STSM Final project report - Biotailorable 3D bone tissue scaffolds

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1. Purpose & Objectives

The main aim of this STSM was to incorporate discrete, uniform microscale beads of alginate into highly porous but mechanically strong polymeric scaffolds made using 3D printing techniques. This approach was chosen to both organise the spatial distribution of alginate beads in 3 dimensional space and provide physiologically relevant mechanical properties to the hydrogel constructs. The proposed basic design of the hybrid composite scaffold is shown in Figure 1.

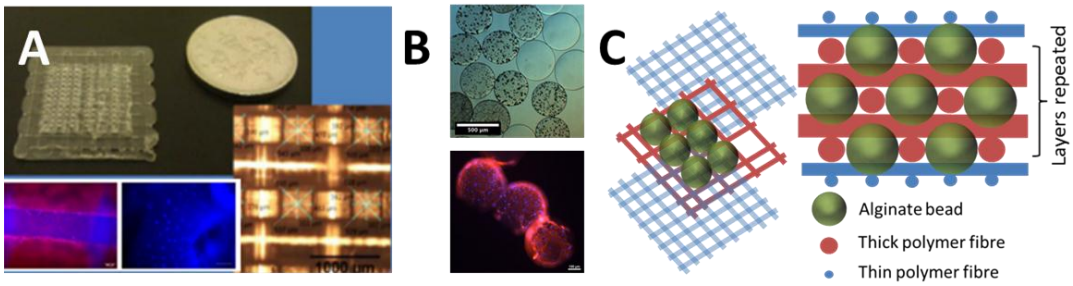


Figure 1: A: 3D printed tissue engineering scaffold, with a close up of the porous structure, and then with imaging of rat calvarial cells seeded on the structure. B: Alginate microbeads showing CaP mineralised and non-mineralised beads (top panel) and mesenchymal stem cells growing on mineralised alginate beads that have differentiated into osteoblast cells (blue) and are depositing a collagen matrix (red) (lower panel). C: Exploded and cross section view of proposed scaffold design to incorporate alginate beads into the polymer scaffold.

2. Experimental approach

2.1 Scaffold design and 3D printing:

Models were designed using Autodesk Inventor Professional 2015 and exported as STL files. STL files were converted to gcode files to be printed using an Ultimaker2 3D printer. All scaffolds were printed in poly lactic acid (PLA).

2.2 Mechanical testing:

15 mm cubic samples were used for mechanical testing in compression. Compressive testing was carried out using a Tinius Olsen H25KS Universal testing machine fitted with a 25 kN load cell. Crosshead speed was set at 2 mm min^{-1} and samples were tested to failure and compressed up to a deflection of 5 mm (1/3 of the original sample height). Six samples were tested in each group.

2.3 Surface functionalization:

PLA scaffolds were modified with three types of coating: plasma, heparin and collagen. Plasma treatment was carried out using a Quorum K1050X plasma barrel reactor (Quorum Technologies Ltd. East Grinstead, UK). Samples were treated in air for 2 periods of 4 minutes at 100 W plasma power, samples were turned between treatments to ensure even coating. Heparin was coated onto the PLA surface by first immersing the scaffolds in a solution of 1,6-hexanediamine/2-propanol for 15 minutes, then washing in water and placing in a solution of heparin (1 mg mL^{-1}) containing N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) and buffered at pH 5 using 2-morpholinoethane sulfonic acid (MES) for 20 minutes. Collagen coating was carried out by immersing heparin coated scaffolds in an aqueous collagen solution (1 mg mL^{-1}) at pH 5 for 1 hour. Heparin and collagen coated samples were stained with Alcian Blue and Sirius Red respectively to visualise the coatings.

2.4 Light microscopy:

Light microscopy was carried out using a Mitutoyo Quickscope stereomicroscope (Mitutoyo (UK) Ltd. Andover, UK) and dimensional measurements were made using QSPAK 7.0 software. 25 measurements were made of each feature and an average taken

3. Results

3.1 Learning 3D printing techniques and identifying constraints:

The first phase of the project focussed on familiarisation with the 3D computer aided design (CAD) software (Autodesk Inventor), and the 3D printing software (Cura) and hardware (Ultimaker 2). Basic "log pile" test specimens were designed and printed to test the capabilities of the 3D printer and to monitor the accuracy of CAD design reproduction. At this stage, it became apparent that it would be difficult to reproduce solid features in the x-y axis below approximately 400 µm, a limitation imposed by the printer nozzle internal diameter (400 µm). Resolution in the z axis, i.e. the minimum thickness of each layer, was found to be approximately 60 µm, however a layer thickness of 80 µm was used in most cases as this was found to produce good results. The resolution limit of hollow structures, i.e. gaps or holes between printed filaments that could be reliably reproduced, was found to be approximately 100 µm.

