.0020 strain bin size.
2.3.5 3D-DIC static performance

This section presents the 3D-DIC error and uncertainty in displacements and strains under static conditions, calculated as described in section 2.2.5. The results under static conditions showed a mean and standard deviation of the displacements of -4.2e-5 ± 1.9e-5 mm in the circumferential direction and -7.7e-5 ± 5.5e-5 mm in the meridional direction. Additionally, the static results showed a mean and standard deviation of the absolute strains of 1.1e-5 ± 5.6e-6 in the circumferential direction and 1.4e-4 ± 9.3e-5 in the meridional direction.

2.3.6 3D-DIC performance with inflation

Finally, this section presents the results from the numerical strain calculation described in section 2.2.6. The results showed that a 5 µm error and 13.4 µm uncertainty in displacements for a 200 µm applied inflation translated into a mean and standard deviation of the absolute strain field of 0.0149 ± 5.2707e-4 in the circumferential direction and 0.0151 ± 6.7605e-4 in the meridional direction (Figure 2.7).

2.4 Discussion

In this study, the 2D-DIC and 3D-DIC outcomes for the inflation of a latex membrane were compared. First, the baseline error and uncertainty were measured for the static membrane under a near zero pressure. The baseline absolute error and uncer-
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

Figure 2.7: Maps of absolute circumferential (a) and meridional (b) strains, averaged over a 3 mm wide annulus, for a sphere subjected to a $195 \pm 13.4 \mu m$ uniform inflation. SD = standard deviation.

Uncertainty in the horizontal displacement were larger for 2D-DIC than 3D-DIC. This could be due to a higher static noise within the correlation window at the membrane boundary, as it was partially on the background and on the speckle pattern, which probably affected the subset correlation and therefore the displacement measurements. In addition, 2D-DIC showed a larger baseline uncertainty in the horizontal displacement than in the vertical displacement. This might be due to the contrast of the membrane boundary imaged in profile being lower, and therefore more susceptible to static noise, in the meridional direction (along the membrane) than in the vertical direction, where approximately half of the correlation window contained the black background. The baseline uncertainty in the vertical displacement was larger for 3D-DIC than 2D-DIC. In addition, 3D-DIC showed a larger baseline absolute error and uncertainty for the vertical displacement than for the horizontal displacement, which was consistent with other studies. Both findings suggest that static noise has a greater effect on
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

the out-of-plane measures compared to the in-plane measures for 3D-DIC, possibly due to a higher sensibility of the perspective transformation to noise. The effect of measuring the displacements at the membrane boundary, where the correlation window partially contains some background may explain why a 2D-DIC displacement uncertainty up to 0.068 pixels was measured, which was 10 times higher than the maximum 0.006 pixels position uncertainty measured by Ke et al.\textsuperscript{200} for a static planar object. Similarly for 3D-DIC, the 0.0322 pixels and 0.128 pixels horizontal and vertical displacement uncertainties reported here were about 10 times higher than the maximum 0.0030 pixels and 0.0093 pixels position uncertainties reported by Ke et al.\textsuperscript{200} for a planar object. However, Hu et al.\textsuperscript{195} numerically and experimentally reported significantly higher positional uncertainties than those reported here, for static cylinders. This suggests that the baseline positional uncertainty of 3D-DIC due to static noise is higher for curved objects than planar objects. The baseline uncertainty in the meridional strain was higher for 2D-DIC than 3D-DIC. This can be explained by the interpolation of displacements in the 3D-DIC analysis, whereas no interpolation was performed in the 2D-DIC analysis. A 4400 \(\mu\)strain uncertainty from 2D-DIC and a 818 \(\mu\)strain from 3D-DIC were obtained, whereas Ke et al.\textsuperscript{200} reported a 3D-DIC in-plane strain uncertainty of 90 \(\mu\)strain from static tests of a planar object. In addition to using a planar object, the smaller strain uncertainty obtained by Ke et al.\textsuperscript{200} can be attributed to the use of a spatial averaging scheme for the strain calculation.
The variation in displacements and meridional strain increased significantly for 2D-DIC and 3D-DIC with inflation. This could be partly attributed to the variations in the material properties and thickness of the membrane shown in Fig. 2.1. The membrane exhibited a distinct texture, appearing as parallel lines in Fig. 2.1, that may have originated from the manufacturing process of the rubber sheet. Under inflation, 3D-DIC also showed a larger variation in the vertical (out-of-plane) displacement than in the horizontal (in-plane) displacement, as reported in this study and others under static conditions.\textsuperscript{195,200} In contrast, 2D-DIC showed similar variations for both displacement components. The variation in both displacement components were generally larger for 3D-DIC than 2D-DIC, and the difference was significantly larger for the vertical displacement, suggesting again additional sources of variability in the 3D-DIC out-of-plane measures. Moreover, the absolute difference between the average 3D-DIC and 2D-DIC displacements across the meridians were within the range of 3D-DIC for both displacement components. Interestingly, the uncertainty in the meridional strain was nearly identical for both DIC methods. This could be due to the interpolation of the displacements on a spherical grid for the 3D-DIC data analysis. For 3D-DIC, the raw displacement data were interpolated onto a spherical grid, which introduced a degree of smoothing to the displacement and strain calculations. Interpolation of the data onto a coarser spherical grid resulted in a smoother strain field. A reasonable interpolation grid seemed to be a grid slightly more sparse than the array of data points given by DIC, to guarantee the accurate interpolation of
the displacements while preventing over-smoothing and the loss of local information.

For this study, an interpolation grid spacing of $\Delta \varphi = 1^\circ$ corresponded to a grid size of 0.27 mm in the meridional direction, which was twice the 0.125 mm step size used for the DIC analysis. However, the absolute difference between the mean of the 3D-DIC and 2D-DIC strain distributions was within the range of the 3D-DIC strain uncertainty. The strain uncertainty of 8000 $\mu$strain reported here for both DIC methods was an order of magnitude larger than the 800 $\mu$strain uncertainty reported by Sutton et al.\textsuperscript{186} for the translation of a cylinder. Although Sutton et al.\textsuperscript{186} used a smoothing scheme to calculate strains, this might suggest that 3D deformation generates higher uncertainties than translation. The variation in displacements and meridional strain did not show an increasing or decreasing trend from the membrane apex towards the holder for the central 8.0 mm apical region for 2D-DIC and 3D-DIC.

In this study, a telecentric lens was not used in the 2D-DIC imaging system and lens distortions were not corrected, which naturally introduced some bias in the 2D-DIC measurements. 2D-DIC was probably affected by the change in magnification with depth due to the membrane curvature, especially without the use of a telecentric lens for the image acquisition.\textsuperscript{207} However, the 2D-DIC displacements were extracted at the membrane boundary to limit the effect of curvature and the focus of the 2D-DIC camera system was set to the membrane boundary at the baseline pressure to minimize the effect of defocus with inflation. The maximum out of the ($\xi$-$z$) plane dimension of the bulged membrane at maximum pressure that was included in the correlation
window was calculated to be 4 mm, which was within the depth of field of the 2D-DIC system. Thus, the effect of defocusing on the 2D-DIC measurements at the membrane boundary could be considered small. 2D-DIC was probably also affected by lens distortions. The main lens distortions include radial and tangential distortions, though tangential distortion effects can usually be ignored. However, the portion of the membrane considered in this study was far away from the image edges where the radial distortion is greater. Moreover, no increase or decrease in the 2D-DIC variations or difference with the 3D-DIC measurements was observed along the meridians, which favors a small effect of lens distortion compared to other effects.

The results suggest that 3D-DIC should be used to fully characterize the mechanical behavior of heterogeneous materials and investigate the anisotropy of their response to inflation. However, 2D-DIC might be used as an alternative to 3D-DIC under certain experimental conditions, such as an inflation, where the deformation can be imaged in profile in the plane of the 2D-DIC camera system. Since 3D-DIC has a larger error and uncertainty in the vertical displacement than in the horizontal displacement, 2D-DIC may provide a comparatively accurate alternative method to measure the deformation of stiff membranes, for which the vertical displacements are comparable to the errors of the 3D-DIC system, and small specimens, where large deformations would exceed the small depth of field of a high magnification 3D-DIC camera system. In addition, 2D-DIC can be used to overcome the camera synchronization issue in high-speed experiments and space limitation for two-angled
CHAPTER 2. INVESTIGATING THE ACCURACY AND UNCERTAINTY OF DIGITAL IMAGE CORRELATION

Cameras in highly controlled experimental environments were used to investigate the 3D-DIC error and uncertainty in displacements and strains under static conditions, and the results from the numerical strain calculation are now discussed. The 3D-DIC error and uncertainty in displacements and strains under static conditions were at least one order of magnitude smaller than the displacements and strains measured for the porcine posterior scleral shells in chapter 3. Under inflation, the mean strain values in both directions were close to the 0.0151 theoretical strain for a 200 µm uniform inflation and the standard deviations were small compared to the strains measured in the porcine specimens (see chapter 3). In addition, the standard deviations were within the 0.03-0.08% strain uncertainty range obtained by Sutton et al. for the 3D translation of a cylinder.

2.5 Conclusion

In conclusion, the 2D-DIC and 3D-DIC outcomes were compared for a simple 3D deformation state, a membrane under inflation. It was found that the baseline uncertainty in the horizontal displacement and meridional strain were smaller for 3D-DIC than 2D-DIC, but the opposite was observed for the vertical displacement, for which 2D-DIC showed a smaller baseline uncertainty. The baseline absolute error was similar for both DIC methods for the vertical displacement and strain, but it was larger for 2D-DIC than 3D-DIC for the horizontal displacement. Inflation generally
produced higher variations than static conditions for both methods. Under inflation, the variability in both displacement components were larger for 3D-DIC than 2D-DIC, but the uncertainty in the meridional strain was similar for both DIC methods. The absolute difference between the average displacement and strain data from 2D-DIC and 3D-DIC were in the range of the 3D-DIC variability. A new methodology was developed to evaluate the error and uncertainty in strains due to the 3D-DIC displacement error and uncertainty for a specimen under inflation. The strain error and uncertainty obtained with the imaging setup described in this study were small compared to the strains measured in the porcine scleral specimens in this work.
Chapter 3

Alterations in the posterior sclera following glycosaminoglycan degradation: a porcine experimental study

This chapter investigates the structural and mechanical effects of experimental s-GAG degradation in the posterior porcine sclera. The effects of s-GAG removal on the s-GAG content, hydration, thickness and inflation response of the posterior sclera in enucleated eyes of 6-9 month-old pigs were measured. The s-GAG content and hydration were evaluated in the 4 anatomical quadrants of some scleral specimens, away from the ONH, under 3 conditions: no treatment (control), after treatment in
buffer alone, and after treatment in buffer with ChABC to digest the s-GAGs. Other specimens were mechanically tested by pressure-controlled inflation with full-field deformation mapping using 3D-DIC and their thickness was measured in 2 experimental groups: as controls and after buffer-treatment, or after buffer-treatment and then after enzyme-treatment. This chapter is reprinted from Acta Biomaterialia, Barbara J. Murienne, Joan L. Jefferys, Harry A. Quigley and Thao D. Nguyen, “The effects of glycosaminoglycan degradation on the mechanical behavior of the posterior porcine sclera”, Vol. 12, Pages 195-206, 2015, with permission from Elsevier.

3.1 Introduction

The sclera, the white outer shell of the eye, is responsible for maintaining the eye shape in the presence of intraocular pressure and protecting the internal ocular structures from external forces. It is the main load-bearing tissue of the eye and studies have suggested that alterations to its mechanical properties, specifically at the posterior pole, contribute to the development of glaucoma and myopia. Glaucoma is the second leading cause of blindness worldwide and glaucoma damage is considered to be dependent on the scleral mechanical properties. Myopia is a common refractive error that is ubiquitous in certain populations and the associated abnormal axial elongation of the globe involves dramatic changes in the scleral mechanical properties.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Changes in the ECM structure of the eye wall have been reported with both glaucoma and myopia. These include variations in the GAG content. GAGs are polysaccharide chains of various lengths, the majority of which binds to core proteins to form PGs. The human posterior sclera is particularly rich in chondroitin sulfate GAG side chains, from the abundant presence of aggrecan, and in dermatan sulfate chains. An abnormal accumulation of GAGs, including chondroitin sulfates, was reported in some portions of the anterior segment and in the lamina cribrosa of glaucomatous human eyes. Similarly, the rat and monkey lamina cribrosa showed a higher chondroitin sulfate content when subjected to IOP elevation. A decrease in the overall GAG content was found in the posterior sclera of human myopic eyes, while a decrease in the s-GAG content was measured in tree shrew eyes with form-deprivation myopia. A decrease in GAG synthesis was also reported in the posterior sclera of form-deprived myopic tree shrew and monkey eyes.

Changes in the tensile and viscoelastic properties of the sclera also occur in glaucoma and myopia. The posterior sclera of glaucoma eyes showed an increase in stiffness and a decrease in creep rate. Myopic posterior scleras, in which there is a decrease in GAG content, had the opposite biomechanical changes in some parameters, with an increase in the strain at maximum pressure and creep rate but no change in stiffness.

GAGs play an important role in the structure and mechanical behavior of collagenous ECM tissues. GAGs fill the space between the collagen fibrils and the collagen-
elastin network in the ECM. Although the core protein of some PGs is found at regular intervals on collagen fibrils, it seems that PG interactions with collagen not only occur through binding of the core protein but also through electrostatic interaction of the GAGs themselves as shown in vitro. The majority of GAGs are highly polar and attract water, due to their negatively charged carboxyl and sulphated groups. Their fixed charge density also creates repulsion and attraction forces among themselves and with the collagen fibrils, respectively. As a result, the GAG content regulates the tissue hydration by determining the number of polar sites for water binding as well as the tissue osmotic pressure. However, tissue swelling is limited by the hydration state of the tissue, the stiffness of the collagen network as well as the repulsion and interactions between the GAGs that determine the space for free water uptake. GAGs have been hypothesized to regulate the collagen interfibrillar spacing through their water-binding capacity and osmotic pressure GAG-GAG charge repulsion and GAG-GAG antiparallel interactions. GAGs have also been shown to regulate collagen fibrillogenesis in vitro under certain conditions, specifically affecting the rate of fibril formation and fibril diameter.

The mechanical functions of GAGs have been studied in various tissues, with tissue-specific and sometimes conflicting results, by comparing their mechanical behavior before and after GAG removal. In cartilage, GAG removal produced a faster creep rate. However, in aortic heart valve leaflets where native creep is negligible,
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

no change in the creep behavior was observed after GAG removal.\textsuperscript{113} GAG removal did not change the hysteresis observed during cyclic loading in ligaments\textsuperscript{113} nor at the high stress range in heart valve leaflets.\textsuperscript{113} In contrast, it decreased the hysteresis in aortic valve leaflets in the low stress range.\textsuperscript{113} A faster stress relaxation was observed after GAG removal in the aorta,\textsuperscript{109} but no change in relaxation was observed in ligaments.\textsuperscript{115} The role of GAGs in the tissue stiffness is still controversial. No change in the uniaxial loading stiffness was found after GAG removal in cartilage\textsuperscript{112} and tendons.\textsuperscript{119} However, an increase in stiffness occurred in mesenteric arteries\textsuperscript{110} and lungs.\textsuperscript{117} In the aorta, the small-strain stiffness was higher and the high-strain stiffness lower after GAG removal, and similar changes were measured for specimens incubated in buffer with or without a GAG-targeting enzyme compared to controls.\textsuperscript{109} Similarly, ligaments showed a higher small-strain stiffness after GAG depletion\textsuperscript{108} whereas no change in stiffness was measured in another study.\textsuperscript{114}

None of the studies comparing the biochemical and biomechanical characteristics of normal and pathological sclera have addressed the mechanical function of GAGs. In this study, the effect of s-GAG removal on the mechanical behavior of the posterior porcine sclera was investigated to infer the mechanical relevance of pathological changes in the scleral s-GAG content.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

3.2 Methods

This section describes the methods used to evaluate the mechanical behavior and structural characteristics of the posterior porcine sclera.

3.2.1 Experimental design and glycosaminoglycan removal

All mechanical and structural outcomes were measured in 4 quadrants of the posterior scleral cup surrounding the ONH (superior-temporal (ST), superior-nasal (SN), inferior-temporal (IT), inferior-nasal (IN)). Different specimens were used for either hydration measurement (n=18), GAG staining (n=3) or s-GAG quantification (n=18) and they were divided into 3 experimental groups. In the first group, the specimens were processed immediately after preparation (control) (n=6 for hydration and s-GAG quantification, n=1 for GAG staining). In the second group, they were incubated for 18 h at 37°C in a modified Trizma buffer at pH 8.0 (buffer-treated) (n=6 for hydration and s-GAG quantification, n=1 for GAG staining). In the third group, they were incubated for 18 h in Trizma buffer containing ChABC (C2905, Sigma-Aldrich, St. Louis MO) at 2 units/ml (enzyme-treated) (n=6 for hydration and s-GAG quantification, n=1 for GAG staining). The detailed protocol including the solutions used for s-GAG degradation can be found in section A.2.1 of the Appendix.

Other specimens used for both mechanical testing and thickness measurement
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

(n=12) were divided into 2 groups only. In one group, the specimens were mechanically tested after dissection and again after incubation in buffer alone for 18 h (protocol 1: control/buffer-treated) (n=5). In another group, the specimens were soaked in buffer alone for 18 h after dissection, mechanically tested, soaked in enzyme solution for 18 h, and tested again (protocol 2: buffer-treated/enzyme-treated) (n=7). Thickness data for 3 additional control/buffer-treated specimens and 2 additional buffer-treated/enzyme-treated specimens that were not inflation tested were added for statistical analysis. In addition, 2 specimens were incubated in buffer with protease inhibitors (protease inhibitor cocktail tablets S8830, Sigma-Aldrich, St. Louis MO) for 18 h after dissection, inflated, incubated in enzyme solution with protease inhibitors for 18 h, and inflated again. After incubation, the samples were always rinsed for 30 min in fresh Trizma buffer without enzyme. Table 3.1 summarizes the number of specimens used in each experiment.

The control specimens represented a baseline hydrated state in that the interior surface of the specimens was exposed to Dulbecco’s Phosphate Buffered Saline (DPBS) during the inflation test and the specimens were tested in a humidity chamber at greater than 90% humidity. Comparing controls to specimens soaked in buffer alone allowed the effect of an increase in hydration on the scleral mechanical properties to be measured. The comparison between the control (baseline hydrated) and buffer-treated (swollen) states allowed the effect of hydration to be distinguished from that of s-GAG degradation when the specimens were treated with buffer containing
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

<table>
<thead>
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<th>Control</th>
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<th>Enzyme-treated</th>
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<td>n=1</td>
<td>n=1</td>
</tr>
<tr>
<td>s-GAG quantification</td>
<td>n=6</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td>Hydration</td>
<td>n=6</td>
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</table>

<table>
<thead>
<tr>
<th>Number of specimens used for the measurement of thickness and mechanical behavior changes</th>
<th>Control/Buffer-treated/Buffer treated/Enzyme-treated with inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical changes</td>
<td>n=5/7/n=2</td>
</tr>
<tr>
<td>Thickness change</td>
<td>n=8*/n=9#/n=2</td>
</tr>
</tbody>
</table>

Table 3.1: Number of specimens used for the measurement of GAGs, hydration and changes in thickness and mechanical behavior. The * denotes that 5 of the 8 specimens used to measure the thickness change were used for mechanical testing. Similarly, the # denotes that 7 of the 9 specimens used to measure the thickness change were subjected to mechanical testing.

ChABC.

3.2.2 Specimen preparation

Eyes from 6-9 month-old pigs (Animal Technologies Inc., Tyler TX) were received on wet ice 24 h after enucleation and used upon arrival. The skin, extraocular fat and muscles were first carefully removed from all eyes using fine dissection scissors to obtain a clean scleral surface. The eyes used for mechanical testing, thickness measurement, GAG staining and s-GAG quantification were mounted on a custom-made acrylic holder, using a cyanoacrylate-based glue (Permabond 910, Electron Microscopy Sciences, Hatfield PA). Details about the holder designs and dimensions can be found in section A.1.1 of the Appendix. The specimens were positioned such that the posterior scleral cup protruded through the holder opening centered about
the ONH (Fig. 3.1(a)). After the glue polymerized, a cut was made through the cornea using a scalpel and the intraocular structures including the retina and choroid were removed with fine curved dissecting forceps, leaving only the scleral shell. The remaining anterior sclera was then scored through its thickness and glued to the back side of the holder. The eyes used for hydration measurement were prepared using an 8 mm biopsy punch to extract scleral discs 2 mm away from the ONH in each of the 4 quadrants (Fig. 3.3). Care was taken not to include the ciliary arteries or other apparent vessels in these punch specimens. The scleral shells and discs were either processed directly or first incubated in solution, depending on their experimental group.

3.2.3 Hydration measurement

Scleral hydration was defined as the wet over dry tissue weight ratio. To measure the wet weight, the 8 mm scleral discs (Fig. 3.3) were blotted dry on a Whatman filter for 1 min, transferred to pre-weighed Eppendorf centrifuge tubes to prevent evaporation and weighed on a precision balance (XP26DR, Mettler-Toledo LLC, Columbus OH). The dry weight was obtained in the same way after the samples were dried for 48 h in an oven at 60°C. The detailed protocol used for the hydration measurement can be found in section A.2.4 of the Appendix.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Figure 3.1: (a) Scleral shell mounted on an acrylic holder. (b) 3D-DIC image of a speckled scleral shell. Side (c) and top (d) views of the mechanical testing setup.
3.2.4 Glycosaminoglycan staining

Staining of acidic GAGs was performed using Alcian Blue (A3157, Sigma-Aldrich, St. Louis MO). The posterior scleral shells were first fixed in 4% paraformaldehyde containing 1% cetylpyridinium chloride (C0732, Sigma-Aldrich, St. Louis MO) for 15 h at 4°C. Cetylpyridinium chloride was used to prevent leakage of GAGs outside the tissue during the fixation process. The samples were then cryo-preserved in a phosphate buffer containing sucrose and cryo-embedded in the same buffer mixed with an optimal cutting temperature compound, following the procedure from Barthel and Raymond. 10 µm thin sections were cut through the scleral thickness, 2 mm away from the ONH, along the nasal-temporal plane, in the same anatomical region for all shells. The sections were then stained in an Alcian Blue solution containing either 0.06 M or 0.5 M MgCl₂ (M8266, Sigma-Aldrich, St. Louis MO) for 30 min. 0.06 M of MgCl₂ was shown to stain for all acidic GAGs, while 0.5 M of MgCl₂ stained for strongly sulphated GAGs only. Details about the experimental procedure used for GAG staining can be found in section A.2.2 of the Appendix. The tissue sections were then imaged with a light microscope fitted with a 20x objective.

