The use of small angle light scattering in assessing strain induced collagen degradation in arterial tissue ex vivo

Gaul, R.T.[1,2], Nolan, D.R.[1,2], Lally, C.[1,2]

[1]Trinity Centre for Bioengineering, Trinity College Dublin, Dublin, Ireland.

[2]School of Engineering, Trinity College Dublin, Dublin, Ireland.

Abstract

Collagen is the predominant load bearing component in many soft tissues including arterial tissue and is therefore critical in determining the mechanical integrity of such tissues. Degradation of collagen fibres is hypothesized to be a strain dependent process whereby the rate of degradation is affected by the magnitude of strain applied to the collagen fibres. The aim of this study is to investigate the ability of small angle light scattering (SALS) imaging to identify strain dependent degradation of collagen fibres in arterial tissue *ex vivo,* and determine whether a strain induced protection mechanism exists in arterial tissue as observed in pure collagen and other collagenous tissues. SALS was used in combination with histological and second harmonic generation (SHG) analysis to determine the collagen fibre architecture in arterial tissue subjected to strain directed degradation. SALS alignment analysis identified statistically significant differences in fibre alignment depending on the strain magnitude applied to the tissue. These results were also observed using histology and SHG. Our findings suggest a strain protection mechanism may exist for arterial collagen at intermediate strain magnitudes between 0% and 25%. These findings may have implications for the onset and progression of arterial disease where changes in the mechanical environment of arterial tissue may lead to changes in the collagen degradation rate.

1 Introduction

Collagen is the most abundant protein in mammals and is responsible for providing much of the load bearing capacity in connective tissue. In soft tissues, collagen fibres along with elastin are, in general, responsible for resisting tensile loading. Remodelling of this collagen network, which is influenced by degradation, is therefore crucial for the integrity of soft tissues, such as arteries.

Existing literature has established that collagen fibres exhibit a strain dependent degradation response where the rate of degradation is governed by the magnitude of strain applied along the fibre axis. Interestingly, literature has identified conflicting degradation responses, including strain induced protection (Robitaille et al., 2011; Wyatt et al., 2009), strain induced degradation (Adhikari et al., 2011; Willett et al., 2008) and a combination of both, dependent on the strain magnitude (Gaul et al., 2018; Ghazanfari et al., 2016; Huang and Yannas, 1977). A number of techniques have been utilised to identify these degradation responses across a range of tissues including stress relaxation experiments (Gaul et al., 2018; Huang and Yannas, 1977; Wyatt et al., 2009), creep experiments (Ellsmere et al., 1999; Flynn et al., 2013; Zareian et al., 2010), imaging of single collagen fibres (Wyatt et al., 2009) and fibrils (Flynn et al., 2013), and tissue alignment analysis using small angle light scattering (SALS) (Robitaille et al., 2011). A number of studies have also identified strain induced degradation (Adhikari et al., 2012, 2011) as well as protection (Camp et al., 2011; Chang et al., 2012) occurring at the molecular level. Although links have been made between the molecular level response and the fibril and tissue level response (Camp et al., 2011; Flynn et al., 2013), this relationship still remains unclear (Chang and Buehler, 2014). SALS is a technique which has been used to characterise collagen orientation, alignment and diameter across large regions of soft tissue without the need for time consuming tissue preparation steps (Billiar and Sacks, 1997; Ferdman and Yannas, 1993; Hiester and Sacks, 1998; Sacks et al., 1997). Using SALS, Robitaille *et al.* identified an increase in collagen fibril alignment in corneal tissue over time along the direction of loading with the application of a 4% strain and in the presence of bacterial collagenase. This finding suggests that strain induced protection occurs in loaded fibrils while unloaded fibrils are preferentially degraded. Unfortunately, only 0% and 4%

strain were assessed using SALS, making it unclear as to whether degradation once again increases with further strain as identified in reconstituted collagen tapes (Huang and Yannas, 1977) and bovine pericardium (Ghazanfari et al., 2016). It is likely that a similar degradation response exists in arterial tissue (Gaul et al., 2018), which consists predominantly of type I and III collagen fibres arranged circumferentially in the arterial wall. (Gaul et al., 2017; Holzapfel, 2008; Shahid et al., 2017).

