Structural Diversity of Decellularized Ovine Forestomach Extracellular Matrix

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Introduction

Constructive remodelling of damaged tissue using the application of collagen-based wound management relies on the material being recognised by and able to integrate into damaged tissue, without posing a foreign body response[1]. The structural analysis of a material can demonstrate its ability to recapitulate the intricacies of native tissue. This work investigated the structural composition of an ovine extracellular matrix technology (OFM†) compared with unprocessed ovine forestomach tissue (OFT), oxidised regenerated cellulose with collagen (ORC/C^) and a Pure Collagen Dressing# using a variety of methods.

Methods

Structural organization of biomaterials was explored at four levels:

1) Global structure using DSC and polarized light collagen bifringency.

2) Macro structure (mm) using dye perfusion and microCT.

3) Microstructure (μm) using SEM.

4)Nano structure (nm) using AFM.

Conclusion

Visual and quantitative approaches to structural analysis demonstrate vast discrepancies between reconstituted and native collagen biomaterial structure. These differences provide mechanistic insights of the in situ responses to these materials during clinical use.

Global Structure

OFM, OFT, Pure Collagen and ORC/C samples were analyzed via Differential Scanning Calorimetry (DSC), to quantify thermal stability and correlating collagen structural integrity (Figure 1). Denatured collagen (ORC/C) readily melted at low temperatures, indicating complete loss of native collagen structure. Pure collagen, which has triple helix organization exhibited higher onset temperate than ORC/C but was significantly lower than OFM ($p<0.05$), indicating deterioration of inter-collagen bonds characteristic of native ECM structure.

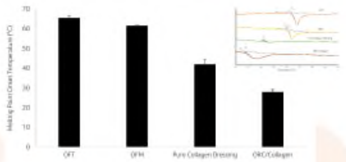
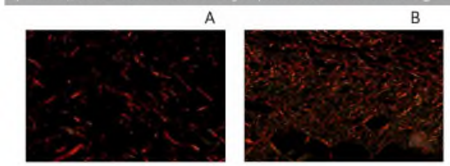


Figure 1. DSC (OFT, OFM, pure collagen and ORC). Differential Scanning Calorimetry was carried out as previously described[2] hydrated samples (5 to 20 mg) were placed in a Tzero Analysis pan (TA, Switzerland) and sealed with a hermetic lid. TA Instruments Q20 (DE, USA) was used with an equilibration step (10 °C, 30 min) and temperature ramp (8 °C/min to 120 °C).

Birefringence is the optical property of a material having a refractive index dependent on the direction of light. Fibrillar collagens stained with Sirius Red appear red or green depending on orientation of collagen bundles[2]. The ratio of green:red fibres is a useful indicator of collagen structure, with highly organized collagen structures composed of different types of collagen such as I and III, exhibiting a high ratio of green:red fibres.

ORC/C, OFM and OFT were analyzed for collagen bifringency by green:red fibre ratio under polarized light. ORC/C demonstrated a significantly lower green:red ratio compared to OFT and OFM ($p<0.05$), which were statistically equivalent, as shown in Figure 2.



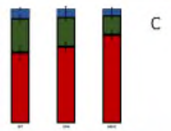


Figure 2. Sirius Red Staining of ORC/C (A) and OFM (B). (OFM, OFT and ORC/C). Collagen bifringency was analysed using hydrated, formalin fixed cross sections (10 um) stained with Sirius red for 60 minutes[3]. Slides (n=3) were imaged at 100x using a Leica DMR microscope with polarized light source and Nikon Digital Sight camera with Nikon NIS Elements . The intensity of red, green, and blue of each image was measured using ImageJ and shown in (C).

Macrostructure

OFM sourced from ovine forestomach tissue retains many macroscopic physiological structures. As shown in Figure 3A, dye perfusion highlights the branching vascular channels into subsidiaries. This demonstrates that the vascular channels remain intact after decelllularization.

MicroCT highlights macro structures of OFM, such as remnants of the luminal basement membrane, well organized and closely packed fibres as well as a large vascular channel (Figure 3B). In contrast, ORC/C at the same scale appears highly porous, with cellulose fibres between amorphous sheets of denatured collagen (Figure 3C).