3.2.5 Glycosaminoglycan quantification

s-GAG quantification was performed on 3x4 mm scleral rectangles, dissected from the scleral cups 2 mm away from the ONH, in each of the 4 anatomical quadrants (Fig.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

3.3), using the Biocolor Blyscan assay (Accurate Chemical & Scientific Corporation, Westbury NY). Prior to the assay, the samples were weighed wet after being blotted dry on Whatman paper for 1 min and then completely digested using Papain (P3125, Sigma-Aldrich, St. Louis MO) in an oven at 60°C, following the protocol used by Boubriak et al. The s-GAG content was reported in \( \mu g/mg \) dry tissue weight by inferring the dry weight from the wet weight using the hydration data for the corresponding experimental group and quadrant. The solutions and detailed protocol used for s-GAG quantification can be found in section A.2.3 of the Appendix.

### 3.2.6 Mechanical testing

For mechanical testing, the scleral shells were placed on a pressure chamber and secured through the holder (Fig. 3.1(c) and (d)). A series of pressure-controlled inflation tests was conducted at room temperature by injection of DPBS inside the pressure chamber using an MTS-actuated syringe (MTS, Eden Prairie MN). The testing protocol was implemented in TestWorks (MTS, Eden Prairie MN) which used pressure readings from a pressure transducer (TJE, 2 psig range, Honeywell, Morris-town NJ) for PID control of the MTS. The specimens were equilibrated at baseline pressure 0.28 kPa for 30 min. Then, they were subjected to a load-unload cycle from baseline to 6 kPa at 0.13 kPa/s, a 30 min recovery period at baseline, a ramp-hold cycle loaded at 1 kPa/s to 6 kPa and held for 20 min, a 40 min recovery period at baseline, and finally a load-unload cycle identical to the first one (Fig. 3.2). Pre-
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

The PID parameters used for each mechanical test are listed in section A.1.2 of the Appendix. The specimens were encapsulated in a clear perspex hydration chamber filled with water saturated humidifier filters (Fig. 3.1(c) and (d)), that guaranteed a humidity level above 90% throughout the duration of the test.

3.2.7 Thickness measurement

The thickness was measured at the baseline pressure, at the end of each mechanical test series. Eight different locations, at equal distance from the ONH and acrylic holder (Fig. 3.3), were measured using an ultrasound transducer with frequency 15 MHz (V260-45, Olympus NDT Inc., Waltham MA), fitted with a Sonopen tip with diameter 1.5 mm (DLP-302, Olympus NDT Inc., Waltham MA) and connected to a pulser-receiver (5073PR-15-U, Olympus NDT Inc., Waltham MA). The ultrasound echoes generated by the external and internal scleral walls were acquired on an oscilloscope (TDS220, Tektronix Inc., Beaverton OR) and the peak-to-peak time difference $\Delta t$ between the echoes was calculated using a custom-written Matlab code. From the
peak-to-peak time difference $\Delta t$ and assuming a speed of sound in the sclera $c_{sclera} = 1597 \text{ m/s}$, the thickness $T$ was calculated as follows:

$$T = \frac{1}{2} c_{sclera} \Delta t$$  \hspace{1cm} (3.1)

The thickness was reported as the average of 2 measurements acquired for each quadrant. Details concerning the thickness measurement apparatus and calculations can be found in section A.1.3 of the Appendix.

### 3.2.8 Digital image correlation

A stereovision system (Fig. 3.1(c) and (d)) composed of two monochrome cameras (GRAS-20S4M-C, Point Grey, Richmond BC, Canada) with a 2 Mpixel resolution, $24^\circ$ stereo angle, objectives with a 35 mm focal length (Xenoplan 1.9/35mm-0901, Schneider Optics, Hauppauge NY) and controlled by Vic-Snap 2009 (Correlated Solutions Inc., Columbia SC) was used to acquire images of the scleral surface every 2 s.
during mechanical testing. The sclera was speckled with black India ink (Fig. 3.1(b)) using an airbrush (ECL4500 HP-CS, Iwata Medea Inc., Portland OR) to allow for 3D-DIC using Vic3D 2009 (Correlated Solutions Inc., Columbia SC).

3.2.9 Strain calculation

3D-DIC provided positions (X, Y, Z) of the specimen surface at the baseline pressure and displacements (U, V, W) at each pressure step for points of a Cartesian ($e_x$, $e_y$) grid, with grid spacing 0.12 mm on average. To calculate the circumferential and meridional strains on the surface (Fig. 3.1 (b)), the (X, Y, Z) positions of the reference surface, for the region starting 1 mm away from the ONH, were fitted to a generalized sphere. A curvilinear grid on the surface of the sphere ($e_\theta$, $e_\phi$) was then created, with grid spacing 3° in the circumferential $e_\theta$ direction and 1° in the meridional $e_\phi$ direction. The average geometric residual of the sphere fit for all specimens ranged between 0.0048% and 0.014% of the radius of the sphere. The 3D displacements of the 3D-DIC Cartesian grid at each pressure step were interpolated to points on the spherical grid using the built-in Matlab function TriScatteredInterp. The stretches, $\lambda_{\theta\theta}$ in the circumferential direction and $\lambda_{\phi\phi}$ in the meridional direction, were calculated from the deformed lengths $l_\theta$ and $l_\phi$ and undeformed lengths $L_\theta$ and $L_\phi$ of material grid lines along the circumferential and meridional directions as follows.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

\[ \lambda_{\theta\theta} = \frac{l_\theta}{L_\theta}; \lambda_{\phi\phi} = \frac{l_\phi}{L_\phi} \]  

(3.2)

For a point \( n \) at position \( \theta \) on the undeformed spherical grid, surrounded by points \( n-1 \) (\( \theta - \Delta \theta \)) and \( n+1 \) (\( \theta + \Delta \theta \)) along the \( e_\theta \) direction, \( L_{\theta n} \) and \( l_{\theta n} \) were calculated using central difference as:

\[ L_{\theta n} = \sqrt{(X_{n+1} - X_{n-1})^2 + (Y_{n+1} - Y_{n-1})^2 + (Z_{n+1} - Z_{n-1})^2} \]  

(3.3a)

\[ l_{\theta n} = \sqrt{(x_{n+1} - x_{n-1})^2 + (y_{n+1} - y_{n-1})^2 + (z_{n+1} - z_{n-1})^2} \]  

(3.3b)

where \( x = X+U \), \( y = Y+V \), \( z = Z+W \). The \( l_{\phi n} \) and \( L_{\phi n} \) were similarly calculated for the \( e_\phi \) direction. The corresponding Lagrangian strains \( E_{\theta\theta} \) and \( E_{\phi\phi} \) were calculated as:

\[ E_{\theta\theta} = \frac{1}{2}(\lambda_{\theta\theta}^2 - 1); E_{\phi\phi} = \frac{1}{2}(\lambda_{\phi\phi}^2 - 1) \]  

(3.4)

The strains were reported in each quadrant as an average over a region spanning 90° in the circumferential direction, 12° in the meridional direction. This corresponded on average to a 3 mm wide region spanning each quadrant located 3 mm away from the ONH (Fig. 3.3). The shear strains \( E_{\theta\phi} \) were also calculated as follows:

\[ E_{\theta\phi} = \frac{1}{2} t_\theta \cdot t_\phi \]  

(3.5)
where \( t_\theta \) and \( t_\phi \) are the tangent vectors of the deformed configuration in the circumferential and meridional directions. The deformed tangent vectors were calculated from the tangent vectors in the reference configuration \( T_\theta = e_\theta \) and \( T_\phi = e_\phi \), as well as the Cartesian displacement vectors \( u(X) = (U,V,W) \) from 3D-DIC interpolated on the undeformed spherical grid and the spherical angles \( \theta \) and \( \phi \) as:

\[
\begin{align*}
t_\theta &= T_\theta + \frac{1}{R \sin \phi} \frac{\partial u(X)}{\partial \theta}; \\
t_\phi &= T_\phi + \frac{1}{R} \frac{\partial u(X)}{\partial \phi}
\end{align*}
(3.6)
\]

For a point \( n \) at position \( (\theta_n, \phi_n) \) on the undeformed spherical grid, surrounded by points \( n - 1 (\theta - \Delta \theta) \) and \( n + 1 (\theta + \Delta \theta) \) along the \( e_\theta \) direction, the reference tangent vectors \( T_\theta \) and \( T_\phi \), as well as the partial derivative \( \frac{\partial u(X)}{\partial \theta} \) in the \( e_\theta \) direction, were calculated as:

\[
\begin{align*}
T_\theta &= (-\sin(\theta_n), \cos(\theta_n), 0); \\
T_\phi &= (\cos(\phi_n) \cos(\theta_n), \cos(\phi_n) \sin(\theta_n), -\sin(\phi_n))
\end{align*}
(3.7)
\]

\[
\frac{\partial u(X)}{\partial \theta} = \left( \frac{U_{n+1} - U_{n-1}}{\theta_{n+1} - \theta_{n-1}}, \frac{V_{n+1} - V_{n-1}}{\theta_{n+1} - \theta_{n-1}}, \frac{W_{n+1} - W_{n-1}}{\theta_{n+1} - \theta_{n-1}} \right)
(3.8)
\]

The partial derivative \( \frac{\partial u(X)}{\partial \phi} \) was similarly calculated for the \( e_\phi \) direction. The shear strains were also reported as an average over a 3 mm wide annulus spanning each quadrant, located 3 mm away from the ONH.

79
3.2.10 Hoop stress calculation

The hoop stresses, $\sigma_{\theta\theta}$ in the circumferential direction and $\sigma_{\phi\phi}$ in the meridional direction, were approximated using the thin shell theory. For all specimens the ratio of thickness to radius satisfied $T/r < 0.1$. For a spherical thin shell, the hoop stresses are:

$$\sigma_{\theta\theta} = \sigma_{\phi\phi} = \frac{Pr}{2T}. \quad (3.9)$$

The stresses were calculated for the 4 quadrants at each level of pressure $P$, based on the radius $r$ and the thickness $T$ obtained for each specimen from the spherical fit of the strain calculation and the thickness measured in each quadrant, respectively.

3.2.11 Mechanical data analysis

Six mechanical outcomes were defined (Fig. 3.4) to compare the tensile and viscoelastic behavior between the quadrants and experimental groups. The slope of the stress-strain loading curves at low and high pressures, and the transition strain at the onset of strain-stiffening were calculated to characterize the stiffness and non-linearity of the tensile response. The low-pressure slope was determined by fitting a line to the data points with strains below 2/3 of the maximum strain. The high-pressure slope was evaluated by fitting a line to the last 30% of the total number of data points. The intersection between the low and high pressure lines defined the transition strain.
Figure 3.4: Schematic of the mechanical outcomes extracted from the load-unload stress-strain and ramp-hold stress-time curves.

The hysteresis of the load-unload stress-strain curves and the normalized creep rate of the ramp-hold strain-time curves were calculated to characterize the viscoelastic response. The hysteresis, defined as the area between the load-unload curves, was calculated by numerical integration using the trapezoidal rule. The initial creep rate of the first 80 s and the final creep rate of the final 1120 s were calculated from the strain-time curves. The creep rate was defined as the slope of the linear fit of the strain-time response, normalized by the strain at the end of the loading curve and plotted on a log time scale.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

### 3.2.12 Statistical analysis

For the GAG staining data, unpaired Student’s t-tests were used to compare the average histogram of gray values over 2 images per specimen between the experimental groups. For hydration and s-GAG quantification data, generalized estimating equation models were used to account for repeat measurements taken in the 4 quadrants of a single specimen. For the thickness data, linear mixed models were used to consider both the clustering of 2 experimental groups within each specimen (control/buffer-treated groups or buffer-treated/enzyme-treated groups) and the repeat measurements in the 4 quadrants for each group. For each outcome, the variogram and Akaike’s Information Criteria were used to determine whether measurements from adjacent quadrants were more highly correlated than measurements from opposing quadrants (Toeplitz structure) or measurements from any 2 quadrants were similarly correlated (compound symmetry structure). For all models, a Bonferroni adjustment allowed for multiple comparisons. A significant interaction between quadrant and group indicated that the group effect depended on the quadrant studied and that the quadrant effect depended on the group studied. When the interaction was not significant, the models were simplified to combine all groups when looking at the quadrants and all quadrants when looking at the groups. For the mechanical data, paired Student’s t-tests were used to compare the control and buffer-treated groups, as well as the buffer-treated and enzyme-treated groups. They were also used to compare the circumferential and meridional responses. Unpaired t-tests allowed
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

for control to enzyme-treated comparisons. Linear mixed models were used to look at the spatial variation of the mechanical outcomes among quadrants. A p-value equal or less than 0.055 was considered significant. All analyses were performed using the Matlab Statistical Toolbox and SAS 9.2 (SAS Institute, Cary, NC).

3.3 Results

3.3.1 Glycosaminoglycan content, hydration and thickness

3.3.1.1 Glycosaminoglycan staining

The scleral GAG content under the 3 experimental conditions was first qualitatively assessed from images of 3 specimens, each belonging to a different group (Fig. 3.5). The image intensity for the enzyme-treated specimen was significantly lower than for the control and buffer-treated specimen for both concentrations of MgCl₂ (p ≤ 0.05). However, there was no significant difference in image intensity between the control and buffer-treated specimen.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Figure 3.5: Images of GAG staining for 3 specimens, each belonging to a different experimental group, and 2 different MgCl₂ concentrations. Scale bars = 100 µm. Unpaired Student’s t-tests were used for statistical analysis.

3.3.1.2 Glycosaminoglycan quantification

The scleral s-GAG content was obtained for the 3 experimental groups and 4 quadrants (Table 3.2). The statistical analysis showed a significant interaction between group and quadrant (p<0.0001). The s-GAG content was not significantly different between control and buffer-treated groups in all quadrants, but enzyme-treatment decreased the s-GAG content by 79.7% on average compared to controls and by 81.5% on average compared to buffer-treated specimens (p<0.0001). In the control group, the s-GAG content significantly differed by quadrant (p<0.005), being on average 68.2% higher in the superior quadrants than the inferior quadrants. Similarly, in the buffer-treated group, the s-GAG content in the 2 superior quadrants was on average
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

<table>
<thead>
<tr>
<th></th>
<th>ST</th>
<th>SN</th>
<th>IT</th>
<th>IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.34 ± 1.91</td>
<td>8.62 ± 2.26</td>
<td>4.40 ± 1.19</td>
<td>4.49 ± 1.11</td>
</tr>
<tr>
<td>Buffer-treated</td>
<td>7.54 ± 1.68</td>
<td>8.97 ± 2.61</td>
<td>4.93 ± 0.97</td>
<td>4.69 ± 1.01</td>
</tr>
<tr>
<td>Enzyme-treated</td>
<td>1.09 ± 0.23</td>
<td>0.90 ± 0.20</td>
<td>1.20 ± 0.25</td>
<td>1.18 ± 0.24</td>
</tr>
</tbody>
</table>

Table 3.2: s-GAG content [µg/mg dry tissue weight] in the 4 quadrants and 3 experimental groups. Significant interaction between quadrant and group (p<0.0001).

<table>
<thead>
<tr>
<th></th>
<th>ST</th>
<th>SN</th>
<th>IT</th>
<th>IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.04 ± 0.066</td>
<td>3.15 ± 0.093</td>
<td>3.04 ± 0.021</td>
<td>3.10 ± 0.063</td>
</tr>
<tr>
<td>Buffer-treated</td>
<td>3.26 ± 0.098</td>
<td>3.41 ± 0.100</td>
<td>3.25 ± 0.055</td>
<td>3.26 ± 0.100</td>
</tr>
<tr>
<td>Enzyme-treated</td>
<td>3.48 ± 0.080</td>
<td>3.52 ± 0.061</td>
<td>3.46 ± 0.182</td>
<td>3.50 ± 0.174</td>
</tr>
</tbody>
</table>

Table 3.3: Hydration ratio [mg/mg] in the 4 quadrants and 3 experimental groups. Non-significant interaction between quadrant and group (p=0.17).

71.6% higher than in the 2 inferior quadrants (p<0.0001). In the enzyme-treated group, only the SN quadrant was significantly different from the inferior quadrants (p≤0.01). Its s-GAG content was 24.4% lower on average than in the inferior quadrants and not higher, as measured in the control and buffer-treated groups.

### 3.3.1.3 Hydration

The scleral hydration, defined as the wet/dry weight ratio, was obtained for the 3 experimental groups and 4 quadrants (Table 3.3). No significant interaction was found between group and quadrant (p=0.17). Hydration was 6.8% higher in buffer-treated than controls (p=0.001) and 6.0% greater in enzyme-treated than buffer-treated (p<0.005). It was 13.3% higher in enzyme-treated compared to controls (p<0.0001). Hydration was the same in all quadrants except for SN where it was on average 2.8% higher (p<0.05).
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Table 3.4: Thickness [mm] in the 4 quadrants and 3 experimental conditions. Non-significant interaction between quadrant and group (p=0.43).

<table>
<thead>
<tr>
<th>Group</th>
<th>ST</th>
<th>SN</th>
<th>IT</th>
<th>IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.24 ± 0.145</td>
<td>1.10 ± 0.071</td>
<td>0.95 ± 0.115</td>
<td>0.88 ± 0.121</td>
</tr>
<tr>
<td>Buffer-treated</td>
<td>1.32 ± 0.094</td>
<td>1.23 ± 0.105</td>
<td>1.00 ± 0.110</td>
<td>0.97 ± 0.111</td>
</tr>
<tr>
<td>Enzyme-treated</td>
<td>1.32 ± 0.121</td>
<td>1.21 ± 0.117</td>
<td>1.04 ± 0.114</td>
<td>1.04 ± 0.110</td>
</tr>
</tbody>
</table>

3.3.1.4 Thickness

The scleral thickness was measured for the 4 quadrants and the buffer-treated data from both groups 1 and 2 repeat measurements were combined to reflect the 3 conditions: control, buffer-treated and enzyme-treated (Table 3.4). No significant interaction was found between group and quadrant (p=0.43). The thickness was 8.3% larger in buffer-treated than controls (p<0.0001) and 2.1% (p=0.01) larger in enzyme-treated than buffer-treated. It was 10.6% greater in enzyme-treated than controls (p<0.0001). In all groups, the superotemporal sclera was thickest and the inferonasal was thinnest (p<0.0001). On average, the thickness was 26.4% higher in the superior quadrants than inferior quadrants.

3.3.2 Mechanical behavior

3.3.2.1 Comparing the experimental groups

First, the average data for all scleral regions for each of the 6 mechanical outcomes were compared between the 2 inflation test sessions in each of the 2 protocols: control condition compared to buffer-treated (Table 3.5), and buffer-treated compared to
enzyme-treated (Table 3.6). In the circumferential direction, for protocol 1, treatment in buffer alone increased the low-pressure stiffness by 28.4% (p<0.005), hysteresis by 16.7% (p<0.01), initial creep rate by 45.1% (p<0.005), and final creep rate by 42.7% (p<0.05) compared to the baseline control values. In addition, buffer-treatment decreased the high-pressure stiffness by 22.3% (p<0.0001) over controls, but there was no significant change in the transition strain. In protocol 2, treatment in enzyme led to a significant decrease in the low-pressure stiffness by 51.5% (p<0.0001) and hysteresis by 24.9% (p<0.0001) compared to the values after treatment in buffer alone. Moreover, enzyme-treatment increased the high-pressure stiffness by 23.0% (p<0.05) and the transition strain by 25.7% (p=0.001) over buffer-treated specimens but there was no difference in the creep rates.

In the meridional direction, for protocol 1, buffer-treatment increased the low-pressure stiffness by 32.8% (p<0.05), hysteresis by 20.8% (p<0.05), and final creep rate by 288.2% (p<0.005) compared to control values. Buffer-treatment also decreased the high-pressure stiffness by 27.6% (p<0.005) over controls but there was no significant change in the transition strain or the initial creep rate. Compared to the effect seen with buffer-treatment in protocol 1, enzyme-treatment in protocol 2 led to nearly the opposite effects with decreased low-pressure stiffness (-54.5%, p<0.0005), hysteresis (-31.2%, p<0.0001), initial creep rate (-57.5%, p<0.001), and final creep rate (-42.9%, p=0.055), as well as increased high-pressure stiffness (+55.4%, p<0.0005) and transition strain (+36.5%, p<0.0005) over buffer-treated specimens.
The data were then compared between controls and enzyme-treated specimens (Table 3.7), though this comparison must take into account that the enzyme-treatment was performed on eyes that had undergone buffer-treatment first. Compared to controls, enzyme-treated specimens showed a significantly lower low-pressure stiffness in the circumferential (-62.3%, \(p<0.0001\)) and meridional (-61.7%, \(p<0.005\)) directions. They also had a lower high-pressure stiffness circumferentially (-30.7%, \(p<0.0005\)) and higher final creep rate meridionally (+163.9%, \(p<0.05\)). In addition, the transition strain was significantly higher in enzyme-treated than controls in the circumferential (+71.0%, \(p<0.0001\)) and meridional (+111.5%, \(p<0.001\)) directions.

