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Investigation of Cell Viability and Morphology in 3D Bio-printed Alginate Constructs with Tunable Stiffness

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Abstract: In this article, mouse fibroblast cells (L929) were seeded on 2%, 5% and 10% alginate hydrogels, and they were also bio-printed with 2%, 5% and 10% alginate solutions individually to form constructs. The elastic and viscous moduli of alginate solutions, their interior structure and stiffness, interactions of cells and alginate, cell viability, migration and morphology were investigated by rheometer, MTT assay, scanning electron microscope (SEM) and fluorescent microscopy etc. The 3 types of bio-printed scaffolds of distinctive stiffness were prepared, and the seeded cells showed robust viability either on the alginate hydrogel surfaces or in the 3D bio-printed constructs. Majority of the proliferated cells in the 3D bio-printed constructs weakly attached to the surrounding alginate matrix. The concentration of alginate solution and hydrogel stiffness influenced cell migration and morphology, moreover the cells formed spheroids in the bio-printed 10% alginate hydrogel construct.

Keywords: Alginate, Bio-printing, Three-dimensional (3D) printing, Additive manufacturing, Rapid prototyping

1. Introduction:

Three-dimensional (3D) bio-printing technology is emerging as a novel and useful technique for complex 3D biomimetic architectures creation in order to enhance tissue repair of skin, heart, bone and retina etc. [1-6]. The biomimetic architectures **can** mimic the natural state of organ that is providing correct extracellular matrix (ECM) compositions, localization and biological functions for the cells during *in vivo* study compared to the conventional 2D and

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3D culturing techniques, thus they further enhance cellular differentiation, gene expressions, proliferation, migration and cell material interactions etc. [7, 8]. The bio-printing technology can be subdivided into inkjet printing, extrusion, microvalve based printing etc. [9-11]. These methods have been developed to print scaffolds and live cells in accurate and efficient ways. The technology has made significant progress for tissue and organ regenerations, for example ear, bone, skin, cardiovascular and nose tissue regenerations [7, 9, 12, 13]. Moreover, biomaterials and cells are vital elements for successful applications of bio-printing technology in tissue regeneration [14-16]. Selection of biomaterials for scaffold fabrication is very critical for successful tissue repair [11, 14]. The selected biomaterials must have proper biocompatibility, biodegradability, mechanical property that fit the requirements of various organ regenerations, and their degraded components must not be toxic [17, 18]. Moreover, the chemical and physical properties of the biomaterials will have significant influences on cell biology [7, 9, 14, 16].

Hydrogels can be considered as ideal biomaterials for cell storage, culture and delivery, and investigations of cellular interactions, due to their natural similarities to the ECM [19, 20]. The hydrogels are 3D hydrophilic, cross-linked polymeric networks capable of absorbing magnificent amount of water or biological fluids, thus are providing ideal 3D microenvironment for cell proliferation and differentiation [19, 21]. **The polymer chains** within the hydrogel can be cross-linked by chemical and physical manners to offer geometric control of the microenvironment guiding and controlling cell orientation. Alginate (ALG) can form tunable and versatile natural degradable hydrogels to be applied as artificial 3D cellular matrix, meanwhile it has been researched in an injectable cell delivery system for decades, and the ALG has certified benign biocompatibility for majority of cells [22, 23]. The ALG forms hydrogel under physiological conditions without using toxic solvents, and is shown to be a commonly used material in biomedical engineering due to its advanced property for cell encapsulation and delivery [22, 23]. Moreover, stiffness of ALG can be tuned in order to fit a range of mechanical property of various native tissues. The compressive modulus of ALG