The aim of this study was to investigate the feasibility of SALS to observe changes in collagen fibre distributions in arterial tissue *ex vivo*, subjected to different magnitudes of strain whilst in a collagenase solution and to determine whether a strain induced protection or degradation mechanism can be identified. If successfully applied to arterial tissue *ex vivo*, SALS offers a valuable technique for quickly and objectively assessing strain induced collagen fibre degradation.

2 Methods

2.1 Tissue preparation and collagenase treatment

Fresh common carotid arteries were excised from 6-month-old Large White pigs (n = 10) and transported on ice before undergoing cryopreservation in the presence of a cryoprotectant to maintain vessel structural properties (Müller-Schweinitzer, 2009). Prior to testing, samples were thawed in a water bath at 37°C, cut open longitudinally and opened out flat. Circumferential strips were next cut using a 4 mm wide punch for experimental testing. The intima and adventitia were then carefully removed; the media consists of predominantly circumferentially orientated fibres, and the reduction in thickness ensured light transmission through the sample for SALS analysis (Gaul et al., 2017). Samples were then clamped in a custom stretching jig before being placed under a nominal circumferential strain of 0% (n = 6), 5% (n = 8) or 25% (n = 7). Strained samples were allowed to relax for approximately 30 minutes in PBS and placed in bacterial collagenase (Type I, 87.5 U/ml; Sigma-Aldrich, Ireland) at 37°C for a period of 4 hours before post degradation analysis. Controls (n = 6) for each strain condition were incubated at 37°C for 4 hours in PBS.

2.2 Histology

Samples chosen for histological analysis were fixed in 4% paraformaldehyde overnight, dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Embedded samples were sectioned at 8 μm using a microtome (RM- 2125RT, LEICA, Germany), affixed to microscope slides and mounted with a coverslip using DPX (Sigma-Aldrich, Ireland). The resulting sections were stained with picrosirius red to assess collagen fibre alignment after the degradation process. Picrosirius red significantly enhances collagen fibre birefringence when viewed under polarised light without negatively impacting perceived fibre orientation (Junqueira et al., 1979). Histological images were taken under crossed polarisers at two polarising angle configurations, 45° apart, using an Olympus BX-41 microscope equipped with a QImaging MicroPublisher 5.0 RTV camera. The resulting images were merged to create a complete picture of the fibre architecture, showing diagonal and off-diagonal fibres.

2.3 Second Harmonic Generation

Second harmonic generation (SHG) imaging was conducted with a Carl Zeiss LSM 710 NLO multiphoton microscope (Carl Zeiss, Germany) and Coherent Chameleon tuneable laser (Coherent, USA) to identify differences in fibre architecture due to strain mediated degradation. Samples were excited at 840 nm and the resulting collagen SHG signal collected using non-descanned detectors with a 485 nm short pass emission filters for the 420 nm SHG signal. Tiled image stacks (4 tiles, 12 sections, 6 μm spacing) were taken of the intimal side of the sample at 20X magnification to assess fibre architecture to a depth of 72 μm. Changes in fibre distribution due to degradation were determined in both histological and SHG images by identifying pixel gradients using the OrientationJ plugin (Rezakhaniha et al., 2012) for ImageJ (Schneider et al., 2012).

2.4 SALS

Changes in fibre alignment due to strain controlled degradation were analysed using an in-house SALS system optimised for arterial tissue (Gaul et al., 2017). The system consists of an unpolarised 5 mW HeNe (λ = 632.8 nm; JDSU, Newbury, UK), focusing lens (f_l = 150 mm; Edmund Optics Ltd, York, UK), automated sample positioner, projection screen and a CMOS USB camera. In SALS, a laser is

passed through a fibrous sample and the incident light is scattered orthogonally to the central axis of the constituent fibres to form an ellipse. Similar to early work on bovine pericardium (Hiester and Sacks, 1998; Sacks et al., 1997), SALS analysis was found to be a suitable technique for describing arterial structure, provided individual layers were separated (Gaul et al., 2017). Samples were interrogated pre- and post-degradation using a 150 μm beam diameter at 64 interrogation regions using a scanning grid size of 125 x 125 μm, informed by previous work (Gaul et al., 2017). The results from the 64 interrogation regions were then averaged to get a single measurement per sample. The eccentricity of the scattered beam is a measure of alignment (Gaul et al., 2017), and was recorded pre- and post-degradation for each strain condition. The relative change in SALS eccentricity, ΔE, was calculated by determining the percentage change in eccentricity, E, from the 0 hour time point when no degradation had occurred using Equation 1 and 2.