Figure 3.6 shows the average stress-strain and strain-time curves for the 3 experimental groups, for the IN quadrant, in the circumferential direction. The curves for the buffer-treated group were generated with the specimens from protocol 1.
### Table 3.5: Comparison of the mechanical outcomes, averaged over all quadrants, between the control and buffer-treated groups, in the circumferential and meridional directions. Paired Student’s t-tests were used for statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Circumferential</th>
<th>Meridional</th>
<th>Buffer-treated</th>
<th>Control</th>
<th>Buffer-treated</th>
<th>p-value</th>
<th>Control</th>
<th>Buffer-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-pressure stiffness [kPa]</td>
<td>496.3 ± 251.6</td>
<td>637.4 ± 348.1</td>
<td>&lt;0.005</td>
<td>953.0 ± 724.8</td>
<td>1265.5 ± 1192.1</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-pressure stiffness [kPa]</td>
<td>6747.3 ± 2045.9</td>
<td>5244.3 ± 1589.4</td>
<td>&lt;0.0001</td>
<td>5595.2 ± 2604.4</td>
<td>4048.2 ± 1635.4</td>
<td>&lt;0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition strain [mm/mm]</td>
<td>0.028 ± 0.012</td>
<td>0.027 ± 0.013</td>
<td>0.50</td>
<td>0.022 ± 0.016</td>
<td>0.025 ± 0.020</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hysteresis [kPa]</td>
<td>0.18 ± 0.06</td>
<td>0.21 ± 0.08</td>
<td>&lt;0.01</td>
<td>0.18 ± 0.07</td>
<td>0.22 ± 0.10</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial creep rate (first 80 sec) [s⁻¹]</td>
<td>0.033 ± 0.020</td>
<td>0.048 ± 0.021</td>
<td>&lt;0.005</td>
<td>0.033 ± 0.057</td>
<td>0.064 ± 0.078</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final creep rate (after 80 sec) [s⁻¹]</td>
<td>0.062 ± 0.047</td>
<td>0.088 ± 0.040</td>
<td>&lt;0.05</td>
<td>0.041 ± 0.058</td>
<td>0.16 ± 0.16</td>
<td>&lt;0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.6: Comparison of the mechanical outcomes, averaged over all quadrants, between the buffer-treated and enzyme-treated groups, in the circumferential and meridional directions. Paired Student’s t-tests were used for statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Circumferential</th>
<th>Meridional</th>
<th>Buffer-treated</th>
<th>Enzyme-treated</th>
<th>p-value</th>
<th>Buffer-treated</th>
<th>Enzyme-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-pressure stiffness [kPa]</td>
<td>386.2 ± 206.7</td>
<td>187.2 ± 102.4</td>
<td>&lt;0.0001</td>
<td>800.8 ± 759.5</td>
<td>364.7 ± 590.2</td>
<td>&lt;0.0005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-pressure stiffness [kPa]</td>
<td>3798.9 ± 1326.6</td>
<td>4673.5 ± 1677.6</td>
<td>&lt;0.05</td>
<td>2881.1 ± 821.3</td>
<td>4477.1 ± 2131.0</td>
<td>&lt;0.0005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transition strain [mm/mm]</td>
<td>0.038 ± 0.014</td>
<td>0.048 ± 0.017</td>
<td>0.001</td>
<td>0.034 ± 0.022</td>
<td>0.046 ± 0.026</td>
<td>&lt;0.0005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hysteresis [kPa]</td>
<td>0.27 ± 0.10</td>
<td>0.20 ± 0.06</td>
<td>&lt;0.0001</td>
<td>0.29 ± 0.11</td>
<td>0.20 ± 0.08</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial creep rate (first 80 sec) [s⁻¹]</td>
<td>0.041 ± 0.020</td>
<td>0.037 ± 0.019</td>
<td>0.29</td>
<td>0.071 ± 0.079</td>
<td>0.030 ± 0.028</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final creep rate (after 80 sec) [s⁻¹]</td>
<td>0.11 ± 0.12</td>
<td>0.096 ± 0.077</td>
<td>0.66</td>
<td>0.19 ± 0.25</td>
<td>0.11 ± 0.11</td>
<td>0.055</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.7: Comparison of the mechanical outcomes, averaged over all quadrants, between the control and enzyme-treated groups, in the circumferential and meridional directions. Unpaired Student’s t-tests were used for statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Circumferential</th>
<th>Meridional</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Enzyme-treated</td>
</tr>
<tr>
<td>Low-pressure stiffness [kPa]</td>
<td>496.3 ± 251.6</td>
<td>187.2 ± 102.4</td>
</tr>
<tr>
<td>High-pressure stiffness [kPa]</td>
<td>6747.3 ± 2045.9</td>
<td>4673.5 ± 1677.6</td>
</tr>
<tr>
<td>Transition strain [mm/mm]</td>
<td>0.028 ± 0.012</td>
<td>0.048 ± 0.017</td>
</tr>
<tr>
<td>Hysteresis [kPa]</td>
<td>0.18 ± 0.06</td>
<td>0.20 ± 0.06</td>
</tr>
<tr>
<td>Initial creep rate (first 80 sec) [s⁻¹]</td>
<td>0.033 ± 0.020</td>
<td>0.037 ± 0.019</td>
</tr>
<tr>
<td>Final creep rate (after 80 sec) [s⁻¹]</td>
<td>0.062 ± 0.047</td>
<td>0.096 ± 0.077</td>
</tr>
</tbody>
</table>

### Table 3.8: Comparison of the mechanical outcomes, averaged over all quadrants for 2 specimens, between the buffer-treated and enzyme-treated groups with protease inhibitors, in the circumferential and meridional directions. NA = not applicable, as no statistical analysis could be performed on 2 specimens.

<table>
<thead>
<tr>
<th></th>
<th>Circumferential</th>
<th>Meridional</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer-treated with inhibitors</td>
<td>Enzyme-treated with inhibitors</td>
</tr>
<tr>
<td>Low-pressure stiffness [kPa]</td>
<td>404.3</td>
<td>193.4</td>
</tr>
<tr>
<td>High-pressure stiffness [kPa]</td>
<td>3938.2</td>
<td>3907.0</td>
</tr>
<tr>
<td>Transition strain [mm/mm]</td>
<td>0.033</td>
<td>0.039</td>
</tr>
<tr>
<td>Hysteresis [kPa]</td>
<td>0.24</td>
<td>0.18</td>
</tr>
<tr>
<td>Final creep rate (after 80 sec) [s⁻¹]</td>
<td>0.088</td>
<td>0.073</td>
</tr>
</tbody>
</table>
## Table 3.9: Comparison of the absolute values of the shear strains, averaged over all quadrants and pressure levels of the loading regime, between the control and buffer-treated groups, and buffer-treated and enzyme-treated groups. Paired Student’s t-tests were used for statistical analysis.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Figure 3.6: Average stress-strain (a) and strain-time (b) behaviors for the control, buffer-treated and enzyme-treated groups, for the IN quadrant, in the circumferential direction. The curves for the buffer-treated group represent the average responses of the specimens from protocol 1.

The mechanical data were also compared between 2 inflation test sessions performed on specimens after treatment in buffer with protease inhibitors, and after treatment in enzyme solution with inhibitors (Table 3.8). In the circumferential direction, enzyme-treatment with inhibitors decreased the low-pressure stiffness (-52.2%), hysteresis (-26.5%), final creep rate (-17.4%), and increased the transition strain (+19.4%) over specimens treated with buffer containing inhibitors. In the meridional direction, enzyme-treatment with inhibitors on specimens treated with buffer containing inhibitors decreased the low-pressure stiffness (-66.6%), hysteresis (-29.6%), final creep rate (-21.8%), and increased the high-pressure stiffness (+13.4%) and transition strain (+6.3%). No statistical analysis could be performed as only 2 specimens were tested.

Finally, the absolute values of the shear strains were averaged over all quadrants
and pressure levels of the loading regime, and compared between the 2 experimental protocols (Table 3.9). The absolute values of the shear strains were at least one order of magnitude smaller than normal strains. Although statistically non-significant, the average of the absolute values of the shear strains decreased after buffer-treatment on control specimens (-53.6%), and increased after enzyme-treatment on buffer-treated specimens (+28.8%).

### 3.3.2.2 Comparing the circumferential and meridional directions

The 6 mechanical outcomes were also examined for significant differences between the circumferential and meridional directions within each of the 3 conditions, control, buffer-treated and enzyme-treated (Table 3.10). Buffer-treated specimens from protocols 1 and 2 were combined for this analysis. In the control condition, the meridional low-pressure stiffness was 92.0% higher than the circumferential (p<0.005), while the meridional transition strain was 21.4% lower than the circumferential (p<0.05). No significant differences by direction were found for the other mechanical outcomes among controls. The buffer-treated group was similar to the controls in the differences observed, also having a higher low-pressure stiffness (+100.2%, p<0.0001) and lower transition strain (-9.2%, p=0.054) meridionally. In addition, the high-pressure stiffness was 24.2% lower (p<0.0001), initial creep rate 56.2% higher (p<0.05) and final creep rate 79.4% higher (p=0.001) in the meridional direction compared to the
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Buffer-treated</th>
<th>Enzyme-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-pressure stiffness</td>
<td>&lt;0.005</td>
<td>&lt;0.0001</td>
<td>0.09</td>
</tr>
<tr>
<td>High-pressure stiffness</td>
<td>0.13</td>
<td>&lt;0.0001</td>
<td>0.65</td>
</tr>
<tr>
<td>Transition strain</td>
<td>&lt;0.05</td>
<td>0.054</td>
<td>0.75</td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.60</td>
<td>0.55</td>
<td>0.79</td>
</tr>
<tr>
<td>Initial creep rate (first 80 sec)</td>
<td>0.90</td>
<td>&lt;0.05</td>
<td>0.24</td>
</tr>
<tr>
<td>Final creep rate (after 80 sec)</td>
<td>0.39</td>
<td>0.001</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Table 3.10: p-values for the comparison of the mechanical outcomes, averaged over all quadrants, between the circumferential and meridional directions, in the control, buffer-treated and enzyme-treated groups. Paired Student’s t-tests were used for statistical analysis.

circumferential direction. Interestingly, no significant differences in any mechanical outcome were found between the meridional and circumferential directions in the enzyme-treated group.

3.3.2.3 Group/quadrant interaction

The significance of the interaction between quadrant and experimental group for all mechanical outcomes and directions is shown in Table 3.11. The interaction between group and quadrant was significant for the low-pressure stiffness in the circumferential and meridional directions (p<0.05), and for the transition strain (p<0.005) and hysteresis (p=0.01) in the meridional direction only.

3.3.2.4 Comparing the quadrants

To investigate spatial differences, the variability of each mechanical parameter among quadrants was investigated, in the circumferential and meridional directions (Fig. 3.7). For the parameters and directions with a significant interaction between
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

<table>
<thead>
<tr>
<th></th>
<th>Circumferential</th>
<th>Meridional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-pressure stiffness</td>
<td>0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>High-pressure stiffness</td>
<td>0.43</td>
<td>0.19</td>
</tr>
<tr>
<td>Transition strain</td>
<td>0.19</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Hysteresis</td>
<td>0.73</td>
<td>0.01</td>
</tr>
<tr>
<td>Initial creep rate (first 80 sec)</td>
<td>0.20</td>
<td>0.09</td>
</tr>
<tr>
<td>Final creep rate (after 80 sec)</td>
<td>0.99</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Table 3.11: p-values for the interaction between group and quadrant for all mechanical outcomes. Linear mixed models were used for statistical analysis.

group and quadrant, the results were presented for each group separately. Otherwise, all groups were combined. For the transition strain and hysteresis, the interaction between group and quadrant was not significant in the circumferential direction as opposed to the meridional direction, hence the difference in representation. The number of significant differences in the mechanical parameters among quadrants was higher in buffer-treated specimens than controls. However, enzyme-treated specimens showed fewer differences than those treated with buffer alone. Overall, the maximum number of differences occurred between SN/IT and ST/IN, followed by the nasal/temporal and finally the superior/inferior quadrants. The largest changes in the mechanical parameters after buffer- or enzyme-treatment were generally measured in inferior quadrants and the smallest changes in superior quadrants (data not shown here). The quadrants with the lowest and highest parameter value varied with the mechanical parameter and direction as well as group (if applicable). For all mechanical parameters, the difference between the 2 most different quadrants was always larger in the meridional direction than circumferential direction (data not presented here).
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Figure 3.7: Schematic of the spatial variations for all mechanical outcomes, in the circumferential and meridional directions, assessed using linear mixed models. For the mechanical outcomes and directions having a significant interaction between group and quadrant, these variations were presented for each group. Each segment linking 2 quadrants indicates a significant difference between them. The plus and minus signs indicate the quadrants with the highest and lowest parameter value, respectively.
3.4 Discussion

To provide a better understanding of the role that s-GAGs play in the mechanical function of the sclera, the scleral s-GAG content, hydration, thickness, as well as 6 mechanical features of the scleral stress-strain and strain-time behavior were measured before and after treatments that could affect the s-GAGs and scleral function.

GAG staining was a first attempt to evaluate the effect of treatment in buffer alone and in buffer containing ChABC on the scleral s-GAG content. The similar image intensity between control and buffer-treated sclera showed that the buffer itself did not remove s-GAGs, while the lower image intensity for enzyme-treated sclera compared to control and buffer-treated sclera suggested a decrease in the s-GAG content in the tissue following ChABC treatment. However, the s-GAG content could not be quantified with the staining method, which led to the assay method. While the s-GAG content was greater in control superior sclera than inferiorly, the values were generally consistent with those reported by Schultz et al.\[^{86}\] in the posterior porcine sclera and by Boubriak et al.\[^{220}\] in the posterior human sclera. Buffer-treatment did not affect the s-GAG content, but enzyme-treatment reduced the s-GAG content by 73-90%, which was consistent with the GAG staining results. Failure to remove all s-GAGs could be caused by insufficient time for digestion, lack of penetration of enzyme into some scleral areas, or the presence of s-GAGs that were not susceptible to ChABC digestion, such as keratan sulfates.

The ratio of the wet to the dry weight of control sclera was approximately 3
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

to 1, with a slightly higher hydration in the superior nasal region. These data are consistent with those measured in the posterior porcine sclera by Schultz et al.\textsuperscript{86} The control thickness results, showing a larger thickness in superior sclera than inferiorly, are comparable to those of Chen et al.\textsuperscript{221} in the posterior porcine sclera. Buffer-treatment significantly increased hydration over the control values. This most likely resulted from additional water entering in the sclera, rather than a change in the other constituent molecular components. In support of this conclusion, there was no significant change in s-GAGs per dry weight after incubation in buffer alone. It was hypothesized that this increased hydration was in the form of bound and free water, due to the charge and osmotic effects of the s-GAGs (Figure 3.8(a)). The increased thickness in buffer-treated compared to control sclera was similar in magnitude to the percent increase in hydration, again probably due to added water in the wet state of the tissue. Enzyme-treatment led to an additional increase in hydration over buffer-treatment that was 13\% greater than in control sclera. This is particularly interesting given the fact that the s-GAGs were largely removed by the enzyme. A similar increase in hydration from a hydrated swollen state was observed in lungs\textsuperscript{117} and in articular cartilage\textsuperscript{222} after ChABC treatment, in intervertebral discs\textsuperscript{223} after the \textit{in-vivo} injection of ChABC and in corneas after s-GAG depletion following edema\textsuperscript{224} However, previous studies have shown contradictory findings on the effect of s-GAG content on hydration in the human sclera. Boubriak et al.\textsuperscript{220} found no significant change in the scleral hydration following s-GAG degradation with ChABC,
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

whereas Brown et al.\textsuperscript{70} measured a lower hydration with a lower s-GAG content in the anterior sclera. The increase in hydration after s-GAG removal can be explained by distinguishing “bound” and “free” water in a matrix composed of charged polymer chains.\textsuperscript{86} When the polymer chains are abundant and long, which is the case of chondroitin sulfates, they tend to interact with each other creating a tight matrix. The resulting matrix has a large number of polar sites to which water can bind but not much space for free water. However, if a large majority of the polymeric chains is removed, the matrix loses water-binding sites but increases its capacity for free water absorption. This explanation is consistent with the study by Boubriak et al.,\textsuperscript{220} where a higher partition coefficient was measured in the human sclera after s-GAG removal. Therefore, it was hypothesized that bound water was lost after s-GAG removal but that the overall tissue hydration was increased by the intake of more free water, due to the increased free space in the tissue (Figure \textsuperscript{3.8}(b)). The effect of s-GAGs on the hydration should vary greatly depending on the amount, type, and organization of s-GAGs in the tissue relative to collagen fibrils.\textsuperscript{225} Therefore, differences between the results presented here and those reported by Boubriak et al.\textsuperscript{220} might be attributed to differences in species and incubation medium. Similarly, differences between the present results and those reported by Brown et al.\textsuperscript{70} may be caused by differences in species and scleral location, in addition to the fact that GAGs were not enzymatically degraded in their study. The thickness was only mildly increased in enzyme-treated compared to buffer-treated sclera. This suggests that the removal of s-GAGs allowed
the entry of more water in the sclera, but that this was accompanied by a minimal volume increase, as the free water occupied less volume than had the s-GAGs and their associated water molecules.

s-GAG removal had significant effects on the tensile and viscoelastic behavior of the posterior sclera. Moreover, these effects were in the opposite direction than those caused by an increase in hydration with treatment in buffer alone. Buffer-
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

treatment from a control state significantly increased the low-pressure stiffness, final creep rate and hysteresis, but it decreased the high-pressure stiffness. The increased low-pressure stiffness suggests an increased network viscosity. This could be due to a higher number of GAG-associated water molecules that need to reorganize as the collagen fibrils uncrimp at low pressures (Figure 3.9(a)). The lower high-pressure stiffness and faster final creep rate point to an increased lubrication between the collagen fibrils. This could be explained by the increased amount of bound water which forms a lubrication layer between the fibrils as they stretch at high pressures (Figure 3.9(b)). It should be noted that 2 distinct regimes are hypothesized here: one regime where the GAG-associated water molecules are rearranged during fibril uncrimping, and another regime where the bound water provides a lubrication layer between the fibrils as they are being stretched. A faster creep rate following an increase in hydration was also observed in ligaments.\textsuperscript{226} The increased hysteresis could be due to an increased flow-induced dissipation of the increased free water and/or an increased network viscosity from the increased bound water. In comparison, enzyme-treatment from a buffer-treated state significantly decreased the low-pressure stiffness, final creep rate, and hysteresis, but it increased the transition strain and high-pressure stiffness. The combined mechanical effects of s-GAG removal produced a significantly more compliant response of the posterior sclera. The decreased low-pressure stiffness could be explained by a decreased bound water in the tissue or a change in the initial connections of adjacent collagen fibrils. Fibrils initially connected through the
s-GAGs were not linked anymore after s-GAG digestion. The increased transition strain suggests an alteration in the crimp morphology of the collagen fibrils (Figure 3.9(c)). If the fibrils are initially more crimped following s-GAG removal, more strain is needed to straighten and mechanically recruit them. This is consistent with the increased fibril crimp observed in the posterior sclera of experimental myopic eyes where the GAG content is known to decrease. The slower final creep rate in the meridional direction and higher high-pressure stiffness are consistent with an increased interfibrillar friction with s-GAG removal. Although hydration was higher after s-GAG degradation, it was hypothesized that this increased water content was in the form of free water. Free water could easily move out of the tissue during deformation, reducing the lubrication between the collagen fibrils (Figure 3.9(d)). Additionally, less s-GAGs prevented the collagen fibrils from independently sliding past each other. A similar stiffening was observed in the annulus fibrosus upon s-GAG depletion. The decreased hysteresis could be explained by the decreased network viscosity due to the loss of s-GAGs, which prevailed on the higher flow-induced viscosity of the increased free water. In addition, only 2 mechanical parameters showed the same change after enzyme-treatment compared to control and buffer-treated scleras: the low-pressure stiffness decreased and the transition strain increased. Those results indicate that pathological changes in the scleral s-GAG content might significantly contribute to the development of an altered scleral mechanical behavior.

The effect of s-GAG removal on the scleral mechanical behavior is consistent with
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Figure 3.9: Model of the arrangement of the GAG-associated water molecules (a) during fibril uncrimping at low pressures and (b) during fibril stretching at high pressures, in buffer-treated sclera. (c) Model of the increased initial fibril crimp after enzyme-treatment from a buffer-treated state. (d) Model of the displacement of the free water molecules during fibril stretching in enzyme-treated sclera. The collagen fibrils are represented in grey, the s-GAGs in blue and the water molecules in red and white.
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

Experimental and human myopia. The sclera of high myopes\textsuperscript{[166]} and experimental myopia eyes\textsuperscript{[169]} has a reduced s-GAG content and exhibits a more compliant load response\textsuperscript{[82,99,106]} However, the mechanical effect of s-GAG removal is not consistent with the overall stiffer mechanical behavior measured in human\textsuperscript{[8]} and experimental\textsuperscript{[173]} glaucoma, where a change in the GAG content has not been studied. Therefore, a decrease in the GAG content might not occur in the posterior sclera of glaucoma eyes or if it does, additional microstructural features might inhibit its softening mechanical effect. Current studies are investigating the remodeling of the collagen structure as the source of altered scleral mechanical behavior observed in glaucoma and myopia. The findings of this study suggest that alterations in the s-GAG content may be an additional significant contributor to the altered mechanical behavior observed in those pathologies.

Looking at the mechanical differences between the circumferential and meridional directions, the most interesting feature was that enzyme-treatment dramatically diminished the mechanical differences between the circumferential and meridional directions observed in the control and buffer-treated groups. A similar phenomenon was reported in aortic valve cusps, where the significant difference in the relaxation constant between the circumferential and radial directions disappeared after ChABC treatment\textsuperscript{[229]} Given that s-GAGs are attached to collagen fibrils through the PG core protein and that they can directly interact with their adjacent fibrils and s-GAGs, it was speculated that the s-GAG arrangement is either anisotropic or enhances the
anisotropy of the collagen fibril arrangement or connectivity.

The same phenomenon was observed when comparing the mechanical differences among the quadrants. Differences among quadrants increased with buffer-treatment from a control state but decreased with enzyme-treatment from a buffer-treated state. These observations are consistent with the result that the s-GAG content was significantly different among quadrants in the control sclera. Therefore, these regional differences in the s-GAG content induced different degrees of swelling upon incubation in buffer alone, thus increasing the regional differences in the mechanical behavior. Moreover, the s-GAG content became more uniform among quadrants after incubation in enzyme solution, thus fewer regional differences in the mechanical behavior were measured. This suggests that the spatial heterogeneity of s-GAG organization contributes to that of the scleral mechanical behavior. More differences in the mechanical parameters were measured between SN/IT and ST/IN, than between neighboring quadrants. This is consistent with the fact that the thickness and s-GAG content varied gradually from one quadrant to the other, making neighboring quadrants more similar than opposite quadrants. The largest changes in mechanical outcomes usually occurred in the inferior quadrants. This suggests that while inferior quadrants had lower s-GAG content, they were more susceptible to s-GAG removal than superior quadrants.