Two different size ranges of both mineralised and non-mineralised alginate beads were produced at NTNU and shipped to Newcastle to evaluate ease of incorporation (**Table 1**). Given the dimensional constraints established early on in the project, the larger sized beads were favoured and scaffolds were designed to accommodate this size of beads accordingly.

Table 1: Size measurements of alginate microbeads produced by electrostatic extrusion.

Sample	Axis 1 (µm)	Axis 2 (µm)
Large Non-mineralised	560 +/- 47	451 +/- 52
Small Non-mineralised	233 +/- 11	216 +/- 9
Large mineralised	540 +/- 35	486 +/- 22
Small mineralised	259 +/- 31	247 +/- 32

3.2 Scaffold design development

Once dimensional constraints of the printed structures had been established, design of a 3D scaffold able to accommodate large alginate microbeads was carried out. Designs began with a basic "log pile" arrangements of struts with a square or rectangular cross section (Design 1 – Figure 2). Dimensions of the struts and gaps between the struts were adjusted to match the alginate microbead diameter. These designs were reproduced faithfully, and while beads fitted within the holes of the scaffold, the simple arrangement of the struts did not retain the beads, since they were able to fall through the edges of the scaffold and also through the gaps between the layers.

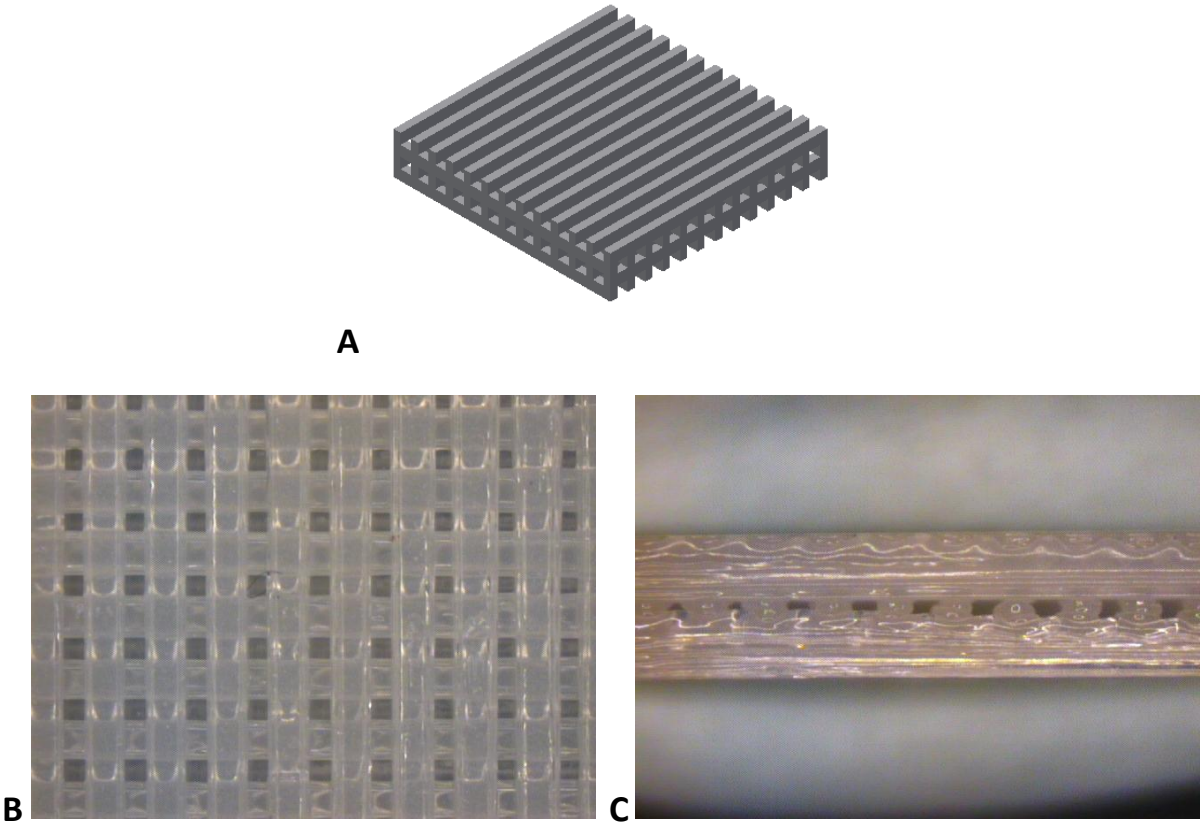
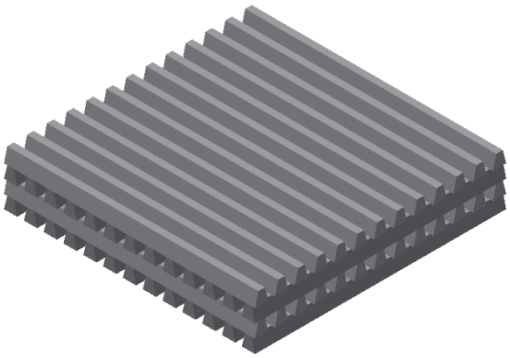
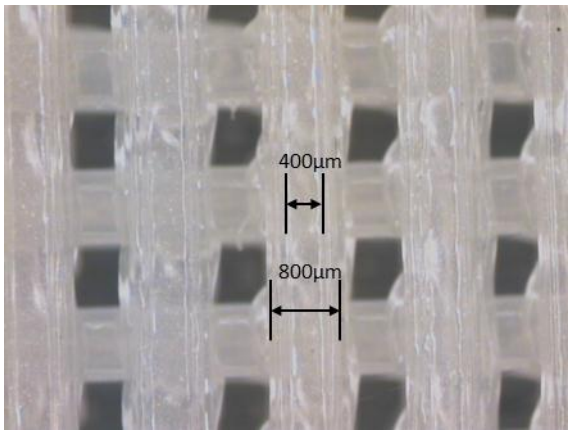