ranges from 1 to 1000 kPa, and the shear modulus of ALG varies from 0.02 to 40kPa [22-25]. Its stiffness can be modified by tuning several parameters, including polymer source, molecular weight, concentration, chemical modification and types/density of crosslinking. Cells in the ALG gel can be routinely analyzed by normal microscope, and the cells can be retrieved in a convenient way without damage. The most common manner to prepare ALG gel by crosslinking is to substitute the sodium ions from guluronic acid units with divalent cations such as calcium (Ca^{2+}), strontium (Sr^{2+}), zinc (Zn^{2+}) and barium (Ba^{2+}) [25]. These ions have different affinities towards ALG, subsequently to modify gel stability, permeability and strength. The stability, permeability and mechanical property of hydrogels heavily rely on the ALG concentration, source, degree, molecular weight and crosslinking agents [23, 25]. In most biomedical applications, Ca^{2+} is applied to crosslink the hydrogel[23]. The Ca^{2+} crosslinked ALG hydrogel can gradually lose its stability by incubation of citrate and phosphate buffers[26]. However, the ALG gel will remain stable in culture media due to sufficient calcium concentration in the media to counteract the deprivation effects[27, 28]. In bone regeneration, ALG and its derivatives have been **widely** applied to deliver cells and growth factors to promote tissue regeneration [29]. Meanwhile, stem cells and chondrocytes are also delivered through ALG beads to assist cartilage regeneration [22, 23, 26]. Further ALG has been applied to other research areas of tissue regeneration including intervertebral disk regeneration, adipose tissue regeneration, cardiac regeneration, skin regeneration and vascularization[27].

The cell behavior and viability in bio-printed hydrogels with stiffness variances is not well understood although many types of hydrogels and cells are used in 3D bio-printing. ALG is an ideal biomaterial for 3D bio-printing due to its benign biocompatibility, convenient and mild gelation approaches [23, 27]. In this article, bio-printed cell laden ALG hydrogel constructs are prepared, and cell viability, cell/material interactions, development of cell morphology and gel structure are investigated and reported. This research may provide useful

references for research and development of other hydrogel based bioinks for 3D bio-printing and tissue regeneration.

2. Materials and Methods

2.1 Material Preparation

ALG was purchased from Sigma-Aldrich (**Product Number: W201502**). 2%, 5% and 10% (w/v) of ALG solutions were prepared by dissolving 0.2, 0.5 and 1 g of ALG in 10 ml double distilled water. 100mM calcium chloride solutions were prepared by adding 0.556g calcium chloride in 50ml double distilled water.

2.2 Cell Culture

Mouse fibroblast cell line (L929) was revived and cultured in DMEM high glucose media with supplementary of 10% fetal bovine serum (FBS, Gibco), and 1% Antibiotic-Antimycotic liquid (Gibco, 100X). The cells were detached upon confluence for future applications.

2.3 Cell Culture and Biological Tests

The ALG powder was exposed and sterilized under **INTELLIRAY UV Flood 400, ($\lambda=320-390\text{nm}$, density: $115\text{mW}/\text{cm}^2$)** for 30 mins, and then the 2%, 5% and 10% ALG solutions were casted on 24 well plates and crosslinked by 100mM calcium chloride for 15 mins. Then fifty thousand (5×10^4) cells were seeded on the ALG hydrogel surface and the same amount of cells were seeded on the original plate as control, the viability of cells was monitored by Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific), and the cells were stained by ActinGreen™ 488 ReadyProbes® and NucBlue® Live ReadyProbes® reagents (Thermo Fisher Scientific), and observed after 24 hours' seeding.

2.4 Extrusion Based ALG Printing

Five million (5×10^6) cells were mixed with 1ml 2%, 5% and 10% ALG solutions respectively, and the cells were stirred mildly inside the ALG solutions to obtain homogenous mixtures. The mixtures were then put in printing cartridges for further applications. 3D Bio-Printer (RegenHU Ltd, Switzerland) was used to print the cell/ALG complex lattice constructs with horizontal and vertical fillings on petri-dish. The cell-laden ALG solution was loaded into cartridge that was connected a 27G needle nozzle. The bio-printed constructs had been

crosslinked by 100mM calcium chloride for up to 5 minutes, and then they were washed by plain DMEM media once and cultured in full DMEM media at 37°C with 5% CO₂ for two weeks.