Limitations of this study include the absence of direct mechanical comparison between control and enzyme-treated sclera. The choice of comparing the mechanical
behavior 1) before treatment (control) and after incubation in buffer alone; and 2) after incubation in buffer and after incubation in ChABC solution, was made to distinguish the effect of incubation in buffer alone, and therefore that of an increase in hydration, from that of s-GAG removal. Additional limitations include the difficulty in obtaining specimens with consistent levels of hydration because of variability in the eye collection procedure, transport, and delivery schedules from week to week. In addition, a higher 3D-DIC correlation noise was observed in scleral areas containing ciliary arteries and vortex veins, which are identified by a darker appearance. These areas are a small proportion of the sclera and their effect was minimized by averaging the strains over a whole quadrant of the sclera. The sclera is known to exhibit large local variations in strains during inflation testing and averaging the strains over a large region does not allow the measurement of the effects of s-GAG removal on local variations of the mechanical behavior. The spherical thin-walled membrane model was used for the stress analysis. The model assumes a constant thickness meridionally, but the scleral thickness can vary significantly along a meridian. The effect of thickness variation in the stress analysis was mitigated by considering a region only 3 mm wide meridionally, over which the variation in thickness was assumed to be small. s-GAGs were degraded with ChABC C2905 from Sigma-Aldrich, which is not guaranteed to be protease-free though the contamination is low (Sigma communication). Harrisson et al. showed that the proteolytic activity of the non protease-free ChABC from Sigma-Aldrich is non-detectable at low concentrations such as the one
CHAPTER 3. ALTERATIONS IN THE POSTERIOR SCLERA FOLLOWING GLYCOSAMINOGLYCAN DEGRADATION: A PORCINE EXPERIMENTAL STUDY

used in this study. A number of studies have used non protease-free ChABC\textsuperscript{119,232–234} and did not find larger effects on the tissue mechanical behavior, compared to studies that used protease-free ChABC\textsuperscript{113,116,117} or non protease-free ChABC with protease inhibitors.\textsuperscript{112,115,235} Specifically, they showed no significant difference in the mechanical behavior upon prolonged incubation in the non protease-free enzyme. This suggests that the impurities themselves do not degrade structural components of the tissues. To confirm that the impurities present in the ChABC used in this study did not affect the mechanical outcomes, a few scleral shells were tested after incubation in buffer with inhibitors and after incubation in buffer with ChABC plus inhibitors. The results showed the same mechanical changes as those observed when comparing buffer-treated and enzyme-treated scleras without inhibitors, confirming that s-GAG degradation is responsible for the observed scleral mechanical changes. The 3D-DIC dynamic uncertainties, 0.05\% strain in the circumferential direction and 0.07\% strain in the meridional direction (see chapter \textsuperscript{2}), were within the 0.03-0.08\% strain standard deviation range reported by Sutton et al.\textsuperscript{186} Details about the 3D-DIC errors and uncertainties in this study, as well as the methodology used to calculate them, can be found in chapter \textsuperscript{2}. 
3.5 Conclusion

In conclusion, the effects of s-GAG removal on the structure and mechanical behavior of the posterior porcine sclera were evaluated and significant differences were found. The main findings were:

1) s-GAG removal increased the scleral hydration.

2) s-GAG removal significantly affected both the viscoelastic and tensile behavior of the posterior sclera, in a way opposite from that of an increase in hydration, and led to an overall more compliant inflation response.

3) s-GAG removal decreased the differences in the mechanical behavior between the circumferential and meridional directions and among the 4 quadrants of the buffer-treated sclera.

In this study, s-GAGs were found to represent on average only 0.6% of the dry weight of the posterior porcine sclera. s-GAGs are often seen as compliant molecules that do not contribute to the mechanical behavior of connective tissues. However, the results showed that s-GAGs had significant effects on the mechanical behavior of the tissue, most likely through their interaction with collagen fibrils and ability to bind water, thus preventing it from flowing through the tissue under load. The results also suggest that alterations to the s-GAG content may contribute to the differences in the mechanical behavior measured in glaucoma and myopia and may play an important role in the development of those ocular pathologies.
Chapter 4

Alterations in the posterior sclera after glycosaminoglycan degradation: a human experimental study

This chapter characterizes the structural and mechanical changes after experimental digestion of s-GAGs in the posterior sclera of human eyes, using ultrasound thickness measurements and inflation testing with 3D-DIC. Each scleral specimen was first incubated in a buffer solution to return to full hydration, inflation tested, treated in a buffer solution containing ChABC, then inflation tested again. The human sclera being much stiffer than the pig sclera, the equipment setup and data analysis from
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

chapter 3 were improved to accommodate for the small displacements measured. This chapter has been submitted to the *Journal of the Royal Society Interface* under the title “The contribution of glycosaminoglycans to the mechanical behavior of the posterior human sclera”, with Michelle L. Chen, Harry A. Quigley and Thao D. Nguyen as co-authors. The strain calculation described in this chapter was developed by Michelle L. Chen.

4.1 Introduction

The mechanical properties of the sclera has been shown to play an important role in the initiation and development of ocular diseases such as glaucoma and myopia in both experimental and modeling analyses. Glaucoma is the second leading cause of blindness worldwide\(^{135}\) and is characterized by the dysfunction\(^{137,138}\) and loss\(^{139,140}\) of the axons of the RGCs at the ONH.\(^{141}\) IOP can produce a high level of stresses and strains in the tissues of the ONH, depending on the mechanical properties of the sclera. This mechanical insult can potentially cause direct damage to the RGC axons, and/or lead to poor vascular nutrition and the activation of astrocytes. These events can occur at any level of IOP, whether higher or lower than that found in non-glaucoma eyes.\(^{144–147}\) Myopia is a common refractive error often characterized by the axial elongation of the eye wall.\(^{236}\) Patients with high myopia are at increased risk for several blinding diseases, such as macular degeneration\(^{155}\) and glaucoma.\(^{156}\) Elon-
ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

The tensile and viscoelastic behavior of the posterior sclera is altered in glaucoma and myopia. The peripapillary sclera of human glaucomatous eyes has a lower creep rate and higher stiffness compared to that of normal eyes. In a monkey model of glaucoma, the posterior sclera also exhibits a higher stiffness and the peripapillary sclera shows a higher equilibrium modulus after stress relaxation following a rapid deformation, but no change in the time-dependent parameters compared to that of normal eyes. The posterior sclera of human myopic eyes reaches a higher strain at maximum pressure than that of normal eyes. In a tree shrew model of myopia, the posterior sclera also shows a greater strain at the peak pressure as well as a higher creep rate but a similar high-pressure elastic modulus than those of normal eyes.

Collagen represents 80% of the dry weight of the sclera whereas elastin fibers and PG core proteins represent 2% of its dry weight. In comparison, GAGs represent only 0.5 to 1% of the scleral dry weight. GAGs are assemblies of disaccharide units into chains of various lengths, most of them attached to a core protein to form PGs. The most abundant GAGs in the posterior sclera are chondroitin sulfates and dermatan sulfates. GAGs attract and retain water through their negative ionic charges as well as their osmotic pressure. They also directly interact with collagen fibrils through electrostatic interactions and have been...
hypothesized to regulate collagen fibril spacing through hydration (swelling),\textsuperscript{56,77,78} GAG-GAG charge repulsion\textsuperscript{79} and GAG-GAG antiparallel interactions.\textsuperscript{69} The changes in the mechanical properties of the posterior sclera are associated with specific changes in the collagen and elastin structure in glaucoma\textsuperscript{9,24,29,161} and myopia.\textsuperscript{166,168,169} In addition, alterations in the s-GAG content have been reported in glaucoma and myopia. There is currently no data on the change in GAG content in the posterior sclera in glaucoma. However, a higher s-GAG content was reported in the iris\textsuperscript{162} and lamina cribrosa\textsuperscript{165} of human glaucomatous eyes, which was consistent with the findings of studies on animal models of glaucoma.\textsuperscript{163,164} A decrease in the overall GAG content was found in the posterior sclera of human myopic eyes,\textsuperscript{106} which was consistent with the results obtained with animal models of myopia.\textsuperscript{169} The mechanical role of GAGs is very debated in the literature. The effect of GAG degradation seems to be tissue-dependent and inconsistent results have even been reported for the same tissue. Studies have measured changes in the viscoelastic behavior of cartilage,\textsuperscript{112} aorta,\textsuperscript{109} and heart valve leaflet\textsuperscript{113} following GAG removal, while others have observed no change in the viscoelasticity of ligaments.\textsuperscript{114,115} The stiffness increased in arteries,\textsuperscript{116} lungs,\textsuperscript{117} ligaments,\textsuperscript{108} and aorta in the small strain range with GAG digestion. GAG degradation decreased the stiffness of the aorta in the high strain range,\textsuperscript{109} but did not affect the stiffness of cartilage,\textsuperscript{112} tendons,\textsuperscript{119} and ligaments in a different study.\textsuperscript{118} In chapter 3, the effects of s-GAG removal on the mechanical behavior of the posterior porcine sclera were studied and changes in
both the tissue tensile and viscoelastic behavior were measured. s-GAG degradation with ChABC from a hydrated state significantly decreased the scleral stiffness at low pressures, the hysteresis and the final creep rate, while increasing the stiffness at high pressures and the strain at the onset of strain stiffening (i.e. length of the toe region). Those changes were found to be opposite to the changes observed from an increase in hydration from a baseline (control) to a hydrated state. In addition, differences in the inflation response between the meridional and circumferential directions were significantly reduced after enzymatic s-GAG degradation. These changes in both the tensile and viscoelastic behavior showed that s-GAGs play an important role in the mechanical behavior of the posterior sclera. Specifically, the observed findings were interpreted to exceed the effects of hydration alone and suggested an interactive effect on the collagen fibrils.

In this study, human posterior scleras were inflated after incubation in buffer alone and after treatment in buffer containing ChABC to degrade the s-GAGs. The inflation behavior for each specimen was compared before and after treatment with ChABC. The results were used to evaluate whether s-GAG degradation induced changes in the mechanical response of the posterior human sclera and whether the changes in humans were comparable to those measured in pigs in chapter 3. The findings were interpreted for their significance to the mechanical and pathological contribution of s-GAGs to the scleral mechanical behavior in glaucoma and myopia.
4.2 Methods

4.2.1 Specimens and glycosaminoglycan degradation

Eleven human donor eyes were obtained from the National Disease Research Inter-
change (NDRI) from 6 donors with an average age of 74.0 ± 9 years, whose medical
records showed no history of glaucoma (Table 4.1). The medical records did not
provide information about myopia. All structural and mechanical outcomes were
measured in the 4 anatomical quadrants of the posterior scleral shell (superior-nasal
(SN), superior-temporal (ST), inferior-nasal (IN), inferior-temporal (IT)), outside the
peripapillary region. To confirm the effectiveness of the s-GAG degradation protocol
of chapter 3, the s-GAG content, in µg per mg wet tissue weight, was first deter-
mined in 2 samples taken in each of the 4 quadrants of a single eye. Both samples
from each quadrant were divided into 2 experimental groups (Fig. 4.2). In the first
group, the specimens were incubated for 18 h at 37°C in a modified Trizma buffer
at pH 8.0 (buffer-treated). In the second group, the specimens were incubated for
18 h at 37°C in Trizma buffer containing ChABC (C2905, Sigma-Aldrich, St. Louis
MO) at 2 units/ml (enzyme-treated). ChABC is known to specifically degrade chon-
droitin and dermatan sulfates at pH 8.0. The s-GAG content in µg per mg dry tissue
weight was then inferred from hydration measurements on 2 samples taken in each
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-GAG quantification</td>
<td>n=1*/1</td>
</tr>
<tr>
<td>Hydration</td>
<td>n=1*/1</td>
</tr>
<tr>
<td>Mechanical testing</td>
<td>n=9#/5</td>
</tr>
<tr>
<td>Thickness measurement</td>
<td>n=9#/5</td>
</tr>
</tbody>
</table>

**Table 4.1:** Number of specimens used for s-GAG quantification, hydration measurement, mechanical testing and thickness measurements. 1/1 indicates that 1 eye was used from 1 donor, 9/5 indicates that 9 eyes were used from 5 donors, * denotes that both eyes were from the same pair and # denotes that the same eyes were used for both experiments.

quadrant of the contralateral eye, and each one incubated in either buffer alone or buffer containing ChABC (Fig. 4.2). The remaining 9 eyes were inflation tested after incubation for 18 h in buffer alone (buffer-treated) followed by incubation for 18 h in the enzyme solution (enzyme-treated). Scleral thicknesses were measured at the end of each inflation test. This work followed the tenets of the Declaration of Helsinki. Details about the s-GAG digestion and hydration measurement protocols can be found in sections A.2.1 and A.2.4 of the Appendix.

### 4.2.2 Specimen preparation

The eyes were received in a closed container, on a gauze pad soaked with saline solution to reduce dehydration, and used within 48 h post-mortem. The extraocular fat and muscles were removed, leaving a clean scleral surface. For 2 eyes, a 8 mm biopsy punch was used to excise samples from each quadrant for s-GAG quantification and hydration measurements (Fig. 4.2). The samples were punched 2 mm away from the ONH and away from visible arteries and veins. Two 3x3 mm square samples were
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

Figure 4.1: (a) Side view and (b) top view of the experimental setup showing the inflation chamber, pressure transducer, MTS, hydration chamber, as well as the imaging and lighting systems.

then cut from each disc, from which the retina and choroid were gently removed (Fig. 4.2).

The eyes used for mechanical testing and thickness measurements were glued to a custom-made acrylic holder centered about the ONH using cyanoacrylate (Permabond 910, Electron Microscopy Sciences, Hatfield PA) (Fig. 4.3(a)). The anterior sclera was cut away and the intraocular structures, including the retina and choroid, were removed from the remaining posterior scleral shell. Details about the holder design can be found in section A.1.1 of the Appendix.
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

4.2.3 Glycosaminoglycan quantification

The protocol for s-GAG content quantification was previously described in chapter 3. Briefly, the s-GAG content was assessed on 3x3 mm samples, taken in each of the 4 quadrants of a single eye (Fig. 4.2) and subjected to either buffer-treatment or enzyme-treatment, using the Blyscan assay (Accurate Chemical & Scientific Corporation, Westbury NY). The samples were first blotted dry on Whatman paper for 1 min, weighed wet and incubated in a solution of Papain (P3125, Sigma-Aldrich, St. Louis MO) for 18 h at 60°C, following Boubriak et al. protocol. The s-GAG content per dry tissue weight in each quadrant was inferred using hydration measurements of the wet over dry sample weight ratio. The wet sample weight was measured after blotting the samples dry on Whatman paper for 1 min using a precision balance (XP26DR, Mettler-Toledo LLC, Columbus OH). The wet samples were weighed inside pre-weighed eppendorf tubes to prevent further evaporation. The samples were then dehydrated by incubation for 48 hours in an oven at 60°C and weighed in the same manner. Details about s-GAG quantification protocol can be found in section A.2.3 of the Appendix.

4.2.4 Mechanical testing

For inflation testing, the specimens that were glued to the custom plastic holder were then clamped to a custom inflation chamber (Fig. 4.1(a) and (b)). Dulbecco’s
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

**Figure 4.2:** Schematic of the human posterior sclera protruding from the holder showing the ONH as well as the locations of the samples used for the s-GAG quantification and hydration, the locations of the thickness measurements, and the regions where the strains were averaged. The letter B indicates the samples incubated in buffer alone, while the letter E indicates samples treated with the enzyme. For the s-GAG quantification and hydration measurement, samples from the same eye were subjected to each condition, while for the mechanical testing the specimens were subsequently treated with the buffer alone and then with the enzyme solution.

Phosphate-buffered saline (DPBS) was injected into the chamber using an MTS-actuated syringe (MTS, Eden Prairie MN) (Fig. 4.1(a)). The pressure inside the chamber was monitored using a pressure transducer (TJE, 2 psig range, Honeywell, Morristown NJ) and fed back into the MTS testing machine to control the motion of the MTS crosshead (MTS, Eden Prairie MN) using a PID controller (Fig. 4.1(a) and (b)). The specimens were first equilibrated at the baseline pressure 0.21 kPa for 30 min, then subjected to a load-unload cycle from baseline pressure to 6 kPa at 0.13 kPa/s, followed by a 30 min recovery period at baseline pressure. The baseline pressure was then maintained for the thickness measurements. Preconditioning cycles were not performed. A previous study showed that the effects of preconditioning are negligible for repeated inflation testing of the posterior sclera. \(^{125}\) The inflation
chamber was enclosed in a clear perspex humidity chamber with 90% humidity to prevent dehydration of the specimens during testing (Fig. 4.1(a) and (b)).

4.2.5 Thickness measurement

The scleral thickness was measured at 8 locations, 2 in each of the 4 quadrants of the eyes (Fig. 4.2) using an 15 MHz ultrasound transducer (V26045, Olympus NDT Inc., Waltham MA) fitted with 1.5 mm diameter Sonopen tip (DLP-302, Olympus NDT Inc., Waltham MA), as described in chapter 3. The ultrasound transducer was linked to a pulser-receiver (5073PR-15-U, Olympus NDT Inc., Waltham MA) and the echoes from the outer and inner scleral surfaces were recorded from an oscilloscope (TDS220, Tektronix Inc., Beaverton OR). The scleral thickness $T$ was calculated as:

$$T = \frac{1}{2}c_{\text{sclera}}\Delta t$$

(4.1)

where $c_{\text{sclera}} = 1597 \text{ m/s}$ is the speed of sound in the sclera and $\Delta t$ is the time difference between the peaks of the outer and inner sclera echoes. The thickness in each quadrant was reported as the average of both measurements. Details about the thickness measurement apparatus and calculations can be found in section A.1.3 of the Appendix.
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

4.2.6 Digital image correlation

The posterior sclera was speckled with black India ink (Fig. 4.3(a)) using an airbrush (ECL4500 HP-CS, Iwata Medea Inc., Portland OR) prior to mechanical testing to enhance the contrast for the 3D surface displacement measurements using 3D-DIC. Images were acquired using a stereoscopic system equipped with 2 monochrome cameras with a 2 Mpixel resolution (GRAS-20S4M-C, Point Grey, Richmond BC, Canada), 26° stereo angle, and objectives with a 35 mm focal length (Xenoplan 1.9/35mm-0901, Schneider Optics, Hauppauge NY) fitted with 5 mm long extension tubes (54-628, Edmund Optics, Barrington NJ). Illumination was provided by circline light bulb (90922L, Commercial Electric, Cleveland OH) to increase image contrast and maintain even lighting across the specimen. Images with a 0.018 mm/pixel resolution were captured every 2 seconds during mechanical testing with Vic-Snap 2009 (Correlated Solutions Inc., Columbia SC) and imported into Vic3D 2012 (Correlated Solutions Inc., Columbia SC) for 3D-DIC analysis.

4.2.7 Strain calculation

For each correlated point at every pressure increment, 3D-DIC provided the 3D surface displacements \((u_x, u_y, u_z)\), from the reference geometry at the baseline pressure, on a 2D Cartesian grid \((e_x, e_y)\) with a 0.090 mm resolution (Fig. 4.3(a)). However, given the geometry of the sclera, strains were calculated along circumferential
and meridional directions, \( e_\theta \) and \( e_\phi \), respectively (Fig. 4.3(a) and (b)). To define the new circumferential-meridional coordinate system, the center of the ONH base was identified as the pole. The separation of the ONH region from the scleral region was found by an iterative least-squares fitting process. A sphere was fitted to the reference position of the posterior scleral shell, and the points with 3% error from the spherical fit radius were removed until the fitting error converged. The remaining points corresponded to the scleral shell over which the strains were calculated, while the points removed corresponded to the ONH. An oblique cylinder was then fitted to the ONH region to determine the center of the ONH (Fig. 4.3(b)).

Surface strains were calculated over triangular domains formed by 3 nearest neighbor points with average grid spacing of 0.10 mm, as described in Genovese et al.237 Triangular domains were constructed using the Delaunay triangulation algorithm in Matlab, and were sufficiently small to be assumed planar. The meridional direction \( e_\phi \) was defined for each triangle by projecting the vector \( \mathbf{v}^{MN} \) connecting the triangle centroid M to the ONH center N onto the plane of the triangular domain. The circumferential direction \( e_\theta \) was calculated as the cross product between the meridional direction and the outward unit normal of the triangular plane \( \mathbf{e}_n \) (Fig. 4.3(b)).

Unit orientation vectors were calculated for the sides of the triangle using the reference positions and transformed to a local spherical coordinate system of the triangle to give \( (\mathbf{n}^{AB}_{\text{sph}}, \mathbf{n}^{BC}_{\text{sph}}, \mathbf{n}^{CA}_{\text{sph}}) \) (Fig. 4.3(b)). The stretches of the sides of the triangular domain \( (\lambda^{AB}, \lambda^{BC}, \lambda^{CA}) \) were calculated using the reference positions
and displacement vectors. They were used to calculate the components of the right Cauchy-Green stretch tensor $C_{θθ}$, $C_{φφ}$ and $C_{θφ}$ using the following system of equations:

\[
\begin{aligned}
(λ^{AB})^2 &= n_{sph}^{AB} \cdot C_{sph}^{AB} \\
(λ^{BC})^2 &= n_{sph}^{BC} \cdot C_{sph}^{BC} \\
(λ^{CA})^2 &= n_{sph}^{CA} \cdot C_{sph}^{CA}
\end{aligned}
\] (4.2)

The Green-Lagrange strains in the circumferential and meridional directions were evaluated as $E_{θθ} = 0.5(C_{θθ} - 1)$ and $E_{φφ} = 0.5(C_{φφ} - 1)$. The shear strain was $E_{θφ} = 0.5(C_{θφ})$. The strain components were reported for each quadrant as an average over a 3 mm region in the meridional direction, spanning the entire quadrant and located 3 mm away from the ONH.