Figure 2: **A**) CAD image of design 1. **B**) Light micrograph of printed design 1 from above. **C**) Light micrograph of printed design 1 from the side.

In order to retain alginate microbeads within the layers, the struts were redesigned with a rhombohedral cross section (Design 2 – Figure 3 A). This design resulted in rectangular holes between the layers with dimensions less than the diameter of the beads, such that they remained in position between the struts. This design was reproduced faithfully (Figure 3 B and C). The rhombohedral cross section of the struts can be observed in figure 3C, however from this view it is only plainly obvious on the top layer due to slight distortion of lower layers following deposition of further layers.



A



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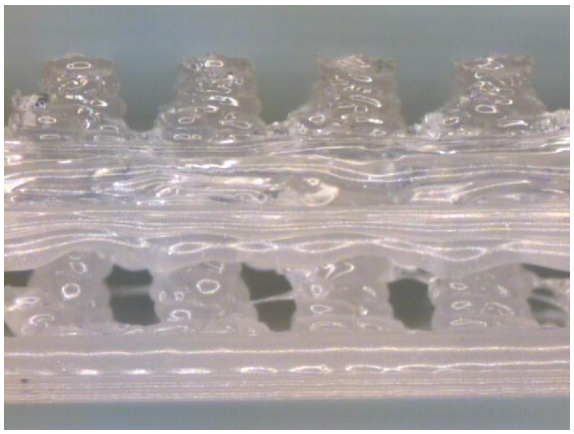

C

Figure 3: **A**) CAD image of design 2. **B**) Light micrograph of printed design 2 from above with indicated approximate dimensions of upper and lower surfaces of struts. **C**) Light micrograph of printed design 2 from the side.

Design 2 was further refined with the addition of "end caps" to the struts to prevent beads from falling out of the edges of the scaffold. This design (Design 3 - Figure 4 A-D), was found to be highly effective at retaining the beads in three dimensional space (Figure 4 E,F) and was chosen as the design of choice for further testing. Accurate measurements of the holes and struts were made with the aid of a light microscope and digital measurement software; the holes were found to be slightly rectangular in shape having dimensions of 296 ± 46 µm x 337 ± 37 µm. The rhombohedral struts had a lower width of 817 ± 44 µm, and an upper width of 366 ± 27 µm.