$$\mathrm{E} = \frac{\sqrt{\mathrm{r}_{maj}{}^2 - \mathrm{r}_{min}{}^2}}{\mathrm{r}_{maj}}, \tag{1}$$

$$\Delta E_t = \frac{E_t - E_{t_0}}{E_{t_0}} \times 100 \tag{2}$$

where r_{maj} and r_{min} are the major and minor axis of the SALS scattered light ellipse respectively, and E_{t_0} and E_t is the eccentricity at 0 and t hours respectively.

3 Results

3.1 Histological analysis of strain dependent degradation

Distinctly different fibre architectures were identified across each strain group using orientation analysis of picrosirius red stained histology samples, viewed under polarised light. This was also observed visually (Figure 1). The greatest degree of collagen fibre alignment is observed at 5% when the sample is strained at 5% circumferential strain.

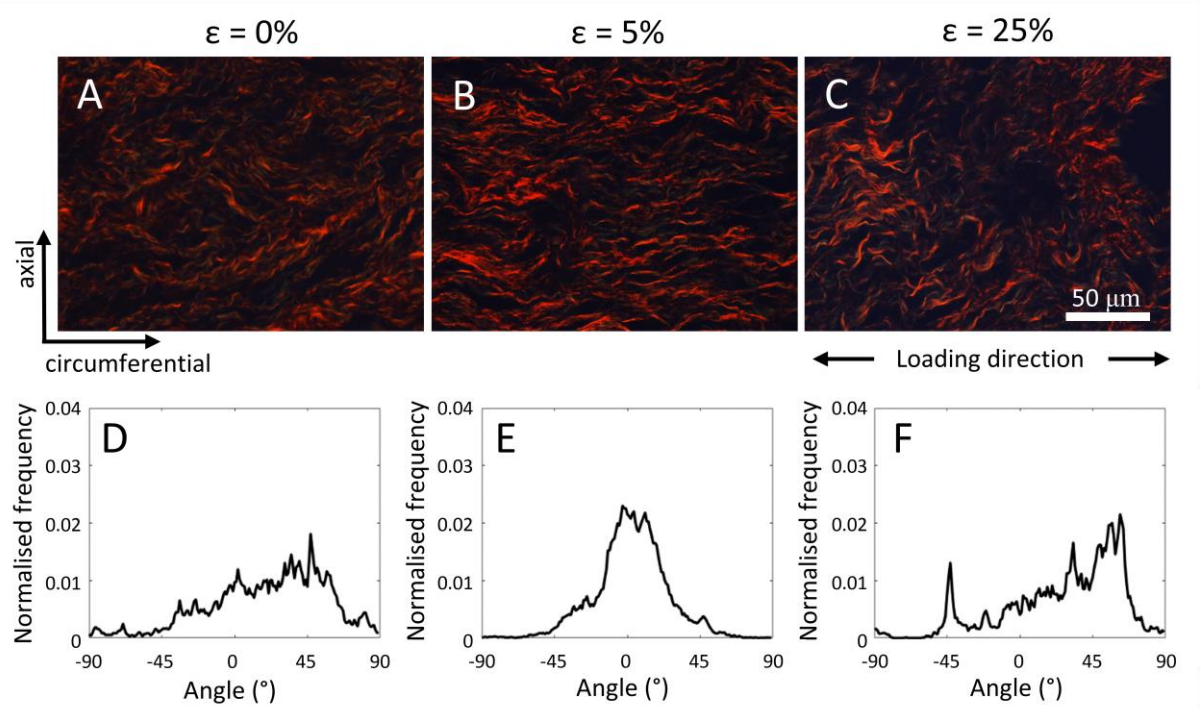

Figure 1: Top panel: Representative histology images of picrosirius red stained artery wall after 4 hours of collagenase treatment, showing collagen in red viewed at 40x under polarised light for A) 0%, B) 5% and C) 25% circumferential strain. Bottom panel: Corresponding normalised frequency distribution plots of fibre angle (D, E, F) for the histological images presented in the top panel.