### 4.2.8 Hoop stress calculation

The hoop stresses $σ_{θθ}$ in the circumferential direction and $σ_{φφ}$ in the meridional direction were reported at each pressure level for each quadrant as:

\[
σ_{θθ} = σ_{φφ} = \frac{Pr}{2T}
\] (4.3)

where $P$ is the pressure, $r$ is the radius of the shell obtained by fitting a sphere to the 3D-DIC reference position data and $T$ is the scleral thickness in a particular quadrant, averaged over 2 measurements. All 3 parameters were specific to the specimen.
Figure 4.3: (a) Image of a left human scleral shell mounted on a holder and speckled for 3D-DIC tracking, showing the eye anatomical quadrants and directions. (b) Schematic showing how the local basis was defined for each Delaunay triangle. For each triangle, the vector $\mathbf{v}_{MN}$ was defined from the centroid of the triangle M to the center of the ONH base N. This vector was then projected onto the plane of the triangle and normalized to find the meridional direction $\mathbf{e}_\phi$. The circumferential direction $\mathbf{e}_\theta$ was defined as the cross product $\mathbf{e}_\phi \times \mathbf{e}_n$, where $\mathbf{e}_n$ is the outward normal unit vector to the plane of the triangle. $(\mathbf{n}_{sph}^{AB}, \mathbf{n}_{sph}^{BC}, \mathbf{n}_{sph}^{CA})$ represent the unit orientation vectors for the sides of the triangle in the local spherical coordinate system of the triangle.
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

treatment state.

4.2.9 Mechanical data analysis

Four mechanical outcomes were extracted from the stress-strain curves for each scleral shell, quadrant and experimental condition: buffer-treated and enzyme-treated. The low-pressure stiffness was defined as the slope at low pressure calculated by fitting the data points of the loading curves that were within the first 25% of the strain at maximum pressure. The high-pressure stiffness was defined as the slope at high pressure calculated from the fit of the last 30% of the data points of the loading curves. The hysteresis was defined as the area between the loading and unloading curves, calculated by numerical integration using a trapezoidal rule. Outliers were locally treated using the built-in Matlab function smooth with the rlowess and rloess methods for the loading and unloading curves, respectively. The function rlowess performs a local linear interpolation, while rloess applies a second-order polynomial local interpolation. Different smoothing methods were used because the loading stress-strain curves for all the specimen were nearly linear, while the unloading curves were significantly more nonlinear (Figs. 4.4 and 4.5). Since the loading curves were linear and did not show a clear transition between the toe and linear regions, the strain at maximum pressure rather than the strain at the onset of strain stiffening was extracted as a measure of the tissue extensibility at the maximum pressure.
4.2.10 Statistical analysis

To evaluate the effect of treatment with the enzyme from a buffer-treated state on the mechanical outcomes and thickness data, a repeated measures ANOVA was implemented with the built-in Matlab function *rmanova*, using the data for all eyes and quadrants. Repeated measures ANOVA not only indicated whether the mechanical outcomes were different between the experimental groups or between the quadrants, but also if there was an interaction between group and quadrant. Similarly, a repeated measures ANOVA was used to evaluate the difference in mechanical outcomes between the circumferential and meridional directions in both the buffer-treated and enzyme-treated groups.

4.3 Results

4.3.1 Glycosaminoglycan degradation

The s-GAG content in all 4 quadrants of a single eye for samples subjected to either buffer-treatment or enzyme-treatment are shown in Table 4.2. The decrease in s-GAG content after treatment with the enzyme was >98% in all quadrants. No statistics were performed as a single specimen was studied only to confirm the s-GAG degradation protocol previously developed for pig scleras in chapter 3 was as effective in humans.
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

<table>
<thead>
<tr>
<th></th>
<th>ST</th>
<th>SN</th>
<th>IT</th>
<th>IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer-treated</td>
<td>5.29</td>
<td>5.87</td>
<td>6.47</td>
<td>3.22</td>
</tr>
<tr>
<td>Enzyme-treated</td>
<td>0.074</td>
<td>0.066</td>
<td>0.026</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Table 4.2: s-GAG content [µg/mg dry tissue weight] in each quadrant of a single eye, for samples in the buffer-treated and enzyme-treated groups. No statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Buffer-treated</th>
<th>Enzyme-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness</td>
<td>0.92 ± 0.26</td>
<td>0.80 ± 0.18</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.3: Thickness [mm] averaged over all specimens and quadrants in the buffer-treated and enzyme-treated groups. Repeated measures ANOVA used for the statistical analysis.

4.3.2 Thickness data

The thickness averaged over all eyes and quadrants after treatment with buffer alone and after treatment with enzyme are presented in Table 4.3. The average thickness from the buffer-treated state decreased by 13.3% with enzyme-treatment (p<0.001). There was no interaction between experimental group and quadrant. No statistically significant spatial differences were detected in the thickness measurements.

4.3.3 Mechanical behavior

4.3.3.1 Comparing the buffer-treated and enzyme-treated groups

First, the mechanical outcomes averaged over the 4 quadrants and specimens were compared after buffer-treatment and after enzyme-treatment (Table 4.4). In
the circumferential direction, treatment with the enzyme from a buffer-treated state increased the low-pressure stiffness by 30.3% (p<0.005), high-pressure stiffness by 11.0% (p<0.05) and hysteresis by 41.6% (p<0.001). In the meridional direction, the low-pressure stiffness increased by 24.2% (p<0.05), the high-pressure stiffness by 11.4% (p<0.05) and the hysteresis by 16.5% (p=0.28) after enzyme-treatment. Treatment with the enzyme had no statistically significant effect on the strain at maximum pressure. There was no interaction between experimental group and quadrant for any of the mechanical outcomes. In addition, no statistically significant spatial differences were detected in any of the mechanical outcomes measured.
### Table 4.4: Comparison of the mechanical outcomes, averaged over all quadrants, between the buffer-treated and enzyme-treated groups, in the circumferential and meridional directions. Repeated measures ANOVA used for the statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Buffer-treated</th>
<th>Enzyme-treated</th>
<th>p-value</th>
<th>Buffer-treated</th>
<th>Enzyme-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-pressure stiffness [kPa]</td>
<td>7331.1 ± 3478.8</td>
<td>9552.2 ± 5345.2</td>
<td>&lt;0.005</td>
<td>4641.1 ± 3376.0</td>
<td>5763.0 ± 2828.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>High-pressure stiffness [kPa]</td>
<td>15544.2 ± 6172.2</td>
<td>17254.4 ± 5510.9</td>
<td>&lt;0.05</td>
<td>6819.4 ± 4031.7</td>
<td>7599.1 ± 4739.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Hysteresis [kPa]</td>
<td>0.030 ± 0.016</td>
<td>0.043 ± 0.019</td>
<td>&lt;0.001</td>
<td>0.120 ± 0.129</td>
<td>0.140 ± 0.088</td>
<td>0.28</td>
</tr>
<tr>
<td>Strain at max pressure [mm/mm]</td>
<td>0.0038 ± 9.77e-4</td>
<td>0.0038 ± 8.96e-4</td>
<td>1.0</td>
<td>0.010 ± 0.0046</td>
<td>0.0090 ± 0.0032</td>
<td>0.10</td>
</tr>
</tbody>
</table>
Figures 4.4 and 4.5 show the stress-strain curves for all human specimens in the circumferential and meridional direction, respectively, after buffer-treatment and after enzyme-treatment.

4.3.3.2 Comparing the circumferential and meridional directions

The differences between the mechanical outcomes in the circumferential and meridional directions, averaged over all specimens and quadrants, were also compared after incubation in buffer alone and after incubation with the enzyme. In both the buffer-treated and enzyme-treated groups, the high-pressure stiffness, hysteresis and strain at maximum pressure were significantly different in the circumferential and meridional directions (p<0.001 for all mechanical outcomes and experimental groups). In the buffer-treated group, the circumferential low-pressure stiffness was 58.0% higher and its high-pressure stiffness was 127.9% larger than meridionally. Furthermore, the circumferential strain at maximum pressure was 62.1% smaller and its hysteresis was 74.7% lower than meridionally. These findings were similar in the enzyme-treated group, with the circumferential low-pressure stiffness 65.8% higher, high-pressure stiffness 127.1% larger, strain at maximum pressure 57.6% smaller, and hysteresis 69.3% lower than in the meridional direction.
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

Figure 4.4: Stress-strain curves in the circumferential direction for all human specimens, averaged over the 4 anatomical quadrants, after buffer-treatment and after enzyme-treatment. The outliers were locally treated with the Matlab function smooth.
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

Figure 4.5: Stress-strain curves in the meridional direction for all human specimens, averaged over the 4 anatomical quadrants, after buffer-treatment and after enzyme-treatment. The outliers were locally treated with the Matlab function *smooth*. 
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

4.4 Discussion

To better understand the role of s-GAGs in the tensile properties of the posterior human sclera, the scleral thickness and mechanical behavior under inflation were compared after buffer hydration with a s-GAG depleted state after treatment with ChABC. This comparison provided a better understanding of the role of the s-GAGs in the tensile properties of the posterior human sclera.

The s-GAG content of the hydrated sclera reported here is similar to that previously reported in the posterior sclera of human eyes and pig eyes (chapter 3). Schultz et al. also reported a similar s-GAG content in the posterior sclera of pig and human eyes. The degradation procedure used here for the human sclera removed nearly all s-GAGs, as it had in the porcine sclera. The minimal remaining s-GAGs quantified after enzyme-treatment could represent s-GAGs that are: 1) not sensitive to ChABC, such as keratan sulfates, 2) incompletely degraded but normally sensitive to ChABC, or 3) degraded but incompletely removed from the tissue.

The human scleral thicknesses in this investigation were comparable to those reported in other studies. Interestingly, the scleral thickness significantly decreased after enzyme-treatment from a buffer-treated state in human eyes, whereas it increased in pig eyes as measured in chapter 3. The opposite change in scleral thickness following s-GAG degradation between humans and pigs could result from several features of their scleral content. The decrease in thickness in the human sclera after enzyme-treatment could result from an overall loss of water with s-GAGs degrada-
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

The amount and type of negative ionic charges determine the scleral capacity for water absorption through charge and osmotic effects. However, the water absorption capacity of the tissue is also limited by the collagen interfibrillar spacing, itself governed by GAG-GAG charge repulsion and GAG-GAG anti-parallel interactions. Although the pig and human sclera have a similar s-GAG content as reported in this work and by Schultz et al., the s-GAGs remaining in the human sclera after enzyme-treatment might carry fewer negative charges. This could result in a weaker repulsion between the s-GAGs, a decreased interfibrillar spacing and a smaller space for free water to enter the sclera. Enzymatic digestion of chondroitin sulfates was shown to reduce swelling in the cornea and to reduce the “re-expansion” capability of collagen fibrils in dentin upon rehydration. Therefore, the amount of bound water lost after s-GAG removal might not be fully replaced by free water in the human sclera. Additionally, the human sclera has 53% less collagen per dry tissue weight but 1.6 times more collagen non-enzymatic “glycation-type” crosslinks than the pig sclera. This is likely due to the older age of the human donors compared to the pigs. A higher collagen crosslink density could lead to a stiffer extracellular matrix, and therefore an increased resistance to swelling and to the intake of more free water. However, no significant decrease in hydration was measured in the hydrated human posterior sclera after s-GAG removal with ChABC, suggesting that a microstructural rearrangement could explain the decrease in thickness observed in the human sclera. The removal of s-GAG leads to the fusion and thinning of collagen fibrils in
cartilage and in decorin and biglycan deficient mice. Such microstructural rearrangement could lead to a smaller tissue thickness without necessarily involving a decrease in hydration. Therefore, 2 processes may underlie the decrease in thickness measured in the human sclera after enzyme-treatment: 1) a lower hydration due to the partial replacement of the bound water lost after s-GAG digestion by free water, and/or 2) a microstructure rearrangement such as the fusion or thinning of the collagen fibrils (Figure 4.6).

Enzyme-treatment from a buffer-treated state significantly increased the low-pressure stiffness, high-pressure stiffness and hysteresis. The combined effect was a significantly stiffer mechanical response of the posterior sclera after s-GAG degradation, though the effect was modest. The increased low-pressure stiffness could be due to a lower hydration and/or the fusion of the collagen fibrils following the loss of s-GAGs. The increased high-pressure stiffness could be explained by a lower tissue hydration and/or an increased free versus bound water in the tissue, which might contribute to a higher friction between the collagen fibrils that are not kept apart by the s-GAGs and their associated water anymore. It may also arise from the fusion of the collagen fibrils. The reason for the increased hysteresis after s-GAG removal remains unclear. It could be due to an increased flow-induced viscosity of the additional free water in the tissue and/or an increased network viscosity despite the loss of s-GAGs. More structural studies are needed to explain this result.

Comparing the mechanical results between pigs (chapter 3) and humans, the hu-
CHAPTER 4. ALTERATIONS IN THE POSTERIOR SCLERA AFTER GLYCOSAMINOGLYCAN DEGRADATION: A HUMAN EXPERIMENTAL STUDY

Human sclera was significantly stiffer than the porcine sclera under inflation, which agreed with uniaxial strip tests\textsuperscript{86} and is consistent with the higher number of non-enzymatic collagen crosslinks in the human sclera.\textsuperscript{86} The overall effect of s-GAG degradation was to make the scleral inflation response slightly stiffer in humans, whereas the same treatment dramatically reduced the stiffness of the porcine sclera. In addition, compared to the pig sclera, s-GAG digestion in the human sclera did not change the strain at maximum pressure or difference in the mechanical behavior between the circumferential and meridional directions, suggesting no alteration to the fibril crimp morphology or anisotropy of the collagen fibrils or of their connections, respectively. This could be due to the abundance of non-enzymatic crosslinks in the human sclera. Therefore, differences in the s-GAG degradation effects observed between humans and pigs could be attributed to differences in their scleral collagen crosslink structure and s-GAG types.

The results presented here are not consistent with the reduction in the scleral GAG content\textsuperscript{169} and overall more compliant response of the posterior sclera\textsuperscript{82,99} measured in experimental myopia and in chapter 3 of this work. Compared to the studies on experimental myopia and pig eyes following s-GAG depletion, this study tested the posterior sclera from elderly donors rather than young animals. Aging might change the scleral tissue, especially through the addition of non-enzymatic crosslinks, in such a way that the tissue softening due to s-GAG degradation is inhibited. This might explain why the results surprisingly follow the same trend as the overall stiffer
mechanical behavior measured in human\textsuperscript{114} and experimental\textsuperscript{117} glaucoma. Interestingly, another study on human myopic eyes showed an increased strain at maximum pressure and a decreased GAG content compared to normal eyes\textsuperscript{106}. This increased scleral compliance could be due to the younger eyes tested compared to those in the present study. Therefore, the biomechanical effects of s-GAG degradation depend on age and hence, on the microstructure of the scleral tissue.

There were several limitations to this study. No hydration data were obtained as mechanical testing and hydration measurement before and after enzyme-treatment could not be performed on the same eyes. In addition, no control group (native eyes) was considered as the eyes were received at various times after death and, while in humidified jars, their hydration state surely varied. Finally, the small strains mea-
sured would only have found large regional differences, therefore preventing regional mechanical comparisons.

4.5 Conclusion

In conclusion, nearly complete s-GAG digestion of the human posterior sclera decreased its thickness and led to a small, significant increase in the overall stiffness and hysteresis of the scleral inflation response. While others have reported minimal effects of GAGs on the tensile or viscoelastic mechanical behavior of connective tissue, this study showed that s-GAGs play a measurable role in the mechanical behavior of the posterior human sclera. s-GAGs most likely contribute to the scleral mechanical behavior through their effects on hydration and collagen-collagen interaction, but their effects might be limited by the tissue age and therefore microstructure.
Chapter 5

The effects of glycosaminoglycan degradation on the scleral matrix and collagen: a numerical approach

This chapter probes the effect of s-GAG degradation on the mechanical properties of the matrix and collagen components of the posterior porcine sclera. The results from chapter 3 suggested that s-GAG degradation may affect the type of hydration in the matrix, as well as the collagen initial crimp, orientation and connections. Three-dimensional specimen-specific finite element models were developed for the posterior scleral shells which were inflation tested as controls, after buffer-treatment, and after ChABC-treatment in chapter 3. A Cauchy traction was applied to the inner surface of the model according to the experimentally applied pressure and 4 parameters of a
CHAPTER 5. THE EFFECTS OF GLYCOSAMINOGLYCAN DEGRADATION ON THE SCLERAL MATRIX AND COLLAGEN: A NUMERICAL APPROACH

distributed fiber constitutive model were determined by minimizing the error between the displacements calculated for the top surface of the mesh and the displacements measured experimentally by 3D-DIC. A surrogate-model accelerated random search algorithm was used for the inverse material characterization problem. The algorithm consisted of a genetic algorithm performed on a neural-network surrogate model obtained from the numerical model, therefore accelerating the convergence to a global solution.

5.1 Introduction

The mechanical properties of the sclera play an important role in the pathophysiology of glaucoma and myopia. In glaucoma, altered scleral mechanical properties can generate pathological levels of stress and strain at the ONH, where the insult to the RGC axons occur, even at physiological levels of IOP. Elevated stresses and strains at the ONH can simultaneously impair the axonal transport and blood supply of the RGC axons, and induce the activation of astrocytes. In myopia, the axial elongation of the globe could be due to alterations in the scleral mechanical properties, caused by tissue remodeling in response to changes in the visual environment. Human and animal model studies have shown that the scleral ECM structure and mechanical properties are remodeled by the progression of glaucoma and myopia. The effects of a change in the collagen and
elastin content on the mechanical behavior of the sclera can be understood from enzymatic degradation studies and are consistent among tissues. However, the mechanical role of GAGs is tissue-dependent and has not been studied in the sclera until the work described in chapters 3 and 4. Although the experiments demonstrated the effects of s-GAG digestion on the mechanical behavior of the posterior porcine and human sclera, the results also suggested that s-GAG digestion could alter the collagen as well as the matrix components of the tissue.

GAGs are chains of repeating disaccharide units of various lengths that are covalently attached to a core protein to form PGs. PGs have been observed in close association with collagen fibrils in the sclera and shown to interact with them via their core protein as well as GAG side chains. GAG-collagen fibril interactions are electrostatic in nature and have mostly been studied in vitro, showing that GAGs themselves can alter the fibrillogenesis, including the collagen fibril diameter and rate of formation. GAGs have also been hypothesized to regulate the interfibrillar distance through their water-binding capacity and osmotic pressure. GAG-GAG charge repulsion and GAG-GAG antiparallel interactions.

The inflation experiments in chapter 3 showed that the structure as well as tensile and viscoelastic mechanical behavior of the posterior porcine sclera were influenced by incubation in buffer alone and incubation in ChABC solution. Buffer-treatment and ChABC-treatment significantly increased the scleral hydration and thickness over
CHAPTER 5. THE EFFECTS OF GLYCOSAMINOGLYCAN DEGRADATION ON THE SCLERAL MATRIX AND COLLAGEN: A NUMERICAL APPROACH

the control and buffer-treated values, respectively, however they showed opposite mechanical effects. Specifically, incubation in buffer alone from an untreated control state significantly increased the low-pressure stiffness, hysteresis and creep rate but it decreased the high-pressure stiffness. In comparison, incubation in ChABC from a hydrated swollen state significantly decreased the low-pressure stiffness, hysteresis and creep rate but it increased the high-pressure stiffness and transition strain. In addition, the difference in the mechanical parameters between the circumferential and meridional directions were less after s-GAG degradation, indicating that the anisotropy of the collagen fibril arrangement and/or of their connections might have been altered.

In this study, inverse FEM was applied to determine the matrix and collagen properties of the inflation tested specimens of chapter 3. Three-dimensional specimen-specific finite element geometries were created from the sphere fit of the 3D-DIC reference positions and the thickness measurements, for the specimens divided into 2 experimental groups: inflated as controls and after buffer-treatment, or inflated after buffer-treatment and subsequently after ChABC-treatment. A Cauchy traction corresponding to the experimentally applied pressure was applied to the bottom surface of the mesh. The sclera was described by a distributed fiber model. Despite a lower-level physical description of the tissue compared to micro-mechanical models, distributed fiber models are computationally less expensive and describe some interesting aspects of the collagen properties. For each posterior scleral shell and treatment
state, parameters describing the matrix shear modulus as well as the collagen fiber stiffness, recruitment and degree of anisotropy were determined by minimizing the difference between the 3D-DIC experimental surface displacements and the displacements on the top surface of the mesh. In this study, the properties of the collagen fibers, which correspond to the scleral collagen lamellae, were evaluated. Differences in the parameters between the experimental groups were analyzed for the effects of buffer-associated swelling and s-GAG degradation on the matrix and collagen properties.

5.2 Methods

5.2.1 Experiments

The experiments were described in chapter 3. Briefly, a protocol was developed to degrade the s-GAGs from the posterior sclera of porcine eyes. Eyes from 6-9 month-old pigs (Animal Technologies Inc., Tyler TX) were received on wet ice 24 h after enucleation and used upon arrival. The posterior scleral shells were dissected and subjected to a series of pressure-controlled inflation tests, to compare the mechanical behavior of scleras within 2 experimental groups. In one group, the specimens were inflated after dissection and again after incubation in Trizma buffer alone for 18 h (protocol 1: control/buffer-treated) (n=5). In another group, the specimens were soaked in Trizma buffer alone for 18 h after dissection, inflated, soaked in enzyme so-
lution for 18 h, and inflated again (protocol 2: buffer-treated/enzyme-treated) (n=7).

The mechanical tests included a load-unload cycle from baseline pressure 0.28 kPa to 6 kPa at 0.13 kPa/s followed by a 30 min recovery period, which data were used in this study. Two cameras acquired top-down images of the specimen surface during testing and the full-field displacements on the scleral surface were obtained from 3D-DIC. Twelve thickness measurements equally spaced in the circumferential direction, away from the peripapillary sclera, were acquired using a 15 MHz ultrasound transducer at the end of each test.