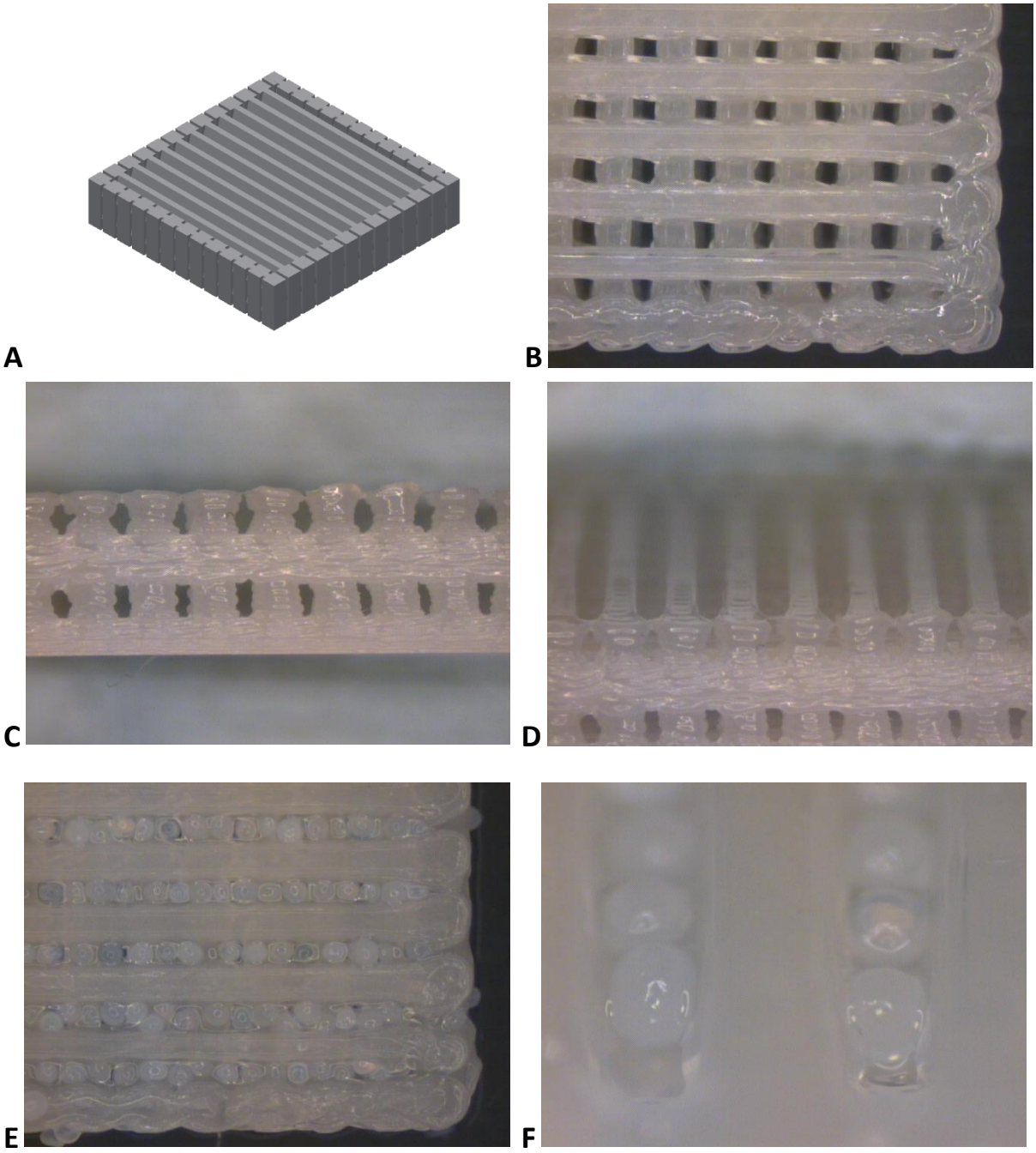

Figure 4: **A**) CAD image of design 3. **B**) Light micrograph of printed design 3 from above showing the end caps. **C**) Light micrograph of printed design 3 from the side. **D**) Light micrograph of printed design 3 from an oblique angle showing the change in groove dimensions between the edge and centre portion of the scaffold. **E**) Light micrograph showing mineralised beads loaded into the scaffold. **F**) High magnification light micrograph taken from an oblique angle showing beads loaded into the scaffold.