3.2 SHG analysis of strain dependent degradation

Orientation analysis of collagen in intact samples identified a similar alignment response to that seen histologically with the greatest alignment occurring at 5% strain (Figure 2). Greater fibre dispersion is observed at higher and lower strain conditions.

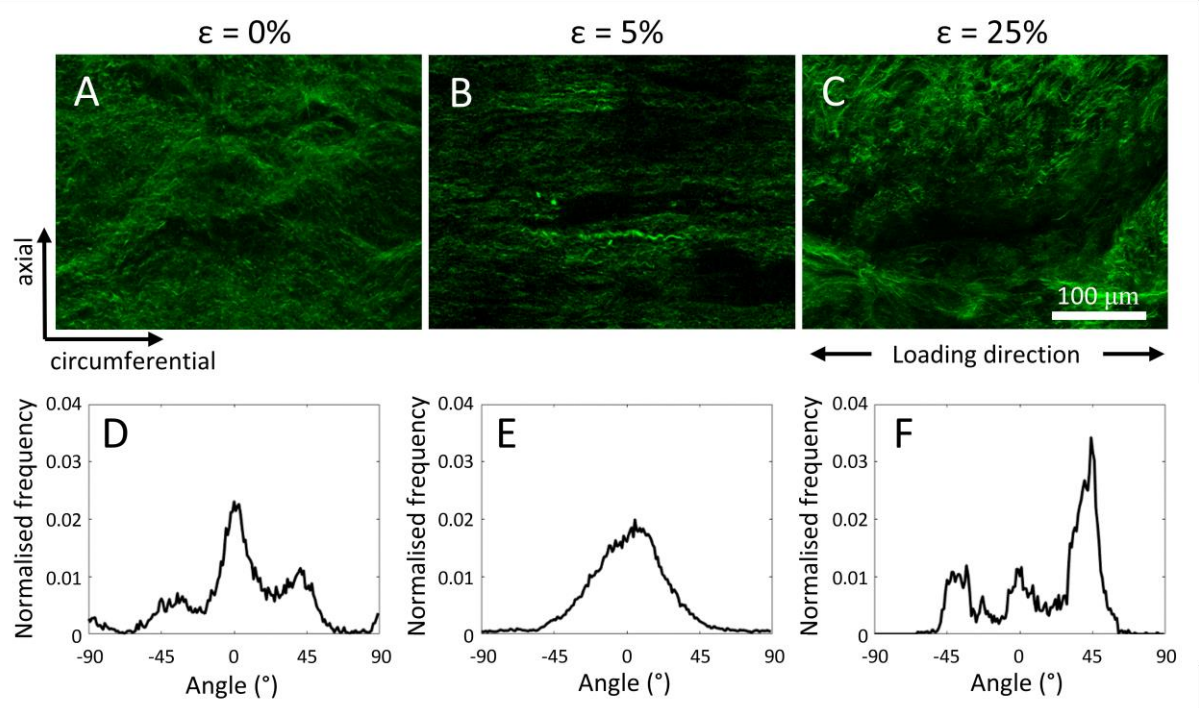

Figure 2: Top panel: Representative SHG images of intact artery wall after 4 hours of collagenase treatment, showing collagen in green viewed at 20x for ***A)*** *0%,* ***B)*** *5% and* ***C)*** *25% circumferential strain. Bottom panel: Corresponding normalised frequency distribution plots of fibre angle* ***(D, E, F)*** *for the SHG images presented in the top panel.*

3.3 SALS analysis of strain mediated degradation

SALS analysis also identified different scattered light distributions occurring in each strain group after 4 hours incubation in bacterial collagenase (Figure 3). Quantitative assessment of relative change in sample eccentricity, a marker of alignment, showed a statistically significant 24% increase in eccentricity at 5% strain compared to 0% and 25% strain (Figure 4). Conversely, 25% circumferential strain led to an 8% reduction in collagen fibre alignment over 4 hours in the presence of bacterial collagenase. Unpaired t-tests confirmed that the eccentricity changes identified in collagenase treated samples were statistically significantly different to the control condition where no collagenase was present for each strain condition.