5.2.2 Anisotropic hyperelastic constitutive model

The scleral tissue was modeled as an anisotropic distribution of collagen fibers embedded in an isotropic matrix of hydrated PGs. The distributed fiber hyperelastic model was applied to describe the anisotropic behavior of the sclera. The matrix and collagen fibers are assumed to deform affinely with the continuum deformation gradient \( \mathbf{F} = 1 + \frac{\partial \mathbf{u}}{\partial \mathbf{x}} \). The right and left Cauchy-Green deformation tensors are defined from \( \mathbf{F} \) as \( \mathbf{C} = \mathbf{F}^T \mathbf{F} \) and \( \mathbf{B} = \mathbf{F} \mathbf{F}^T \). The strain energy density of the sclera \( \Psi_{\text{sclera}} \) is decomposed additively into an isotropic component \( \Psi_{\text{matrix}} \) and anisotropic component \( \Psi_{\text{fibers}} \) as:

\[
\Psi_{\text{sclera}}(\mathbf{C}) = \Psi_{\text{matrix}}(\mathbf{C}) + \Psi_{\text{fibers}}(\mathbf{C}) \tag{5.1}
\]
The $\Psi_{\text{matrix}}$ represents the contribution of all non-fibrillar collagen components, including elastin fibers, non-fibrillar collagens, GAGs and water, to the strain energy density of the sclera. The matrix was described by a quasi-incompressible Neo-Hookean model as:

$$
\Psi_{\text{matrix}}(C) = \frac{\mu}{2}(\bar{I}_1(C) - 3) + \frac{\kappa}{2}(I_3(C) - \ln(I_3(C)) - 1) \tag{5.2}
$$

where $\mu$ is the matrix shear modulus, $\kappa$ is the matrix bulk modulus, $\bar{I}_1 = I_0 - \frac{2}{3}I_1$ is the deviatoric part of the first invariant $I_1 = \text{tr}(C)$ and $I_3(C) = \text{det}(C)$ is the third invariant. The bulk modulus was set to more than one order of magnitude larger than $\mu$ to enforce near-incompressibility and ensure a volume change of less than 1% across the entire geometry. The $\Psi_{\text{fibers}}$ represents the collagen fiber contribution to the strain energy density of the sclera and was described as:

$$
\Psi_{\text{fibers}}(C) = \int_{-\pi}^{\pi} \Psi_{\text{fiber}}(\lambda_f(\Theta))P(\Theta)d\Theta \tag{5.3}
$$

where $\lambda_f = \sqrt{a_0^0 C, a_0}$ is the stretch of a collagen fiber with reference orientation $a_0 = [\cos(\Theta), \sin(\Theta), 0]$ in the plane of the sclera, $P(\Theta)$ is the probability density distribution for the fibers, and $\Psi_{\text{fiber}}$ is the strain energy density for a single fiber. $\Psi_{\text{fiber}}$ was described by a Fung-type exponential function as:

$$
\Psi_{\text{fiber}}(\lambda_f) = \frac{\alpha}{\beta}[\exp(\beta(\lambda_f^2 - 1)) - \beta\lambda_f^2] \tag{5.4}
$$
where $\beta$ is the collagen fiber strain-stiffening parameter and $4\alpha\beta$ represents the fiber stiffness as shown later on. A lower value of $\beta$ indicates a larger onset of the strain-stiffening of the fibers. The probability density distribution was defined as a semi-circular von Mises distribution:

$$P(\Theta) = \frac{\exp(k \cos(2(\Theta - \Theta_p)))}{2\pi I_0(k)}$$

(5.5)

where $\Theta_p$ is the preferred collagen fiber orientation, $k$ represents the fiber dispersion around $\Theta_p$ and $I_0(k) = \frac{1}{\pi} \int_0^\pi \exp(k \cos(\Theta))d\Theta$ is the modified Bessel function of the first kind of order zero. $k = 0$ represents an isotropic distribution of the fibers, while increasing values of $k$ indicate a progressive alignment of the fibers along their preferred orientation. Therefore, the constitutive model has a total of 5 parameters: $\mu$, $\alpha$, $\beta$, $\Theta_p$ and $k$. The modulus of the collagen fibers can be determined from the anisotropic part of the material tangent modulus $C_{fibers}$ defined as:

$$C_{fibers} = 2 \frac{\partial^2 \Psi_{fibers}}{\partial C^2} = 2 \frac{\partial S_{fibers}}{\partial C}$$

(5.6)

where $S_{fibers}$ is the second Piola-Kirchhoff stress tensor defined as a function of the collagen fiber stretch $\lambda_f$ as:

$$S_{fibers} = 2 \frac{\partial \Psi_{fibers}}{\partial C} = 2 \left( \frac{\partial \Psi_{fibers}}{\partial \lambda_f} \frac{\partial \lambda_f}{\partial C} \right)$$

(5.7)
For the expression of $\Psi_{\text{fibers}}$ of equation \ref{eq:5.3}, the second Piola-Kirchhoff stress tensor and the material tangent modulus can be evaluated assuming all collagen fibers are orientated in a single direction $P(\theta) = \delta(0)$ are:

$$S_{\text{aniso}} = 2\alpha \left[2\beta \lambda_f \exp(\beta(\lambda_f^2 - 1)) - 2\beta \lambda_f\right] \frac{1}{2\lambda_f} \mathbf{a}_o \otimes \mathbf{a}_o$$

$$= 2\alpha \left[\exp(\beta(\lambda_f^2 - 1)) - 1\right] \mathbf{a}_o \otimes \mathbf{a}_o \quad (5.8)$$

$$C_{\text{aniso}} = 4\alpha \left[2\beta \lambda_f \exp(\beta(\lambda_f^2 - 1))\right] \frac{1}{2\lambda_f} \mathbf{a}_o \otimes \mathbf{a}_o \otimes \mathbf{a}_o \otimes \mathbf{a}_o$$

$$= 4\alpha \beta \exp(\beta(\lambda_f^2 - 1)) \mathbf{a}_o \otimes \mathbf{a}_o \otimes \mathbf{a}_o \otimes \mathbf{a}_o$$

When no stretch is applied to the collagen fibers, the material tangent modulus becomes:

$$C_{\text{aniso}} \bigg|_{\lambda_f = 1} = 4\alpha \beta \mathbf{a}_o \otimes \mathbf{a}_o \otimes \mathbf{a}_o \otimes \mathbf{a}_o, \quad (5.9)$$

which shows that $4\alpha \beta$ represents the collagen fiber stiffness.

### 5.2.3 Finite element model

#### 5.2.3.1 Geometry

Three-dimensional FEM meshes were constructed for each scleral specimen and experimental condition (control, buffer-treated or enzyme-treated), from the thickness measurements and spherical fit of the 3D-DIC position data, using Cubit (Sandia National Labs, Livermore, CA). The thickness was assumed to be constant in the
meridional direction, and linearly interpolated between each measurement point in the circumferential direction. Each mesh was composed of 1440 HEX8-type elements, including 3 elements through the thickness (Fig. 5.1(a) and (b)).

### 5.2.3.2 Boundary conditions

The boundary conditions represented those of the experiments conducted in chapter 3. A Cauchy traction corresponding to the experimentally applied inflation pressure was applied to the bottom surface of the mesh, while the upper surface of the mesh was traction free. Angle boundary conditions were applied to the lateral surfaces (Fig. 5.1(b)). The angle boundary condition allows the nodes to displace tangentially to the surface without friction, therefore allowing the sclera to contract through the thickness during inflation.
CHAPTER 5. THE EFFECTS OF GLYCOSAMINOGLYCAN DEGRADATION ON THE SCLERAL MATRIX AND COLLAGEN: A NUMERICAL APPROACH

Figure 5.2: Boundary conditions on the cross-section of a representative mesh.

Figure 5.3: Comparison of the stress-stretch curves for the analytical model and FEM for a cube under equibiaxial stretch and various degree of anisotropy of the collagen fibers.

5.2.3.3 Model validation

The constitutive model, already implemented in Tahoe (Sandia National Labs, Livermore, CA), was validated by comparison of the stress-stretch curves from the analytical model coded in Matlab and FEM for a cube under equibiaxial stretch and various degree of anisotropy of the collagen fibers (Fig. 5.3).
5.2.3.4 Convergence study

The mesh element density was chosen based on a convergence study that compared various mesh densities expressed as a function of the 3D-DIC data in-plane resolution (0.125 mm) (Fig. 5.4). The element density of the reference mesh corresponded to the 3D-DIC resolution in the meridional direction and 4 times the 3D-DIC resolution in the circumferential direction (shown in blue in Fig. 5.4). For each coarser or finer mesh, the \(L_2\)-norm of the displacements was calculated at each node of top surface, and the average \(L_2\)-norm over the mesh top surface was compared to that of the reference mesh. A coarser mesh with twice the 3D-DIC resolution in the meridional direction and 6 times the 3D-DIC resolution in the circumferential direction was chosen as it showed a 0.1% difference in the average norm of the displacements on the top surface compared to the reference mesh, without being too computationally expensive for an optimization problem (encircled in blue in Fig. 5.4).

5.2.3.5 Sensitivity study

The sensitivity of the top surface displacements to changes in the model parameters was assessed by varying each of the 5 model parameters by \(\pm 30\%\). Two sets of test parameters that represented the measured stress-strain response of the control and enzyme-treated groups were chosen (Table 5.1). The matrix and fiber test parameters \(\mu, \alpha\) and \(\beta\) were determined from the analytical fit of the average stress-strain curve obtained experimentally in chapter 3 for each group, in the circumferential
CHAPTER 5. THE EFFECTS OF GLYCOSAMINOGLYCAN DEGRADATION ON THE SCLERAL MATRIX AND COLLAGEN: A NUMERICAL APPROACH

Figure 5.4: Plot showing the percent difference between the average $L_2$-norm of the displacements on the top surface nodes of various meshes and that of a reference mesh, as a function of the smallest element volume. The element density of the reference mesh corresponded to the 3D-DIC resolution in the meridional direction and 4 times the 3D-DIC resolution in the circumferential direction.

direction for the IN quadrant. The dominant fiber direction $\Theta_p$ was arbitrarily set to 0.3, which is close to 0, the circumferential direction, and the fiber dispersion $k$ was chosen to be 10 for the control group and 2 for the enzyme-treated group. The difference in the top surface displacements due to variations in each model parameter by $\pm 30\%$, calculated at each node and averaged over the top surface, was plotted as a function of the pressure level for both the control and enzyme-treated groups (Fig. 5.5). A similar trend was obtained when each parameter was varied by $-30\%$. The sensitivity analysis showed that the displacements on the mesh top surface were most sensitive to $\mu$, followed by $\beta$, $\alpha$ and finally $k$ and $\Theta_p$. Since the displacements on the top surface were not very sensitive to variations in $\Theta_p$ and that the posterior
**Table 5.1:** Table showing 1) 2 sets of test parameters, determined from the fit of the average stress-strain curve obtained experimentally for the control and enzyme-treated group, in the circumferential direction for the IN quadrant, and 2) the corresponding optimized parameters returned by the optimization algorithm. NO = non-optimized parameter.

<table>
<thead>
<tr>
<th>Parameter Type</th>
<th>$\mu$ [kPa]</th>
<th>$\alpha$ [kPa]</th>
<th>$\beta$ [no unit]</th>
<th>$\Theta_p$ [rad]</th>
<th>$k$ [no unit]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control test parameters</td>
<td>180</td>
<td>0.07</td>
<td>103</td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>Optimization parameters</td>
<td>180.5</td>
<td>0.071</td>
<td>103.2</td>
<td>NO</td>
<td>13.0</td>
</tr>
<tr>
<td>Enzyme-treated test parameters</td>
<td>80</td>
<td>0.008</td>
<td>70</td>
<td>0.3</td>
<td>2</td>
</tr>
<tr>
<td>Optimization parameters</td>
<td>79.4</td>
<td>0.012</td>
<td>65.7</td>
<td>NO</td>
<td>1.89</td>
</tr>
</tbody>
</table>

**Figure 5.5:** Difference in the top surface displacements with and without a +30% change in each of the model parameter, calculated at each node and averaged over the top surface for (a) the control group and (b) the enzyme-treated group.

sclera away from the ONH is rather isotropic, $\Theta_p$ was set to 0, which corresponds to the circumferential direction. Thus, only 4 parameters were optimized with a better accuracy.

### 5.2.4 Optimization

The optimization minimized the following cost function:
CHAPTER 5. THE EFFECTS OF GLYCOSAMINOLYCAN DEGRADATION ON THE SCLERAL MATRIX AND COLLAGEN: A NUMERICAL APPROACH

\[ C(u^{exp}, u^{fem}(\mu, \alpha, \beta, k)) = \frac{1}{n_{nodes}n_{steps}} \sqrt{\sum_{p=1}^{n_{steps}} \sum_{n=1}^{n_{nodes}} (u^{exp}_p(x_n) - u^{fem}_p(x_n))^2} \]  

(5.10)

where \( n_{steps} = 22 \) is the number of pressure levels of the loading phase, \( n_{nodes} = 456 \) is the number of nodes on the top surface of the mesh excluding the boundary nodes, \( u^{fem}_p(x_n) \) is the FEM displacement vector for the top surface node \( n \) and level of pressure \( p \) and \( u^{exp}_p(x_n) \) is the 3D-DIC displacement vector at the pressure level \( p \) interpolated at node \( n \). The range of the 4 parameters to be optimized was defined as follows: \( \mu: [0-1000], \alpha: [0.001-1], \beta: [5-500], \) and \( k: [0-30] \). The ranges for \( \mu, \alpha \) and \( \beta \) were defined as \( \pm \) one order of magnitude from the test parameters obtained by fitting the average experimental stress-strain curve for the control and enzyme-treated group (chapter 3) using the analytical model. The range for \( \beta \) was further reduced to remove values below 5 which led to a linear mechanical behavior that was not representative of the scleral behavior in any of the experimental groups, as well as values above 500 for which the behavior was very non-linear and the maximum collagen fiber stiffness was above 1 MPa. The range for \( k \) was defined up to 30, the value for which more than 50% of the fibers are aligned within \( \pm 7.5^\circ \) of their preferred orientation.

The inverse material characterization problem was solved using a surrogate-model accelerated random search (SMARS) algorithm. This algorithm performs a genetic algorithm followed by a local optimization on a neural-network surrogate.
model, which is a low-cost approximation of the numerical model. The neural-network was trained using 200 sets of model parameters defined by a Hammersley sampling sequence. The neural-network was composed of 2 hidden layers, each containing 8 neurons to which additional neurons could be added up to 60 should the neural-network training be unsatisfactory. An initial population of 200 and a mutation rate of 0.14 were used for the genetic algorithm. Those SMARS parameters were found to be optimal when optimizing both sets of test model parameters (Table 5.1). The optimizations were run for each scleral shell and treatment state, and were stopped when the cost function did not change for more than 25 consecutive iterations, or when it did not decrease by more than 10% over 2 weeks. The cost functions obtained were below 1.2e-3 mm (see section B.1 of the Appendix), which was small compared to the experimental displacements measured in the posterior porcine sclera in this work.

5.2.5 Statistical analysis

Paired t-tests were used to compare the optimized model parameters between the control and buffer-treated specimens of protocol 1, as well as those between the buffer-treated and enzyme-treated specimens of protocol 2. Unpaired t-tests were used to compare the parameters from the control versus enzyme-treated specimens of protocols 1 and 2, respectively.
CHAPTER 5. THE EFFECTS OF GLYCOSAMINOGLYCAN DEGRADATION ON THE SCLERAL MATRIX AND COLLAGEN: A NUMERICAL APPROACH

Table 5.2: Table showing the 4 optimized model parameters and collagen fiber stiffness, averaged over all specimens in the control and buffer-treated groups of protocol 1. Paired t-tests were used for statistical analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Buffer-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$ [kPa]</td>
<td>58.6 ± 19.9</td>
<td>64.8 ± 19.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>$\alpha$ [kPa]</td>
<td>0.17 ± 0.36</td>
<td>0.0079 ± 0.015</td>
<td>0.35</td>
</tr>
<tr>
<td>$\beta$ [no unit]</td>
<td>107.1 ± 49.7</td>
<td>110.4 ± 36.9</td>
<td>0.75</td>
</tr>
<tr>
<td>$k$ [no unit]</td>
<td>0.41 ± 0.31</td>
<td>0.79 ± 0.61</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>$4\alpha\beta$ [kPa]</td>
<td>20.9 ± 38.1</td>
<td>1.68 ± 2.62</td>
<td>0.29</td>
</tr>
</tbody>
</table>

5.3 Results

First, the 4 optimized model parameters, averaged over all specimens, were compared for the 2 experimental protocols: control condition versus buffer-treated (Table 5.2) and buffer-treated versus enzyme-treated (Table 5.3). The converged set of parameters and corresponding cost function for all specimens of both protocols can be found in section B.1 of the Appendix. Buffer-treatment from a control state significantly increased the matrix shear modulus $\mu$ ($p<0.05$) and collagen fiber dispersion parameter $k$ ($p<0.05$), but it did not significantly change the fiber stiffness $4\alpha\beta$ ($p=0.29$) or fiber strain-stiffening parameter $\beta$ ($p=0.75$). In comparison, enzyme-treatment from a buffer-treated state significantly decreased the matrix shear modulus $\mu$ ($p<0.01$) and collagen fiber strain-stiffening parameter $\beta$ ($p<0.05$). However, it did not significantly change the fiber dispersion parameter $k$ ($p=0.39$) or fiber stiffness $4\alpha\beta$ ($p=0.38$).

The optimized parameters were also compared between the control and enzyme-treated conditions (Table 5.4). The differences measured were similar to those ob-
CHAPTER 5. THE EFFECTS OF GLYCOSAMINOGlyCAN DEGRADATION ON THE SCLERAL MATRIX AND COLLAGEN: A NUMERICAL APPROACH

<table>
<thead>
<tr>
<th></th>
<th>Buffer-treated</th>
<th>Enzyme-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu ) [kPa]</td>
<td>42.7 ± 19.5</td>
<td>18.5 ± 6.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>( \alpha ) [kPa]</td>
<td>0.12 ± 0.28</td>
<td>0.22 ± 0.20</td>
<td>0.40</td>
</tr>
<tr>
<td>( \beta ) [no unit]</td>
<td>69.0 ± 32.9</td>
<td>38.0 ± 12.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>( k ) [no unit]</td>
<td>0.55 ± 0.99</td>
<td>0.26 ± 0.25</td>
<td>0.39</td>
</tr>
<tr>
<td>4( \alpha \beta ) [kPa]</td>
<td>13.8 ± 30.3</td>
<td>26.1 ± 23.0</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Table 5.3: Table showing the 4 optimized model parameters and collagen fiber stiffness, averaged over all specimens in the buffer-treated and enzyme-treated groups of protocol 2. Paired t-tests were used for statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Enzyme-treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu ) [kPa]</td>
<td>58.6 ± 19.9</td>
<td>18.5 ± 6.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>( \alpha ) [kPa]</td>
<td>0.17 ± 0.36</td>
<td>0.22 ± 0.20</td>
<td>0.75</td>
</tr>
<tr>
<td>( \beta ) [no unit]</td>
<td>107.1 ± 49.7</td>
<td>38.0 ± 12.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>( k ) [no unit]</td>
<td>0.41 ± 0.31</td>
<td>0.26 ± 0.25</td>
<td>0.37</td>
</tr>
<tr>
<td>4( \alpha \beta ) [kPa]</td>
<td>20.9 ± 38.1</td>
<td>26.1 ± 23.0</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Table 5.4: Table showing the 4 optimized model parameters and collagen fiber stiffness, averaged over all specimens in the control and enzyme-treated groups of protocols 1 and 2, respectively. Unpaired t-tests were used for statistical analysis.

...served between the buffer-treated and enzyme-treated conditions. Compared to controls, enzyme-treated specimens showed a significantly lower matrix shear modulus \( \mu \) (p<0.001) and collagen fiber strain-stiffening parameter \( \beta \) (p<0.001), but a similar fiber dispersion parameter \( k \) (p=0.37) and fiber stiffness 4\( \alpha \beta \) (p=0.77).

5.4 Discussion

In this study, inverse FEM was used to optimize parameters of a constitutive model describing matrix and collagen fiber properties in the posterior porcine sclera in 2 experimental groups: after buffer hydration from a control condition, and after s-GAG degradation from a buffer hydrated state. The matrix shear modulus, as well
as the collagen fiber recruitment, stiffness and degree of anisotropy were compared within and between the groups, to probe their change with swelling and s-GAG digestion.

Buffer-treatment from a control state significantly increased the matrix shear modulus and degree of anisotropy of the collagen fibers, but it did not affect the fiber stiffness. The increased matrix shear modulus could explain the significant increase in low-pressure stiffness measured in chapter 3 after buffer-treatment of control scleras. In chapter 3 it was hypothesized that the increase in hydration measured after buffer-treatment was in the form of bound and free water due to the charge and osmotic effects of the s-GAGs. A higher matrix shear modulus may result from such an increase in the amount of bound water molecules, that need to rearrange during collagen fiber uncrimping at low strains, as described in chapter 3. The increased degree of collagen fiber alignment was not consistent with the results from chapter 3 which did not show a significant difference in the mechanical behavior between the circumferential and meridional directions after incubation of control scleras in buffer alone. This suggests that such an increase in the fiber alignment does not significantly alter the anisotropy of the scleral mechanical behavior. A higher degree of fiber alignment could result from an increased tissue hydration as bound and/or free water, which may further constrain the orientation of the fibers by filling the space between them.

As expected, buffer-treatment did not affect the collagen fiber stiffness. This suggests that the significant decrease in high-pressure stiffness measured in chapter 3 following
buffer-treatment of control scleras may be caused by a hydration effect rather than a decrease in fiber stiffness. The more abundant s-GAG-associated water molecules in the tissue might act as a spacer between the fibers, therefore reducing their friction when stretched at high strains, as hypothesized in chapter 3.