Design 3 was slightly modified to create scaffolds suitable for cell culture experiments. The overall dimensions were such that the scaffold would fit in the wells of a standard 12 well cell culture dish. The number of bead containing layers was increased to 6, and an additional thin layer of struts was

added to the top such that beads could be retained in the top layer (Figure 5). In theory such scaffolds should contain approximately 2,500 (or 1 mL) of 450µm average diameter alginate beads, which is a good amount to conduct *in vitro* cell experiments. Multiple copies of this design were produced for future cell culture experiments.

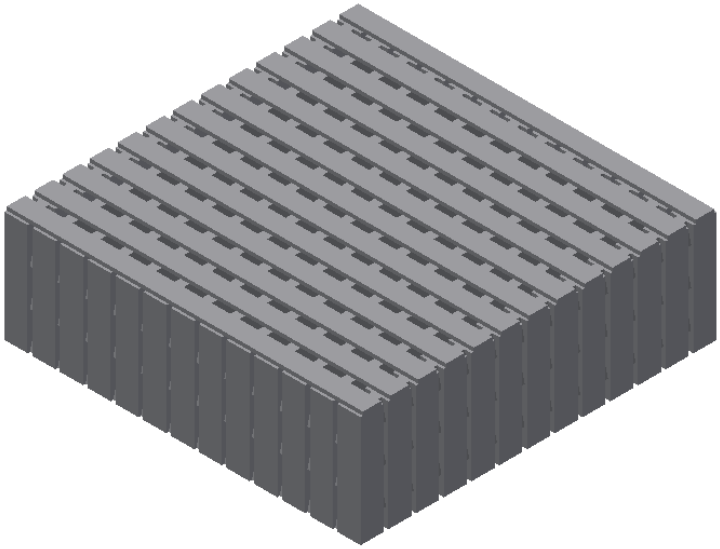

Figure 5: CAD image of the final design produced for cell culture experiments.

3.3 Mechanical testing

To test the mechanical properties of the final scaffold structure, cubic samples were designed and printed based on the structure of design 3 (Figure 6 A). Since this scaffold design is asymmetrical in 2 axes, samples were tested in compression in the 2 different axes (X and Z). A summary of the of the effective compressive (Yield) strength and effective compressive moduli for both axes is shown in Table 2. A typical stress-strain curve is shown in Figure 6 B.

Table 2: Effective compressive mechanical properties of the scaffold design 3 in the X and Z axes.

Axis	Modulus (MPa)	Strength (MPa)
X	317.5 ± 35.9	13.4 ± 1.6
Z	439.5 ± 18.7	16.3 ± 1.2

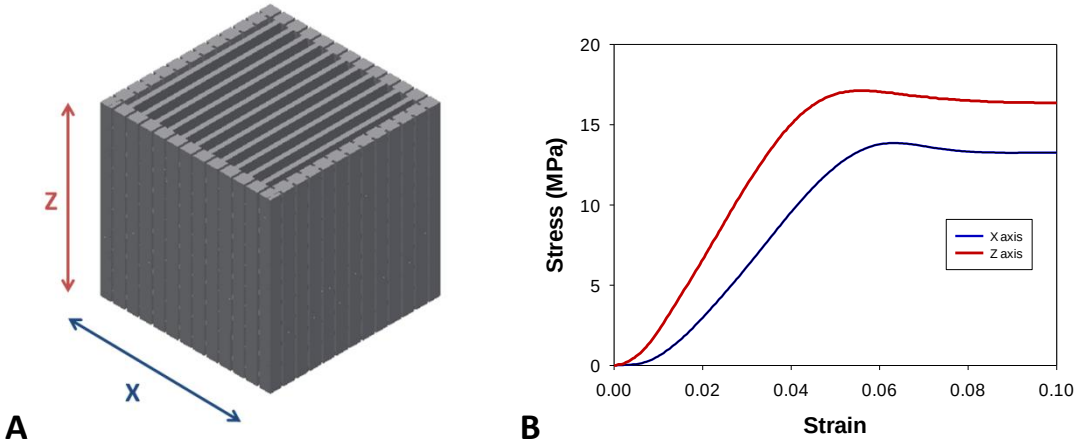

Figure 6: **A**: CAD design of the samples used for compression testing with the axial directions of compression shown (X and Z). The dimension of the samples was 15 x 15 x 15 mm. **B**: Typical stress-strain curves of a scaffold sample under compression recorded in the X and Z axes.