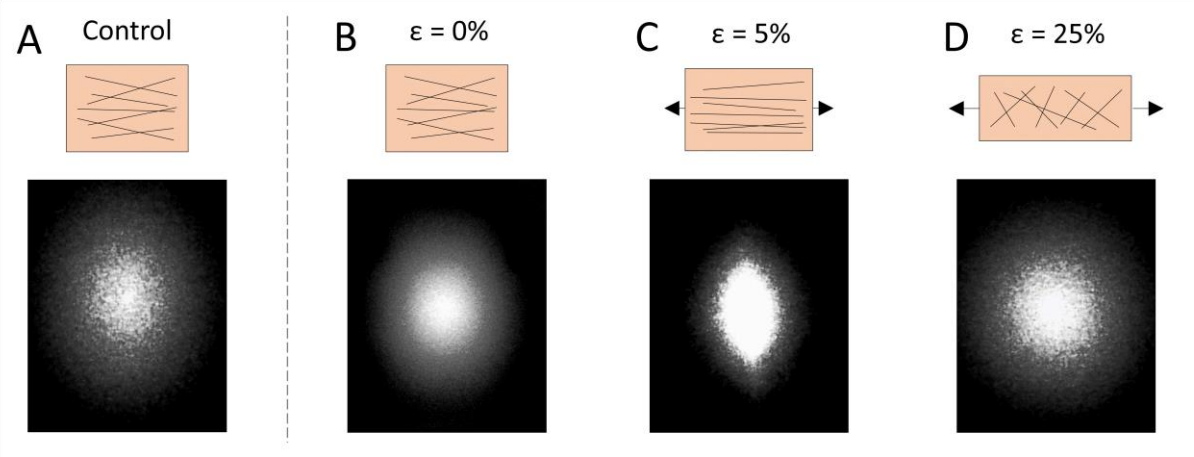

Figure 3: Representative scattered light images at a single location after 4 hours incubation for ***A)*** *0% control,* ***B)*** *0%,* ***C)*** *5% and* ***D)*** *25% circumferential strain showing greater or less fibre alignment determined by the eccentricity of the scattered light distribution. Control samples were incubated in PBS while test samples were incubated in crude bacterial collagenase.*

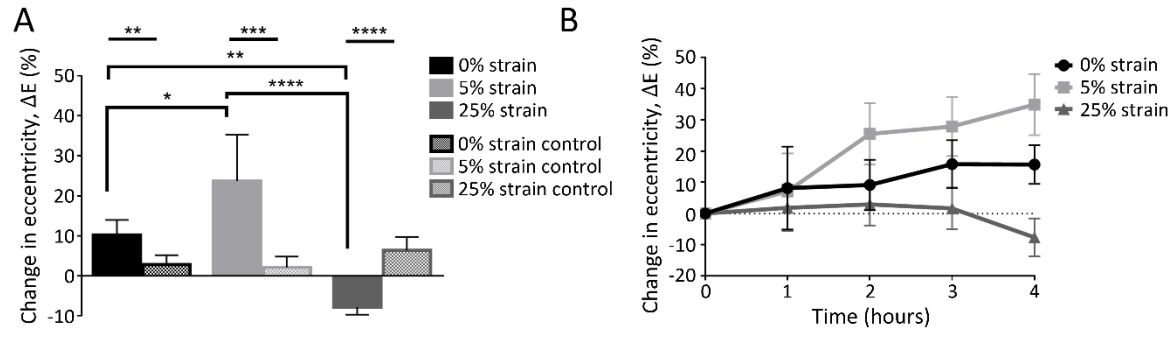

Figure 4: ***A)*** *Relative change in SALS eccentricity in the presence of collagenase after 4 hours for each strain case including their untreated control. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.* ***B)*** *Relative change in eccentricity over time for a single sample at 0%, 5% and 25% strain showing mean and SD.*