Enzyme-treatment from a buffer-treated state significantly decreased the matrix shear modulus and increased the onset of strain-stiffening of the collagen fibers, but it did not affect the fiber dispersion or fiber stiffness. The decreased matrix shear modulus could explain the decrease in low-pressure stiffness measured in chapter 3 after enzyme-treated of buffer-treated scleras. In chapter 3 it was hypothesized that the increase in hydration measured after enzyme-treatment resulted from an increased free space in the tissue, allowing the entry of more free water that compensated the loss of bound water. A lower matrix shear modulus may originate from such a loss of bound water molecules and therefore s-GAG associated tissue viscosity, as described in chapter 3. The increased onset of the collagen fiber strain-stiffening could explain the lengthening of the toe region measured in chapter 3 after incubation of buffer-treated scleras in enzyme solution. The increased onset of strain-stiffening points to an increase in the initial fiber crimp, as hypothesized in chapter 3. This hypothesis is consistent with the increased fibril crimp reported in the posterior sclera of experimental myopic eyes where the GAG content is known to be reduced. The non-significant change in the degree of collagen fiber alignment, compared to the reduced mechanical differences between the circumferential and meridional directions
described in chapter 3 following enzyme-treatment of buffer-treated scleras, suggests
that the s-GAGs themselves account for some of the tissue anisotropic mechanical
behavior by connecting adjacent fibers in a highly ordered manner. As expected,
s-GAG digestion did not affect the collagen fiber stiffness. This suggests that the
significant increase in high-pressure stiffness measured in chapter 3 after enzyme-
treatment from a buffer-treated state may be due to a hydration effect rather than
an increase in fiber stiffness. The free water molecules moving within the tissue
might cause an increased fiber friction and therefore stiffening at high strains, as
hypothesized in chapter 3. A similar discussion holds for the comparison of the
enzyme-treated versus control condition.

Limitations of this study include the choice of the circumferential direction as the
preferred orientation of the collagen fibers. This was chosen to optimize the other 4
model parameters with a better accuracy, and was based on the observation that the
sclera away from the ONH is rather isotropic. If the sclera is isotropic (i.e. $k \rightarrow 0$),
then the choice of the preferred direction does not matter much. Another limita-
tion was the low sensitivity of the displacements of the mesh top surface to the fiber
dispersion parameter. This suggests that small variations in the fiber degree of align-
ment cannot be captured well with the current methodology. However, such small
changes in the degree of collagen fiber alignment might not significantly influence the
anisotropy of the scleral mechanical behavior. The representation of the experiment
was simplified. The specimen-specific geometry did not include the ONH and the
part of sclera closer to the holder. The angle boundary condition at the mesh lateral boundaries assumed no shear through the scleral thickness, an assumption based on the small shear strain values reported for the porcine scleral surface in chapter 3. However, uniform displacement boundary conditions through the thickness at the lateral surfaces induced compressibility issues for the large applied deformations. The simplified model did not capture well the local heterogeneity of the raw 3D-DIC displacement data as shown in section B.2 of the Appendix. Modeling the entire posterior scleral shell, with the ONH, a fully-fixed boundary condition at the edge and additional thickness data could help obtaining more accurate numerical surface displacements. The hyperelastic model used in this study described the pseudoelastic behavior of the posterior sclera but did not incorporate the swelling, poroelastic and electrostatic effects of s-GAG degradation.

5.5 Conclusion

In conclusion, this study indicated that s-GAG degradation induced significant changes in the matrix/collagen components of the posterior porcine sclera. s-GAG degradation decreased the matrix shear modulus and increased the onset of strain-stiffening of the collagen fibers, but it did not alter the collagen fiber stiffness or degree of anisotropy. Together with the results from chapter 3, this study suggests that s-GAGs play an important role in the mechanical behavior of the posterior
porcine sclera, through their effects on the type of hydration in the matrix and the anisotropic way they connect adjacent collagen fibers, rather than by affecting the collagen fiber stiffness. In addition, s-GAGs probably contribute to the collagen fiber crimp structure.
Chapter 6

Conclusions and Future Work

This work developed experimental and numerical approaches to investigate the contribution of s-GAGs to the structure and mechanical properties of the posterior sclera.

6.1 Summary of Findings

Chapter 2 compared the 2D and 3D versions of DIC, a technique extensively used in this work and in the field of experimental mechanics. The use of 2D-DIC is not appropriate for non-planar samples or samples undergoing large amounts of out-of-plane displacement or rotation. However, some experimental conditions can discourage the use of 3D-DIC, such as a high-magnification and therefore small depth of field, high-speed experiments where the camera synchronization is crucial, stiff...
tissues such as the posterior human sclera for which the out-of-plane displacement is small, or a highly controlled environment with limited space for 2 cameras. An experimental setup was designed to compare the 3D-DIC and 2D-DIC displacements and meridional strain for a membrane under inflation. A two-camera stereovision system imaged the membrane top-down, while a single-camera system simultaneously acquired images of the membrane in profile. Under inflation, 2D-DIC had a smaller variability in the displacements than 3D-DIC, especially vertically, but a similar strain uncertainty. Moreover, the absolute difference in the average displacements and strain between 3D-DIC and 2D-DIC were in the range of the 3D-DIC variability. The results showed that 2D-DIC could be an alternative to 3D-DIC to characterize the profile behavior of materials inflated under conditions that discourage the use of a stereovision system. However, 3D-DIC was used in this work to characterize the three-dimensional anisotropic behavior of heterogeneous materials, such as the posterior sclera, under inflation.

Chapter 2 developed a methodology to evaluate the error and uncertainty in strains due to the 3D-DIC displacement error and uncertainty for an object under inflation. The static performance of 3D-DIC can be calculated from multiple images of the specimen under static conditions. However, the error and uncertainty in the 3D-DIC measurements for a specimen under 3D deformation are not easily measured. They cannot be calculated from the synthetic deformation of images as done for 2D-DIC, and experimentally applying a known non-uniform 3D displacement field to an
object is difficult. Therefore, most studies consider static conditions or the effects of rigid body motions and planar deformations, which prevents the calculation of the error and uncertainty in strains with 3D deformation. The method was based on the controlled translation of a rigid spherical object to obtain the 3D-DIC displacement error and uncertainty, which were then applied to a numerical sphere subjected to inflation to calculate the error and uncertainty in strains. This methodology was proven useful for posterior scleral shells under inflation but could potentially be applied to other objects under 3D deformation.

Chapter 3 developed a protocol to experimentally degrade s-GAGs from the posterior sclera of pig eyes using ChABC. Independent studies have reported pathological alterations to the scleral GAG content and mechanical behavior in glaucoma and myopia, but the significance of GAG content variations in the mechanics of those diseases was never studied. The s-GAG content and hydration were measured for specimens under 3 experimental conditions: control (no treatment), buffer-treated (incubation in buffer alone) and enzyme-treated (incubation in buffer containing ChABC). In comparison, the measurement of the thickness and inflation-response with 3D-DIC were repeated on specimens as controls and then after buffer-treatment, or after buffer-treatment and then after ChABC-treatment. Inflation testing was used to mimic the effect of IOP on the scleral shells away from the clamp. Compared to cartilage, tendons or ligaments, for which the role of s-GAGs has been studied for years, no experimental data was available in the literature for the effects of s-GAG removal on the
scleral structure and mechanical behavior. Buffer-treatment on control scleras and ChABC-treatment on buffer-treated scleras significantly increased the scleral thickness, which was consistent with the measured increase in hydration, but had opposite mechanical effects. Buffer-treatment on control scleras significantly increased the low-pressure stiffness, hysteresis, and creep rate, and decreased the high-pressure stiffness. Those results were consistent with an increased scleral hydration hypothesized to be in the form of bound and free water due to the charge and osmotic effects of the s-GAGs. In comparison, ChABC-treatment on buffer-treated scleras significantly decreased the low-pressure stiffness, hysteresis, and creep rate, and increased the high-pressure stiffness and transition strain. Furthermore, s-GAG digestion dramatically reduced the differences between the circumferential and meridional inflation responses compared to the buffer-treated condition. Those changes were consistent with a change in the scleral hydration hypothesized to be in the form of free rather than bound water, due to an increased free space in the tissue after s-GAGs removal.\textsuperscript{220} The results also suggested a change in the collagen fibril initial crimp, orientation and connections following s-GAG degradation. The overall more compliant mechanical response of the posterior porcine sclera following s-GAG digestion is consistent with the altered mechanical behavior measured in the posterior sclera of human\textsuperscript{106} and experimental\textsuperscript{82,99} myopic eyes, in which the GAG content is reduced.\textsuperscript{106,169} However, it is not consistent with the increased stiffness observed in the posterior sclera of experimental\textsuperscript{17,3} and human\textsuperscript{41} glaucoma eyes, for which the change in the GAG content has never
been studied. The main structural and mechanical changes measured in the posterior porcine sclera after s-GAG degradation, along with the current best hypothesis for those changes, are summarized in Table 6.1. It was concluded from this chapter that s-GAGs have a measurable contribution to the structure and mechanical behavior of the posterior porcine sclera, mainly through their effects on hydration and their interactions with the collagen fibrils.

In chapter 4 the protocol from chapter 3 was used to degrade s-GAGs in the posterior sclera of human eyes. The experimental methods were changed to improve the lighting and magnification to capture more accurately the smaller displacements. The strain calculation and stress-strain curve analysis were also modified to use fewer approximations and limit the influence of noise in the data to obtain more accurate mechanical parameters. The thickness and inflation response were repeatedly measured on the same specimens after buffer-treatment and then after ChABC-treatment. ChABC-treatment on buffer-treated scleras significantly increased the low-pressure stiffness, high-pressure stiffness and hysteresis, and decreased the scleral thickness. It was hypothesized that the scleral thinning measured after s-GAG digestion could be due to a lower hydration although probably non-significant\cite{220} or to a microstructure rearrangement, such as the fusion or thinning of the collagen fibrils.\cite{65,239,240} A lower scleral hydration, an increased amount of free water in the tissue and the fusion of collagen fibrils following s-GAG removal could all contribute to the mechanical changes measured. The overall stiffer mechanical response of the posterior human
CHAPTER 6. CONCLUSIONS AND FUTURE WORK

sclera following s-GAG digestion is not consistent with the more compliant mechanical behavior observed in the posterior sclera of human[106] and experimental[52,99] myopic eyes along with a lower GAG content[106,169] as well as in chapter 3 of this work. However, it should be noted that the posterior scleras tested in this chapter were from much older donor eyes compared to the myopic eyes tested by Avetisov et al.[106] and the young animal eyes of chapter 3 and experimental myopia studies. Aging might have affected the scleral microstructure, especially through the addition of collagen non-enzymatic crosslinks, in a way that inhibited the tissue softening effect of s-GAG degradation. This might explain why the results are surprisingly consistent with the increased stiffness measured in glaucoma.[4,173] The main structural and mechanical changes measured in the posterior human sclera after s-GAG degradation, along with the current best hypothesis for those changes, are presented in Table 6.2. It was concluded from this chapter that s-GAG do play a role in the structure and mechanical properties of the posterior human sclera, through their effects on hydration and collagen-collagen interactions. The results also highlighted that the mechanical influence of s-GAGs might be reduced or altered as the scleral microstructure remodels with age. Therefore, the benefits of s-GAG removal in glaucoma/myopia patients and animal models might be different.

Chapter 5 used a numerical approach to extend the investigation of the effects of buffer-associated swelling and s-GAGs degradation on the properties of the matrix and collagen components in the porcine specimens of chapter 3. Inverse FEM was ap-
CHAPTER 6. CONCLUSIONS AND FUTURE WORK

plied to three-dimensional specimen-specific meshes and parameters of an anisotropic
distributed fiber model were optimized based on the 3D-DIC surface displacements
at all pressure levels. Buffer-treatment on control scleras mainly increased the matrix
shear modulus, which was consistent with an increased matrix hydration, especially in
the form of bound water. In comparison, ChABC-treatment on buffer-treated scleras
significantly decreased the matrix shear modulus and increased the onset of the col-
lagen fiber strain stiffening. Those results were consistent with the hypothesis of the
presence of free rather than bound water in the tissue and of an increased collagen
fiber initial crimp after s-GAG degradation, respectively. However, s-GAG degra-
dation did not alter the fiber stiffness or degree of collagen fiber alignment, which
was not consistent with the hypothesis of a rearrangement of the collagen fibers af-
ter s-GAG removal. Table 6.3 links the main mechanical changes measured in the
posterior porcine sclera after s-GAG degradation in chapter 3 to the changes in the
matrix and collagen properties measured in chapter 5 and the current best hypothe-
sis for those changes. It was concluded from this chapter that s-GAGs contribute to
the mechanical behavior of the posterior porcine sclera through their effects on the
type of hydration in the matrix and possibly on the collagen fiber crimp structure,
rather than the alteration of the fiber stiffness. s-GAGs also seem to contribute to the
anisotropy of the scleral mechanical behavior by connecting adjacent collagen fibers
in a highly ordered manner.
### Table 6.1: Summary of the main structural and mechanical changes measured in the posterior porcine sclera after s-GAG degradation and the current best hypothesis for those changes.

<table>
<thead>
<tr>
<th>Change in properties</th>
<th>Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased hydration</td>
<td>Increased free space in tissue allowing the entry of more free water</td>
</tr>
<tr>
<td>Increased thickness</td>
<td>Increased hydration</td>
</tr>
<tr>
<td>More compliant mechanical behavior</td>
<td>Increased initial fibril crimp</td>
</tr>
<tr>
<td>More isotropic mechanical behavior</td>
<td>Change in anisotropy of the fibrils or of their connections</td>
</tr>
</tbody>
</table>

### Table 6.2: Summary of the main structural and mechanical changes measured in the posterior human sclera after s-GAG degradation and the current best hypothesis for those changes.

<table>
<thead>
<tr>
<th>Change in properties</th>
<th>Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased thickness</td>
<td>Lower hydration, non-enzymatic crosslinks, fibril fusion</td>
</tr>
<tr>
<td>Stiffer mechanical behavior</td>
<td>Lower hydration, free rather than bound water, fibril fusion</td>
</tr>
<tr>
<td>Same anisotropic mechanical behavior</td>
<td>No change in anisotropy of the fibrils or of their connections due to non-enzymatic crosslinks</td>
</tr>
</tbody>
</table>

### Table 6.3: Summary of the main changes in the mechanical behavior and corresponding matrix/collagen properties observed in the posterior porcine sclera after s-GAG degradation and the current best hypothesis for those changes.

<table>
<thead>
<tr>
<th>Change in mechanical behavior</th>
<th>Change in matrix/collagen properties</th>
<th>Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased low-pressure stiffness</td>
<td>Decreased matrix shear modulus</td>
<td>Decreased bound water</td>
</tr>
<tr>
<td>Increased high-pressure stiffness</td>
<td>No change in fiber stiffness</td>
<td>Increased free water</td>
</tr>
<tr>
<td>Increased toe region</td>
<td>Increased onset of strain-stiffening of the fibers</td>
<td>Increased fiber crimp</td>
</tr>
<tr>
<td>Decreased tissue anisotropy</td>
<td>No change in fiber dispersion</td>
<td>s-GAG connections between fibrils contribute to the tissue anisotropy</td>
</tr>
</tbody>
</table>
6.2 Key Contributions

This work included 4 key contributions to the field of experimental mechanics and scleral biomechanics:

(1) The comparison between the error and uncertainty in displacements and strain under static conditions as well as under nonuniform displacement of 2D-DIC and 3D-DIC for an inflation test. Although 2D-DIC could be an alternative to 3D-DIC to characterize the profile behavior of materials inflated under conditions that discourage the use of a stereovision system, 3D-DIC should be used to characterize the three-dimensional anisotropic behavior of heterogeneous materials.

(2) The development of a numerical methodology to evaluate how the 3D-DIC displacement error and uncertainty translate into an error and uncertainty in strains for a specimen under a 3D deformation, such as an inflation.

(3) The development of a protocol to effectively degrade chondroitin sulfate and dermatan sulfate GAGs from the posterior sclera of pig and human eyes.

(4) The finding that s-GAGs play important structural and mechanical roles in both the posterior human and porcine sclera, through their effects on hydration and their interactions with the collagen fibrils. s-GAG removal induces an overall stiffening of the posterior sclera from older donors, when it makes
the posterior sclera from young animals more compliant. Therefore, the effect of s-GAG degradation seems to be dependent on the scleral age and hence microstructure. s-GAG digestion also seems to change the collagen fibril structure and collagen network architecture, which needs to be further investigated. The findings also suggest that variations in the scleral s-GAGs content could be involved in the mechanical alterations observed in glaucoma and myopia. In the long-term, new therapies based on the controlled alteration of the scleral s-GAG content could be beneficial for some patients.

6.3 Limitations

The limitations specific to each part of this thesis were detailed in the corresponding chapter. However, several important limitations should be acknowledged here. In the porcine study of chapter 3, no direct mechanical comparison was made between control and ChABC-treated scleras. The direct comparison between control and buffer-treated scleras, as well as buffer-treated and ChABC-treated scleras, were chosen to distinguish the mechanical effects of an increased hydration from those of s-GAG degradation. The human study of chapter 4 did not include hydration measurements as those could not be performed on eyes subjected to inflation. There was no comparison of buffer-treated and ChABC-treated eyes with controls (no treatment), as the control eyes were poor references. The eyes were received at different
times after death, they were not immersed in solution but shipped in humidified jars, therefore their hydration surely varied. The high inter-specimen variability due to differences in age and handling \textit{post-mortem} and the small strains measured did not allow the study of regional variations. In the inverse FEM problem of chapter 5, the numerical model could not capture well the local heterogeneities of the raw 3D-DIC displacement data due to the simplified geometry and boundary conditions. The hyperelastic constitutive model describing the posterior sclera captured the collagen fiber recruitment, stiffness and anisotropy but did not describe the collagen microstructure, such as its crimp morphology and diameter. In addition, this model did not incorporate the swelling, poroelastic and electrostatic effects of s-GAG degradation.

\section*{6.4 Future Directions}

The main future directions include: 1) investigating the structural changes at the microscale following s-GAG degradation, 2) investigating the effects of collagen crosslinks on the mechanical effects of s-GAGs degradation, 3) modeling the role of s-GAGs in the posterior sclera, 4) studying the structural and mechanical effects of s-GAG digestion in the peripapillary sclera, and 5) developing the \textit{in-vivo} treatment of animal models of glaucoma and myopia with ChABC.

In the short term, the primary focus should be to evaluate the microstructural
changes following s-GAG removal in both the pig and human sclera, as they could help explain the changes in the scleral mechanical behavior measured. Those information include the change in the type of hydration in the tissue (amount of bound versus free water), in the collagen fibril structure (fibril crimp) and in the collagen network architecture (interfibrillar/intrafibrillar spacing, fibril fusion/thinning). Those structural properties could also help explain the scleral thinning observed in the posterior human sclera: whether caused by a decrease in hydration or a rearrangement of the tissue, such as the fusion of the collagen fibrils. The amount of bound versus free water in the tissue can be determined using a combination of differential scanning calorimetry and desiccation. The fibril crimp can be observed using second harmonic generation imaging. The fibril crimp could also be indirectly evaluated using a micromechanical model, such as the one developed by Tonge et al or by Grytz and Meschke. The interfibrillar spacing can be evaluated by measuring the partition coefficient of the sclera for molecules of various sizes or the permeability of the tissue using fluorescence recovery after photobleaching. The interfibrillar spacing can also be measured using small-angle x-ray scattering and the change in the intrafibrillar spacing between the collagen molecules can be determined using wide-angle x-ray scattering. The collagen fibril arrangement, including their thinning or fusion, can be observed using transmission electron microscopy.

Another focus should be to determine the influence of collagen crosslinks on the mechanical effects of s-GAG removal. The changes in the mechanical behavior after
s-GAG degradation could be compared between human and crosslinked pig scleras. This could provide information about the role of the additional collagen crosslinks in older human donor eyes on the mechanical differences measured between pig and human scleras with s-GAG removal.

A hyperelastic model was used to describe the pseudoelastic behavior of the posterior sclera in chapter 5. The parameters of the model were determined from inflation tests of ChABC-treated specimens and the results were applied to infer the effects of s-GAG digestion on the mechanical properties of the tissue. However, the model does not consider the swelling, poroelastic and electrostatic effects of s-GAG digestion. Models describing the effects of GAGs in connective tissues exist in the literature. Poroelastic-multiphasic models describe the tissue swelling associated with the presence of charged molecules.\textsuperscript{245–249} Electrostatic models use the Poisson-Boltzmann equation to describe the spatial variations in the electrical potential and obtain the electrostatic free energy due to the presence of charged entities.\textsuperscript{250–257} However, none of the models describe the influence of GAGs on the collagen fibril structure or collagen network architecture. Micro-mechanical models have also been developed. Those models represent GAGs as springs between the collagen fibrils,\textsuperscript{258,259} but do not include the GAG swelling, poroelastic and electrostatic effects. Future work should develop a micro-mechanical model that considers those effects and their influence on the collagen fibril structure and collagen network architecture.

It would also be of great interest to evaluate the structural and mechanical changes
in the human peripapillary sclera, the region of the posterior sclera closest to the lamina cribrosa, the site of axonal damage in glaucoma, due to experimental s-GAG degradation. Compared to the rest of the posterior sclera, the peripapillary sclera is thicker, has a circumferential arrangement of the collagen fibrils on its outer surface (closer to the eye’s cavity) and shows significant changes in its mechanical properties in glaucoma. One of the challenges will be glares in this highly curved region around the ONH that will prevent DIC correlation. Light diffusers placed in front of the light source could help reduce those glares.

In the long term, the in-vivo injection of ChABC in a mouse model of glaucoma or in a tree shrew model of myopia followed by the evaluation of the RGC axonal damage or axial elongation would help determining whether s-GAG removal could be beneficial against either ocular pathology. New ways to affect the s-GAG composition of the posterior sclera could also be developed or evaluated, such as the attachment of s-GAGs to collagen via crosslinking. However, one should keep in mind that the effects measured in those young animals might not be representative of the changes that would occur in older human eyes, as demonstrated in this work.
Appendix A

Appendix: Experimental details

A.1 Experimental setup

The main experimental setup used for DIC imaging and mechanical testing of porcine and human eyes described in chapters 3 and 4 was initially designed for the study of bovine eyes by a former post-doc in our lab, Kristin Myers (now a Professor at Columbia University, NY). However, important personal contributions to the setup were made and are described in the next three sections. Those include the design of holders adapted to the mechanical testing of porcine and human posterior scleral shells, the tuning of the PID parameters for the pressure-controlled mechanical tests of these specimens, and the design of an ultrasonic apparatus for scleral thickness measurement.
A.1.1 Scleral holder design

The scleral holders designed for the mechanical testing of porcine eyes are designs 2a and 2b (Fig. A.1(a) and (b)). The scleral holders 2a were also used for the human eyes. The holders were designed in SolidWorks (Dassault Systmes SolidWorks Corp., Waltham MA) based on the intersection of a sphere with a plate, and machined out of a transparent acrylic sheet. The holder designs have different curvatures at the hole to accommodate for different eye dimensions and ensure a close fit.
APPENDIX A. APPENDIX: EXPERIMENTAL DETAILS

Table A.1: PID parameters used to equilibrate the porcine specimens at baseline pressure 0.28 kPa for 30 min and hold them at baseline pressure while taking the thickness measurements.