3.4 Surface functionalization

The PLA scaffolds as printed were extremely hydrophobic and therefore repelled alginate microbeads and they did not load well within the scaffolds (Figure 7). Plasma treatment in air was used to reduce the surface charge of the PLA scaffolds rendering them hydrophobic and able to be loaded with the alginate microbeads (Figure 4 E & F). 2 plasma treatments of 4 minutes at 100% power was found to be sufficient to coat the samples. Surface functionalization with heparin and collagen was carried out to encourage the attachment of endothelial and osteoblast cells respectively. This was performed to increase scaffold design options with a view to conduct future cell culture studies. Samples were stained with Alcian Blue and Sirius Red to visualise the attachment of heparin and collagen respectively (Figure 8).

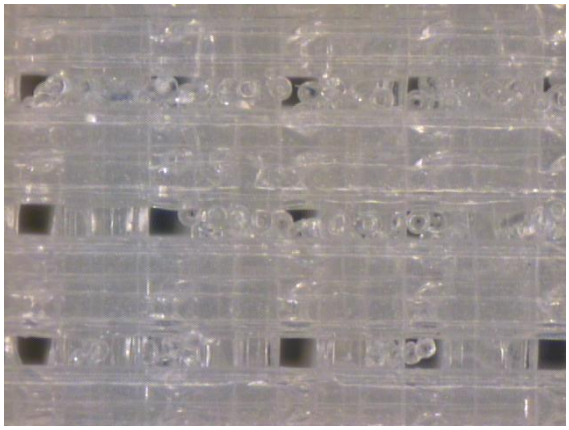

Figure 7: Light micrograph of beads loaded onto untreated scaffold. Bead loading was poor on untreated PLA scaffolds due to the highly hydrophobic surface.

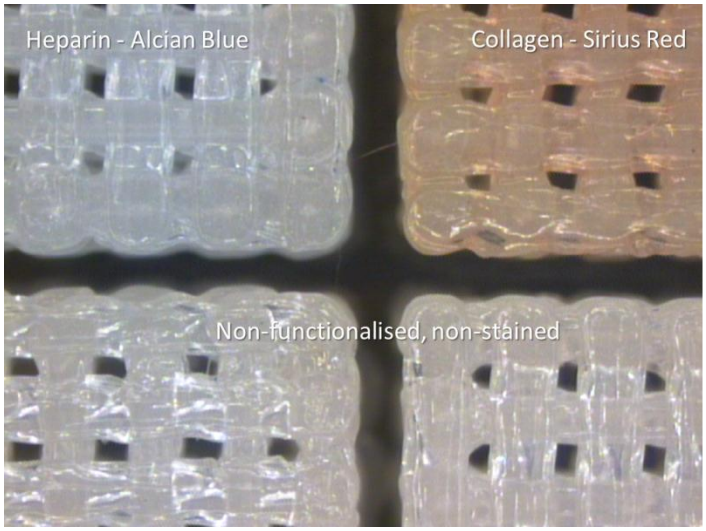

Figure 8: Light micrograph of scaffolds functionalised with heparin and collagen and stained with Alcian Blue and Sirius Red respectively compared to non-functionalised, non-stained samples.

4. Outlook

This STSM resulted in the design and fabrication of a 3D scaffold based on PLA that is mechanically strong and able to accommodate alginate microbeads produced by electrostatic extrusion. This is highly useful since alginate microbeads are excellent cell matrices, but lack the mechanical strength required for application as bone tissue scaffolds. Additionally, functionalization techniques were employed that will aid the attachment of cells to the scaffolds themselves, which could improve vascularisation potential, for example. Follow up experiments to be conducted at NTNU will focus on the 3D culture of cells resident to bone using these scaffolds and monitoring their biological and mechanical performance with time. This STSM has stimulated a collaboration between NTNU and Newcastle University and it is envisaged this will continue to develop along the common interest in bone tissue engineering using this scaffold design as a platform for further work. It is planned to use this work as the basis of a joint publication describing the development of our 3D biotailorable PLA-alginate composite scaffold.