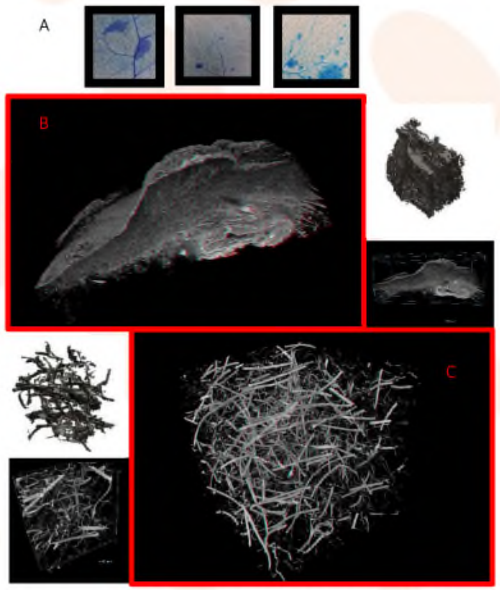


Figure 3. Sterographic representation and 3D modelling of A) OFM and B) ORC/C., put your 3D glasses on.
A) OFM shows distinct macroscopic features such as vascular channels and organised collagen fibres. B) ORC/C appears as a porous mixture of thick cellulose fibres and collagen. Samples (5 x 8 mm) were vapour stained for 3 days above a 1% solution of OsO_4, then mounted for imaging in a Bruker Skyscan 1272 microCT instrument. Instrument parameters: 54kV x-ray voltage, 200 mA beam current, 4000mS exposure, 0.25 Aluminium filter, 4904 x 3280 camera pixels and 0.5 instrument pixel resolution. Datasets were collected using 0.15 degree rotation step, 2x frame averaging over a 3.5-hour period. For dye injection (OFM), a solution of methylene blue (0.4%) with 10% glycerol was injected into vascular channels before the material was photographed

Microstructure

At microscale, the matrix around cells in living tissue is a densely packed fibrous structure containing large (up to 10 μm diameter) collagen fibres and reticular components such as elastin and fibronectin (2-5 um)[4]. At this scale the basement membrane appears smooth with papillae (Figure 4A). Remnants of the basement membrane can be separated to reveal the fibres beneath (Figure 4B). Cross-sectional imaging shows fibres between luminal and abluminal surfaces are tightly packed (Figure 4C). On the abluminal surface (4D), both large collagen fibres and thinner reticular fibres can be clearly observed. Fibre diameter demonstrated a normal distribution ranging from 0.5 to 8.7 μm (Figure 4D). In comparison, ORC/C was less dense, with loose sheets of denatured collagen homogeneously mixed with thick cellulose strands (falsely coloured green) (Figure 4E).

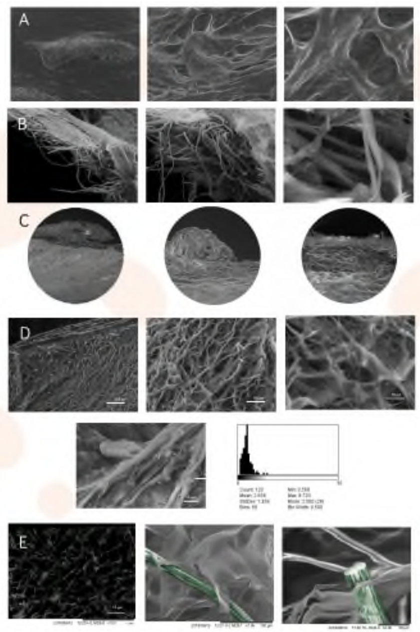


Figure 4 SEM Imaging of OFM and ORC/C Samples were imaged on a Hitachi scanning electron microscope TM3030Plus without staining or sample preparation. ECM samples were oriented on the stand to show either side or a cross-section of the matrix. Fibre diameter was measured using ImageJ on acquired images with a scale set to the appropriate SEM image.

Nanostructure

Collagen fibres visible by SEM are comprised of closely arranged fibrils (~1 μm diameter). Collagen triple helices contain major & minor grooves visible by AFM[5]. Collagen fibres on the abluminal face of OFM are visible, with distinct striations spaced regularly along each fibril (Figure 5A). ORC/C in comparison does not appear to show fibre striation (Figure 5B). The triple helix conformation of collagen molecules is stabilized by hydrogen bonds which are destroyed at temperatures > 60 °C. Consequently, harsh or high temperature processing of collagen leads to disassembly of the fibre structure[6].

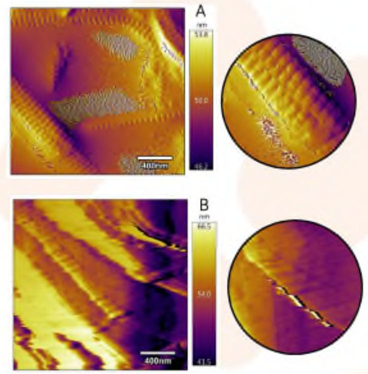


Figure 5 AFM imaging of OFM (A) and ORC/C (B). S amples were imaged dry using a Cypher ES AFM from Asylum Research with a TAP150Al-G to scan samples in tapping mode. Images were acquired using "amplitude" to acquire optimal detail and

References

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^Promogran® Wound Dressing (Acelity, United States).
#Puracol® Plus (Medline Industries, United States).

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