4 Discussion

The objective of this study was to investigate whether SALS could be utilised to determine strain induced degradation in arterial tissue *ex vivo* and if so, determine whether a strain induced degradation or protection mechanism exists for arterial tissue. We have demonstrated for the first time that SALS is capable of identifying changes in arterial tissue architecture due to strain induced degradation. Our

findings also suggest that a V-shaped degradation response occurs in arterial tissue whereby accelerated degradation occurs either side of a region of strain dependent protection.

Histological and SHG analysis of degraded arterial tissue show different collagen fibre distributions depending on the strain condition applied (Figure 1 and 2). Highly aligned fibres were identified in samples experiencing intermediate (5%) circumferential strain, while greater disruption to the native network was observed at low (0%) and high (25%) circumferential strain.

SALS analysis of samples experiencing these same strain conditions showed statistically significant differences in sample eccentricity, an indicator of alignment, with the greatest eccentricity change occurring in samples experiencing 5% circumferential strain (Figure 4). It is hypothesised that the slight increase in eccentricity identified at 0% strain may arise due to a sample thinning and clearing effect as collagen is degraded over time, increasing the signal-to-noise ratio. Interestingly, eccentricity or alignment was found to reduce across samples undergoing 25% strain, suggesting preferential degradation of these highly strained fibres. This strain induced protection mechanism was previously identified in corneal tissue using SALS; however, no higher strain magnitude was investigated to identify whether degradation once again increased with strain (Robitaille et al., 2011). Similar findings, showing both strain induced protection and degradation have been identified through mechanical testing, with a minimum degradation rate occurring at approximately 3% strain in reconstituted collagen tapes (Huang and Yannas, 1977) and closer to 20% in bovine pericardium (Ghazanfari et al., 2016). It is hypothesised that the minor increases in eccentricity identified in control samples after 4 hours incubation in PBS at 37°C may be due to fibres reorienting during viscoelastic stress relaxation, as well as a tissue clearing effect over time, resulting in a greater signal-to-noise ratio (Figure 4). No similar trends were identified in angular results. This is likely due to the highly organised single circumferential collagen fibre family previously identified in porcine carotid tissue (Gaul et al., 2017; Sáez et al., 2016) which is unlikely to vary greatly with degradation. No multiple fibre populations were identified using SALS which is consistent with previous investigations of porcine carotid media (Gaul et al., 2017).

SALS offers a valuable technique for quickly and objectively assessing strain induced collagen fibre degradation across large regions, removing much of the subjectivity associated with histological

analysis and the depth limitations associated with SHG analysis. Diffusion tensor imaging (DTI) may offer a potential alternative for looking at similar processes *in vivo* (Flamini et al., 2013, 2010; Shahid et al., 2017), however expensive and time consuming post-processing has thus far limited such applications. The versatility of SALS enables it to be used for real time analysis of tissues in static and dynamic loading environments which is far more challenging with SHG imaging and other high magnification systems due to field of view and depth of field issues. SALS analysis can also be carried out in a number of minutes in comparison to hours for similar size regions in SHG imaging. SALS analysis is particularly suited to imaging thin fibrous tissues and can also be applied to other tissues such as bovine pericardium and dura mater to assess the load induced degradation response of collagen. Although the current study has only considered the medial arterial layer, SALS analysis can also be performed on other arterial layers (Gaul et al., 2017).

It is important to note that the nominal strains were applied to arterial tissue strips rather than dogbone samples which may lead to a non-uniform strain distribution across the sample. Although force data during degradation was not recorded in the current study, a thorough investigation of the mechanical degradation response of arterial tissue can be found elsewhere (Gaul et al., 2018). The study also only considered a small number of strain conditions and further testing is required in order to find the precise strain magnitude corresponding to a minimum collagen degradation rate in arterial tissue. It's should be noted that collagenase diffusion kinetics through the tissue are likely to influence the degradation response identified (Zareian et al., 2010). This, however, has less influence on our SALS results whereby collagen fibres on the light emitting surface of the vessel, which are in contact with the collagenase, have the greatest influence on the resulting signal. Additionally, the crude bacterial collagenase used in this study was found to also degrade non-collagenous material in the vessel wall which may contribute to fibre reorganisation over time when loaded. Although arterial tissue is known to have an out-of-plane, elevation angle, this has been found to be very minor in the case of porcine carotid artery and assumed to have negligible influence here (Sáez et al., 2016). Despite these limitations, significant differences in the structural response of arterial tissue have been identified due to strain induced degradation in an intact arterial layer. These preliminary findings may have