<table>
<thead>
<tr>
<th>Load</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>10000</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure A.2: Schematic of the loading regime used to equilibrate the porcine specimens and measure their thickness at baseline pressure.

A.1.2 PID control of pressure

The mechanical tests performed on the porcine and human posterior scleral shells were pressure-controlled. However, the pressure inside the inflation chamber was controlled by a PID feedback loop, that actuated the MTS crosshead responsible for the injection of DPBS inside the chamber, based on the pressure transducer readings. The PID parameters had to be finely tuned to avoid inertial effects due to the control of the MTS crosshead displacement rather than the pressure itself, and are listed below for each inflation test performed on the porcine specimens (Tables A.1, A.2, A.3 and corresponding Figures A.2, A.3, A.4). The same PID parameters were used for the inflation of the human specimens from baseline pressure 0.21 kPa.
APPENDIX A. APPENDIX: EXPERIMENTAL DETAILS

Table A.2: PID parameters used to load the porcine specimens from baseline pressure 0.28 kPa to 6 kPa at 0.13 kPa/s, unload them to 0.28 kPa at the same rate and hold them at baseline pressure for 30 min for recovery.

<table>
<thead>
<tr>
<th></th>
<th>Load</th>
<th>Unload</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>25000</td>
<td>200</td>
<td>10000</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure A.3: Schematic of the loading regime for a load-unload cycle followed by a recovery period for the porcine specimens.

Table A.3: PID parameters used to load the porcine specimens from baseline pressure 0.28 kPa to 6 kPa at 1.03 kPa/s, hold them at 6 kPa for 20 min, unload them to 0.28 kPa at the same rate and hold them at baseline pressure for 40 min for recovery.

<table>
<thead>
<tr>
<th></th>
<th>Load</th>
<th>Hold</th>
<th>Unload</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>8000</td>
<td>5000</td>
<td>8000</td>
<td>10000</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>0.05</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure A.4: Schematic of the loading regime for a creep test followed by a recovery period for the porcine specimens.
A.1.3 Thickness measurement apparatus

Basics of ultrasound measurements:

- A pulser-receiver sends an electrical pulse to an ultrasound transducer, which is in contact with the object to be characterized.

- The ultrasound transducer converts the electrical wave into an ultrasound wave that propagates through the object and partly reflects back from interfaces between materials having a different acoustic impedance (product of the material density and ultrasound propagation speed). The larger the difference in acoustic impedance between the materials, the larger the portion of the wave reflected at their interface.

- The reflected ultrasound waves, called echoes, travel back to the ultrasound transducer where they are converted back into electrical signals that are transmitted to the pulser-receiver.

- The number of echoes and the time difference between them provide information about the number, position, and therefore size, of the different material layers within the object.

Setup components:

- Ultrasound transducer: delay line ultrasound transducer, 15 MHz, 3 mm diameter tip (V26045, Olympus NDT Inc., Waltham MA), fitted with a Sonopen delay tip with diameter 1.5 mm (DLP-302, Olympus NDT Inc., Waltham MA)
APPENDIX A. APPENDIX: EXPERIMENTAL DETAILS

-Pulser-receiver: high-frequency ultrasonic pulser/receiver, 75 MHz bandwidth, 39 dB, RF gain (5073PR-15-U, Olympus NDT Inc., Waltham MA)

-Oscilloscope: digital real-time oscilloscope, 100 MHz bandwidth, 1 GS/s sampling rate (TDS220, Tektronix Inc., Beaverton OR)

Setup assembly:

The transmit/receive (T/R) connector of the pulser-receiver was connected to the ultrasound transducer, while the pulser-receiver synchronization (SYNC OUT) and frequency (RF OUT) output connectors were connected to the channel 1 (CH 1) and channel 2 (CH 2) connectors of the oscilloscope, respectively (Fig. A.5(a) and (b)). The T/R connector was used to transmit and receive the electrical signals to and from the ultrasound transducer. The electrical pulse sent to the ultrasound transducer was available at the SYNC OUT connector and displayed on channel 1 of the oscilloscope, while the electrical signal from the ultrasound transducer was available at the RF OUT connector and visible on channel 2 of the oscilloscope. The oscilloscope was linked to a computer for signal recording using the WaveStar software for oscilloscopes (Tektronix Inc., Beaverton OR).

Pulser-receiver parameters:
APPENDIX A. APPENDIX: EXPERIMENTAL DETAILS

-Pulse repetition frequency (PRF): 1 kHz

-Pulse energy: 8 $\mu$J (position 3)

-Damping resistance: 33 $\Omega$ (position 2)

-Gain: +35 dB

-Mode: pulse-echo (position 1)

-High-pass filter (HPF): 1 kHz (OUT)

-Low-pass filter (LPF): 75 MHz (FULL BW)

Thickness measurement:

-Pressurize the posterior scleral shell at baseline pressure to avoid tissue wrinkles.

-Position the ultrasound transducer perpendicular to the scleral surface and record the ultrasound echo trace displayed on the oscilloscope. Do not press down on the specimen surface and check for the presence of water between the transducer tip and specimen surface, as both will produce inaccurate measurements.

-Calculate the scleral thickness $T$ as $T = \frac{1}{2}c_{\text{sclera}}\Delta t$, with $c_{\text{sclera}}$ the speed of sound in the sclera and $\Delta t$ the peak-to-peak time difference between the echo signals from the outer and inner scleral surfaces (Fig. A.5(c)). Assuming a speed of sound in the sclera of 1597 m/s, the thickness resolution of the system was 1.6 $\mu$m.
Figure A.5: (a) Image and (b) schematic of the ultrasound apparatus for the scleral thickness measurement, showing the ultrasound transducer, pulser-receiver, oscilloscope and computer, as well as their connections. The T/R connector is on the front panel of the pulser-receiver, while the SYNC OUT and RF OUT connectors are on the pulser-receiver back panel. (c) Electrical trace from the ultrasound transducer showing the peak-to-peak time difference between the echo signals from the outer and inner scleral surfaces.

A.2 Experimental protocols

Note: All reagents listed in this section that are not buffers or solutions are powder reagents.
A.2.1 Glycosaminoglycan degradation

Solutions:

- Reconstitution solution: 0.01% bovine serum albumin (A3912, Sigma-Aldrich, St. Louis MO) in distilled water
- Dilution solution: 50 mM Trizma base, 60 mM sodium acetate (S8750, Sigma-Aldrich, St. Louis MO), 0.02% bovine serum albumin in distilled water
- GAG degradation buffer (no ChABC, can be stored at 4°C for up to 1 week): 1:4 ratio of reconstitution to dilution solution
- GAG degradation buffer with protease inhibitors (no ChABC): 1 protease inhibitor tablet (S8830, Sigma-Aldrich, St. Louis MO) for 100 ml GAG degradation buffer
- ChABC solution (to be prepared just before use): ChABC (C2905, Sigma-Aldrich, St. Louis MO) at 2 units/ml in GAG degradation buffer
- ChABC solution with protease inhibitors (to be prepared just before use): ChABC at 2 units/ml in GAG degradation buffer containing 3.4% of GAG degradation buffer with protease inhibitors

Protocol:

- Incubate the scleral samples in GAG degradation buffer (buffer-treatment) or freshly prepared ChABC solution with or without protease inhibitors (enzyme-treatment), for 18 h at 37°C, on an orbital shaker, in a way that prevents
evaporation.

-After incubation, rinse the samples in GAG degradation buffer for 30 min, on an orbital shaker, with 4 buffer changes (after 3, 5, 10 and 20 min).

A.2.2 Qualitative assessment of the glycosaminoglycan content

Solutions:

- Fixation solution: 4% paraformaldehyde in 0.1 M PO₄ buffer at pH 7.2. Add 1% cetylpyridinium chloride (C0732, Sigma-Aldrich, St. Louis MO) just before use

- 0.1 M Sorensen’s sodium phosphate buffer at pH 7.2

- 0.1 M PO₄ buffer at pH 7.2 with either 5% or 20% sucrose (A15583, Alfa Aesar, Ward Hill, MA)

- 0.1 M sodium acetate buffer at pH 5.8

- Alcian Blue solutions: 0.1% Alcian Blue (A3157, Sigma-Aldrich, St. Louis MO) in 25 mM sodium acetate buffer (diluted from stock in distilled water) at pH 5.8 with 0.06 M or 0.5 M MgCl₂ (M8266, Sigma-Aldrich, St. Louis MO)

Protocol:

- Fixation:
APPENDIX A. APPENDIX: EXPERIMENTAL DETAILS

- Incubate the sleral samples in fixation solution for 15 h at 4°C.
- Rinse the samples in Sorensen’s phosphate buffer 3x3 min on a rotator.

- Cryo-preservation:
  - Wash the samples 3x10 min in 5% sucrose in PO₄ buffer on a rotator.
  - Wash in 2:1 of 5% sucrose/PO₄ buffer to 20% sucrose/PO₄ buffer for 30 min.
  - Wash in 1:1 (5% sucrose/PO₄ buffer:20% sucrose/PO₄ buffer) for 30 min.
  - Wash in 1:2 (5% sucrose/PO₄ buffer:20% sucrose/PO₄ buffer) for 30 min.
  - Wash in 20% sucrose in PO₄ buffer for 3 h.
  - Wash in 2:1 (20% sucrose/PO₄ buffer:optimum cutting temperature (OCT) compound) for 30 min.

- Cryo-embedding:
  - Embed the samples in the same 2:1 (20% sucrose/PO₄ buffer:OCT compound) fresh solution.
  - Freeze immediately in a dry ice/isopentane bath for 15 min.
  - Keep each block wrapped in aluminum foil in a freezer -80°C until sectioning.

- Cryo-sectioning:
  - Cut 10 μm thin sections from each block in the cryostat at -25°C and place
them on glass slides.

- Let the slides dry at room temperature for about 1 h before boxing them and placing them back in the fridge at -80°C until staining.

- **Staining:**
  
  - Thaw the slides for about 30 min.
  
  - Hydrate the sections in distilled water for 5 min.
  
  - Immerse the sections in Alcian Blue solution with 0.06 M or 0.5 M MgCl₂ for 30 min.
  
  - Wash the sections with running distilled water for 4-5 min until water is clear.
  
  - Dehydrate in 70% (1x2 min), 95% (1x2 min) and 100% ethanol (2x2 min).
  
  - Clear with xylene.
  
  - Mount with a resinous medium.

- **Imaging:**
  
  - Image with a light microscope.
A.2.3 Quantitative assessment of the glycosaminoglycan content

Solutions:

- 100 mM Trizma buffer at pH 7.2 (made from Trizma base and Trizma HCl in distilled water)
- Tissue digestion buffer (no papain, can be stored at 4°C up to 1 week): 1 mM ethylenediaminetetraacetic acid (E4884, Sigma-Aldrich, St. Louis MO), 1 mM cysteine-HCl (C7880, Sigma-Aldrich, St. Louis MO), 50 mM Trizma buffer in distilled water
- Papain solution (to be prepared just before use): 1 µl papain buffered aqueous suspension (P3125, Sigma-Aldrich, St. Louis MO) per 100 µl tissue digestion buffer

Protocol:

Note: The samples should be kept hydrated, in GAG degradation buffer (see section A.2.1) for the buffer-treated and enzyme-treated specimens, or in a hydration chamber for the controls, prior to the procedure.

- Tissue digestion:
  - Weigh the scleral samples immediately after blotting them dry on a Whatman
APPENDIX A. APPENDIX: EXPERIMENTAL DETAILS

paper for 1 min. Record the wet weight of each sample.

-Cut the samples in small pieces, place them in closed plastic tubes (with O-ring caps to prevent evaporation) and incubate them in freshly prepared papain solution for 18 h in an oven at 60°C.

- Blyscan assay (Biocolor Ltd, UK):
  - Remove the tubes from the oven and shake them to mix their content.
  - Centrifuge the tubes at 10,300 rpm for 10 min.
  - Invert the tubes onto new microtubes to decant off the supernatant. Do not pipette the remaining drops from the bottom of the tube but gently tap the tubes against each other. Discard the tubes containing the pellet.
  - For each sample, take duplicate aliquots of the previously recovered supernatant, put them into new colorful microtubes and fill up to 100 µl with distilled water. The aliquots should contain enough s-GAGs to generate a spectrophotometer absorbance >0.1 but <1.5.
  - Add 1 ml Blyscan dye reagent to each tube.
  - Vortex mix the tubes for 30 min.
  - Centrifuge the tubes at 12,500 rpm for 10 min. Add an additional 5 min if the pellet formed is not tightly packed.
  - Drain the tubes carefully by inverting them onto a beaker and gently tapping them against the beaker edge to remove as much supernatant as possible. Be
Appendix A. Appendix: Experimental Details

careful not to lose part of the pellet during this process. Remove the remaining supernatant droplets on the tube cap and opening using a cotton tipped applicator.

- Add 1 ml dissociation agent to each tube.
- Vortex mix the tubes for 15 min.
- Centrifuge the tubes at 12,000 rpm for 5 min.
- Prepare a tube with 1 ml distilled water for the spectrophotometer blank.
- Place the blank and content of tubes sequentially in the spectrophotometer.

For each sample, check that the absorbance is >0.1 and <1.5. Outside this range, the correlation between absorbance and s-GAG concentration might not be linear.

- Perform a wavelength scan and extract the absorbance value at 656 nm.

- s-GAG content calculation:

  - Deduce the s-GAG content per wet tissue weight from the absorbance values using the calibration curve. The calibration curve relating the absorbance to the s-GAG content is produced using the same procedure as described above, but with known quantities of a s-GAG standard (see the Blyscan assay manual\textsuperscript{261}). The duplicate absorbance values should be within ±5% of their mean value. Use the hydration measurements (see section A.2.4) to infer the s-GAG content per dry tissue weight.
A.2.4 Hydration measurement

Protocol:

Note: The samples should be kept hydrated, in GAG degradation buffer (see section A.2.1) for the buffer-treated and enzyme-treated specimens, or in a hydration chamber for the controls, prior to the procedure.

- **Weight measurements:**
  - Take the scleral samples, blot them dry on Whatman paper for 1 min, and immediately weigh them on a precision balance. Record the wet weight of each sample. To measure the sample weight more precisely, place each sample into an eppendorf tube prior to weighing (to prevent further water evaporation). Subtract the tube weight (measured beforehand) from the measured weight to get the sample weight.
  - Place each sample in a microfuge tube resistant to heat, leave the cap open and place all tubes in an oven at 60°C for 48 h.
  - Take the dry samples out their tube using tweezers and weigh them. Record the dry weight of each sample. Be careful not to crush or drop the dried samples as they are very brittle and small pieces might detach, leading to inaccurate weight measurements.
APPENDIX A. APPENDIX: EXPERIMENTAL DETAILS

• Hydration calculation:

  - Calculate the wet over dry tissue weight ratio as a measure of the tissue hydration. This ratio can be used to infer the s-GAG content per dry tissue weight from the s-GAG quantification measurements (see section A.2.3).
Appendix B

Appendix: Numerical details

B.1 Converged model parameters

The converged set of parameters and corresponding cost function $C$ for all porcine specimens of protocol 1 (control/buffer-treated) and protocol 2 (buffer-treated/enzyme-treated) are listed in Tables B.1, B.2, B.3, and B.4.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>$\mu$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$k$</th>
<th>$C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen P.180713</td>
<td>62.11</td>
<td>0.001</td>
<td>136.54</td>
<td>0.66</td>
<td>5.02e-4</td>
</tr>
<tr>
<td>Specimen P.250713</td>
<td>25.93</td>
<td>0.807</td>
<td>27.34</td>
<td>0.36</td>
<td>7.71e-4</td>
</tr>
<tr>
<td>Specimen P.180913</td>
<td>62.73</td>
<td>0.001</td>
<td>136.29</td>
<td>0.26</td>
<td>4.25e-4</td>
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<tr>
<td>Specimen P.291013</td>
<td>61.81</td>
<td>0.040</td>
<td>89.61</td>
<td>4.2e-7</td>
<td>5.54e-4</td>
</tr>
<tr>
<td>Specimen P.211113</td>
<td>80.30</td>
<td>0.001</td>
<td>145.72</td>
<td>0.78</td>
<td>4.07e-4</td>
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</tbody>
</table>

Table B.1: Table showing the 4 optimized model parameters and corresponding cost function for the control porcine specimens from protocol 1.
### APPENDIX B: NUMERICAL DETAILS

<table>
<thead>
<tr>
<th>Specimen</th>
<th>$\mu$ [kPa]</th>
<th>$\alpha$ [kPa]</th>
<th>$\beta$ [no unit]</th>
<th>$k$ [no unit]</th>
<th>$C$ [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen P.180713</td>
<td>62.18</td>
<td>0.001</td>
<td>124.29</td>
<td>1.19</td>
<td>5.90e-4</td>
</tr>
<tr>
<td>Specimen P.250713</td>
<td>32.51</td>
<td>0.035</td>
<td>45.10</td>
<td>0.65</td>
<td>8.71e-4</td>
</tr>
<tr>
<td>Specimen P.180913</td>
<td>71.22</td>
<td>0.001</td>
<td>126.34</td>
<td>0.52</td>
<td>4.43e-4</td>
</tr>
<tr>
<td>Specimen P.291013</td>
<td>72.99</td>
<td>0.001</td>
<td>120.81</td>
<td>1.2e-6</td>
<td>6.73e-4</td>
</tr>
<tr>
<td>Specimen P.211113</td>
<td>84.90</td>
<td>0.001</td>
<td>135.38</td>
<td>1.58</td>
<td>4.73e-4</td>
</tr>
</tbody>
</table>

**Table B.2:** Table showing the 4 optimized model parameters and corresponding cost function for the buffer-treated porcine specimens from protocol 1.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>$\mu$ [kPa]</th>
<th>$\alpha$ [kPa]</th>
<th>$\beta$ [no unit]</th>
<th>$k$ [no unit]</th>
<th>$C$ [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen P.170713</td>
<td>29.89</td>
<td>0.010</td>
<td>48.37</td>
<td>0.36</td>
<td>7.86e-4</td>
</tr>
<tr>
<td>Specimen P.240713</td>
<td>17.82</td>
<td>0.764</td>
<td>26.83</td>
<td>1.7e-8</td>
<td>7.50e-4</td>
</tr>
<tr>
<td>Specimen P.020813</td>
<td>34.04</td>
<td>0.001</td>
<td>68.11</td>
<td>6.5e-5</td>
<td>8.81e-4</td>
</tr>
<tr>
<td>Specimen P.310713</td>
<td>28.92</td>
<td>0.074</td>
<td>38.60</td>
<td>0.61</td>
<td>8.48e-4</td>
</tr>
<tr>
<td>Specimen P.311013</td>
<td>61.36</td>
<td>0.001</td>
<td>106.55</td>
<td>2.73</td>
<td>6.20e-4</td>
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<tr>
<td>Specimen P.131113</td>
<td>67.81</td>
<td>0.001</td>
<td>110.28</td>
<td>5.5e-7</td>
<td>5.06e-4</td>
</tr>
<tr>
<td>Specimen P.201113</td>
<td>59.01</td>
<td>0.001</td>
<td>84.55</td>
<td>0.16</td>
<td>7.32e-4</td>
</tr>
</tbody>
</table>

**Table B.3:** Table showing the 4 optimized model parameters and corresponding cost function for the buffer-treated porcine specimens from protocol 2.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>$\mu$ [kPa]</th>
<th>$\alpha$ [kPa]</th>
<th>$\beta$ [no unit]</th>
<th>$k$ [no unit]</th>
<th>$C$ [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen P.170713</td>
<td>7.57</td>
<td>0.423</td>
<td>22.47</td>
<td>0.42</td>
<td>9.66e-4</td>
</tr>
<tr>
<td>Specimen P.240713</td>
<td>15.00</td>
<td>0.381</td>
<td>25.91</td>
<td>4.3e-4</td>
<td>1.19e-3</td>
</tr>
<tr>
<td>Specimen P.020813</td>
<td>19.04</td>
<td>0.001</td>
<td>53.71</td>
<td>1.2e-4</td>
<td>8.88e-4</td>
</tr>
<tr>
<td>Specimen P.310713</td>
<td>16.68</td>
<td>0.001</td>
<td>52.90</td>
<td>0.31</td>
<td>9.38e-4</td>
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<tr>
<td>Specimen P.311013</td>
<td>20.49</td>
<td>0.399</td>
<td>33.44</td>
<td>0.57</td>
<td>7.16e-4</td>
</tr>
<tr>
<td>Specimen P.131113</td>
<td>22.95</td>
<td>0.310</td>
<td>36.65</td>
<td>1.1e-7</td>
<td>6.21e-4</td>
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<td>Specimen P.201113</td>
<td>27.64</td>
<td>0.037</td>
<td>40.73</td>
<td>0.51</td>
<td>7.83e-4</td>
</tr>
</tbody>
</table>

**Table B.4:** Table showing the 4 optimized model parameters and corresponding cost function for the enzyme-treated porcine specimens from protocol 2.
B.2 Comparison between experimental and FEM 3D surface displacements

Maps of the raw experimental surface displacements and corresponding FEM displacements at maximum pressure, for the converged set of parameters, are shown in Figures B.1, B.2 and B.3 for a representative control, buffer-treated and enzyme-treated specimen, respectively. The mean error was calculated as the difference between the average experimental and numerical displacement across the maps.
Figure B.1: Maps comparing the experimental and FEM displacement components [mm] at maximum pressure for a representative control porcine specimen from protocol 1.
Figure B.2: Maps of the experimental and FEM displacement components [mm] at maximum pressure for a representative buffer-treated porcine specimen from protocol 1.
Figure B.3: Maps of the experimental and FEM displacement components [mm] at maximum pressure for a representative enzyme-treated porcine specimen from protocol 2.
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Vita

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