2.5 Mechanical Study

The 2%, 5% and 10% plain ALG solutions were placed in 40mm parallel plate (Peltier plate steel) of a Discovery Hybrid Rheometer 2 (TA Instruments), and frequency sweep was performed from 0.1 to 100 rad/s, and the running temperature and strain were set at 25°C and 0.5% respectively [30]. Meanwhile, the 2%, 5% and 10% ALG hydrogel cylinders were prepared by filling ALG solutions into dialysis tube (flat width 25 mm, MWCO 12,000 Da, Sigma), then the proper filled dialysis tubes were put into 100mM CaCl₂ solutions for crosslinking and gel formation, and then the gels were gently squeezed out and trimmed into small cylinder disks with approximately diameter of 25mm, and height of 10mm. The samples were placed at the center of metal platens of Instron 5566 testing system, and they were compressed at 0.5 mm/min to a final strain at 10%. The stress-strain curves were plotted, and the compressive stiffness/slopes were calculated.

2.6 Cells and Scaffolds Observation

The bio-printed cell/ALG constructs were stained by LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific) after 24 hour's culture, and the live cells (green) and dead cells (red) were imaged under inverted microscope (ZEISS). The development of cell morphologies in several ALG hydrogel constructs with distinctive stiffness at day 1, 7 and 14 were also observed, and the cells were stained by ActinGreen™ 488 ReadyProbes® and NucBlue® Live ReadyProbes® reagents (Thermo Fisher Scientific). The same samples and plain printed scaffolds were also fixed by 4% formaldehyde, and dehydrated gradually in 50%, 75%, 90% and 100% ethanol before critical point drying and observed under scanning electron microscope (SEM).

2.7 Statistical Analysis

All data are expressed as means ± the standard deviation. Statistical analysis was performed by multiple comparisons using single-factor analysis of variance (ANOVA) and post hoc

Tukey tests, using SPSS Statistics version 19.0, and $p < 0.05$ and 0.01 were considered statistically significant.

3 Results

The rheological analysis was demonstrated in figure 1 a-c, the 2%, 5% and 10% ALG solutions show elastic moduli from 0.082 ± 0.037 to 7.973 ± 0.589 Pa, 0.042 ± 0.005 to 12.993 ± 0.173 Pa and 0.055 ± 0.005 to 8.077 ± 1.379 Pa respectively, while their viscous moduli increased magnificently along with the higher ALG concentrations to a final 1.167 ± 0.099 , 8.826 ± 0.233 and 44.021 ± 1.443 Pa at 100 rad/s individually. Meanwhile the compressive stiffness of 2%, 5% and 10% ALG hydrogels were 6242.42 ± 577.34 Pa, 21451.47 ± 1180.71 Pa and 64596.44 ± 5336.42 Pa separately. SEM images in figure 2 indicated the interior structure of the 3D bio-printed ALG scaffolds. There were obvious chaps within the printed 2% ALG constructs in figure 1a, compared to figure 1b and c (x1000). While in microscopic view as shown in figure 1 a1, b1 and c1, the polymer network structures are similar in 2% and 5% ALG based hydrogels, and the network structure became denser in 10% ALG hydrogel.

In figure 3a, the cells were individually seeded on the 2%, 5% and 10% ALG hydrogels, and their viabilities were monitored closely at day1, 7 and 14 by MTT assay, and no significant difference among cell viability was observed. The cell morphology on hydrogels was also displayed in figure 3, and the difference of width, length and spread area of the cells can be clearly observed. The cells were substantially less spread and elongated on 2% ALG hydrogel (lower stiffness). However, the cell morphology on the 10% ALG hydrogel was similar to that of original cell culture plate (figure 3 d and e). Further, the cell viability and morphology in 3D bio-printed scaffolds were also investigated. In figure 4 a, a representative 3D bio-printed ALG scaffold was cultured in media, and the images of Live/Dead staining demonstrated that majority of cells were alive (green) and robust in all bio-printed ALG constructs (figure 4 b, c and d). Moreover, the cell distribution, morphology and migration were carefully monitored by actin staining. In figure 5 a, a1 and a2, the cells were encapsulated in 2 % bio-printed ALG construct, some of the cells showed elongated shapes

when they interacted with other cells. Meanwhile, the cells massively proliferated from day 1 to day 14, and they almost filled in all the available space within the 2% ALG construct. The cells could still freely migrate within the 5% hydrogel, and proliferated along with the time from day 1 to day 14 (figure 5 b b1 and b2). Further, the cells in 10% ALG hydrogel construct might be completely restrained by the polymer networks leading to disabled cell mobility, and cell spheroids could be observed (figure 5 c c1 and c2).