implications for the onset and progression of arterial disease, such as aneurysms, where strain directed degradation may lead to maladaptive remodelling of the surrounding arterial tissue.

Acknowledgements

This publication has emanated from research conducted with the financial support of the Irish Research Council (GOIPG/2014/515), Science Foundation Ireland under the Grant Number SFI/13/ERC/B2775 and SFI/13/CDA/2145 and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No. 637674)

References

Adhikari, A.S., Chai, J., Dunn, A.R., 2011. Mechanical load induces a 100-fold increase in the rate of collagen proteolysis by MMP-1. J. Am. Chem. Soc. 133, 1686–1689. doi:10.1021/ja109972p

Adhikari, A.S., Glassey, E., Dunn, A.R., 2012. Conformational dynamics accompanying the proteolytic degradation of trimeric collagen I by collagenases. J. Am. Chem. Soc. 134, 13259–13265. doi:10.1021/ja212170b

Billiar, K., Sacks, M., 1997. A method to quantify the fiber kinematics of planar tissues under biaxial stretch. J. Biomech. 30, 753–756.

Camp, R.J., Liles, M., Beale, J., Saeidi, N., Flynn, B.P., Moore, E., Murthy, S.K., Ruberti, J.W., 2011. Molecular mechanochemistry: Low force switch slows enzymatic cleavage of human type I collagen monomer. J. Am. Chem. Soc. 133, 4073–4078. doi:10.1021/ja110098b

Chang, S.-W., Buehler, M.J., 2014. Molecular biomechanics of collagen molecules. Mater. Today 17, 70–76. doi:10.1016/j.mattod.2014.01.019

Chang, S.-W., Flynn, B.P., Ruberti, J.W., Buehler, M.J., 2012. Molecular mechanism of force induced stabilization of collagen against enzymatic breakdown. Biomaterials 33, 3852–3859. doi:10.1016/j.biomaterials.2012.02.001

Ellsmere, J.C., Khanna, R.A., Michael Lee, J., 1999. Mechanical loading of bovine pericardium accelerates enzymatic degradation. Biomaterials 20, 1143–1150. doi:10.1016/S0142-

9612(99)00013-7

Ferdman, A., Yannas, I., 1993. Scattering of light from histologic sections: a new method for the analysis of connective tissue. J. Invest. Dermatol. 100, 710–6.

Flamini, V., Kerskens, C., Moerman, K.M., Simms, C.K., Lally, C., 2010. Imaging Arterial Fibres Using Diffusion Tensor Imaging—Feasibility Study and Preliminary Results. EURASIP J. Adv. Signal Process. 2010, 1–14. doi:10.1155/2010/904091

Flamini, V., Kerskens, C., Simms, C., Lally, C., 2013. Fibre orientation of fresh and frozen porcine aorta determined non-invasively using diffusion tensor imaging. Med. Eng. Phys. 35, 765–776. doi:10.1016/j.medengphy.2012.08.008

Flynn, B.P., Tilburey, G.E., Ruberti, J.W., 2013. Highly sensitive single-fibril erosion assay demonstrates mechanochemical switch in native collagen fibrils. Biomech. Model. Mechanobiol. 12, 291–300. doi:10.1007/s10237-012-0399-2

Gaul, R.T., Nolan, D.R., Lally, C., 2017. Collagen fibre characterisation in arterial tissue under load using SALS. J. Mech. Behav. Biomed. Mater. 75, 359–368. doi:10.1016/j.jmbbm.2017.07.036

Gaul, R.T., Nolan, D.R., Ristori, T., Bouten, C.V.C., Loerakker, S., Lally, C., 2018. Strain mediated Enzymatic Degradation of arterial tissue: Insights into the role of the non-collagenous tissue matrix and collagen crimp. Acta Biomater. doi:10.1016/J.ACTBIO.2018.06.037

Ghazanfari, S., Driessen-Mol, A., Bouten, C.V.C., Baaijens, F.P.T., 2016. Modulation of collagen fiber orientation by strain-controlled enzymatic degradation. Acta Biomater. 35, 118–126. doi:10.1016/j.actbio.2016.02.033

Hiester, E.D., Sacks, M.S., 1998. Optimal bovine pericardial tissue selection sites. I. Fiber architecture and tissue thickness measurements. J. Biomed. Mater. Res. 39, 207–214. doi:10.1002/(SICI)1097-4636(199802)39:2<207::AID-JBM6>3.0.CO;2-T

Holzapfel, G.A., 2008. Collagen in Arterial Walls: Biomechanical Aspects, in: Fratzl, P. (Ed.), Collagen. Springer US, Boston, MA, pp. 285–324. doi:10.1007/978-0-387-73906-9

Huang, C., Yannas, I. V, 1977. Mechanochemical studies of enzymatic degradation of insoluble collagen fibers. J. Biomed. Mater. Res. 11, 137–154. doi:10.1002/jbm.820110113

Junqueira, L.C.U., Bignolas, G., Brentani, R.R., 1979. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem. J. 11, 447–455. doi:10.1007/BF01002772

Müller-Schweinitzer, E., 2009. Cryopreservation of vascular tissues. Organogenesis 5, 97–104. doi:10.4161/org.5.3.9495

Rezakhaniha, R., Agianniotis, a., Schrauwen, J.T.C., Griffa, a., Sage, D., Bouten, C.V.C., Van De Vosse, F.N., Unser, M., Stergiopulos, N., 2012. Experimental investigation of collagen waviness and orientation in the arterial adventitia using confocal laser scanning microscopy. Biomech. Model. Mechanobiol. 11, 461–473. doi:10.1007/s10237-011-0325-z

Robitaille, M.C., Zareian, R., Dimarzio, C. a, Wan, K.-T., Ruberti, J.W., 2011. Small-angle light scattering to detect strain-directed collagen degradation in native tissue. Interface Focus 1, 767–76. doi:10.1098/rsfs.2011.0039

Sacks, M.S., Smith, D.B., Hiester, E.D., 1997. A small angle light scattering device for planar connective tissue microstructural analysis. Ann. Biomed. Eng. 25, 678–89.

Sáez, P., García, A., Peña, E., Gasser, T.C., Martínez, M.A., 2016. Microstructural quantification of collagen fiber orientations and its integration in constitutive modeling of the porcine carotid artery. Acta Biomater. 33, 183–193. doi:10.1016/j.actbio.2016.01.030

Schneider, C. a, Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. doi:10.1038/nmeth.2089

Shahid, S.S., Gaul, R.T., Kerskens, C.M., Flamini, V., Lally, C., 2017. Quantifying the ultrastructure of carotid artery using high-resolution micro-diffusion tensor imaging – comparison of intact vs. open cut tissue. Phys. Med. Biol. 62, 8850–8868. doi:10.1088/1361-6560/aa9159

Willett, T.L., Labow, R.S., Lee, J.M., 2008. Mechanical overload decreases the thermal stability of

collagen in an in vitro tensile overload tendon model. J. Orthop. Res. 26, 1605–1610. doi:10.1002/jor.20672

Wyatt, K.E.-K., Bourne, J.W., Torzilli, P. a, 2009. Deformation-Dependent Enzyme Mechanokinetic Cleavage of Type I Collagen. J. Biomech. Eng. 131, 051004. doi:10.1115/1.3078177

Zareian, R., Church, K.P., Saeidi, N., Flynn, B.P., Beale, J.W., Ruberti, J.W., 2010. Probing collagen/enzyme mechanochemistry in native tissue with dynamic, enzyme-induced creep. Langmuir 26, 9917–9926. doi:10.1021/la100384e