In SEM observation (figure 6&7), the cells were evenly distributed inside the bio-printed scaffold at day 1, and the cell density was magnificently increased during 2 weeks' culture in the three groups. The elongated cells could be observed in 2% ALG construct at week 1 (figure 6&7 a1), while the morphology of cells turned to "spherical" shapes at week 2 (figure 6&7 a2). Meanwhile the cells in 5% ALG hydrogel constructs slightly aggregated during proliferation in 2 weeks (Figure 6&7 b2). The cells formed spheroids in 10% ALG constructs over 2 weeks' culture. Further, the morphology of cells was observed clearly in microscopic images in figure 7. The cells were spherical in all three groups in day 1, and the cell proliferated and spread in 2% ALG and their morphology changed to "sphere like" at week 2 (figure 7 a2). The cells in 5% ALG construct kept "spherical" in the polymer networks over two weeks (figure 7 b2), and cell spheroid assembled by lots of cells could be clearly observed in 10% bio-printed ALG construct (figure 7 c2).

4 Discussion

The cell morphology, proliferation and migration in the bio-printed cell/ALG complex constructs are investigated and reported in this article. In line with most studies, the ALG shows great biocompatibility, meanwhile the ALG based hydrogel also shows non-immunogenic property, and great potential for cell delivery [23, 31-33]. Thus, it has been applied in biomedical applications for decades, for example wound dressing, dental impression and controlled release etc.[27]. Various cell types are utilized with ALG hydrogels for *in vitro*, *ex vivo* and *in vivo* biomedical applications, including human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs) etc. [23, 32]. The

mechanical property of ALG hydrogel is adjustable through polymer source, molecular weight, concentration and types of cross-linkers to fit a variety of tissue requirements [23, 26, 27]. ALG solution shows shear thinning and non-Newtonian flow behavior [24], and the viscoelasticity of 2%, 5% and 10% ALG solutions are investigated by frequency sweep as can be seen in figure 1 a, b and c. The G' and G'' are increasing with the frequency, and the G'' is getting stronger in the higher ALG concentrations at the same frequency. These solutions exhibit typical viscoelastic behavior, and the concentrations of ALG are vital for their viscoelasticity development.

The 2%, 5% and 10% ALG solutions are filled in dialysis tubes and slowly crosslinked by 100 mM calcium chloride solution to form homogenous hydrogels for mechanical testing, and then the hydrogels are collected and compressed. Stiffness is calculated according to the stress-strain curves, as can be seen in figure 1 d. The stiffness differences of the three types of hydrogels are significant, and the range varies from 6242.42 ± 577.34 Pa to 64596.44 ± 5336.42 Pa respectively ($P < 0.01$). The 2%, 5% and 10% ALG solutions show different printability, the 2% ALG solution with low viscoelasticity can only be printed at 1 layer, while the 5% ALG solution can be printed at 3 layers and the 10% ALG solution can be printed at up to 5 layers (figure S1). The printed ALG solution without crosslinking may fill in the printed lattice structure due to poor supports (figure S1 c). However the cells in the printed construct will only be influenced by the concentrations of ALG solutions before crosslinking, subsequently the cells will sense the mechanical stimulation from the surrounding polymer chains to coordinate their own cellular responses in terms of cell morphology, migration and proliferation to different accommodation environments upon crosslinking. Meanwhile, the ALG hydrogel is relatively bio-inert [22, 23, 28], thus the cell behavior in the bio-printed constructs is mainly regulated by the stiffness of the hydrogels, for example the adhesion and morphology of chondrocytes as well as the proliferation, apoptosis and differentiation of pre-osteoblasts are influenced by the stiffness of the hydrogels [34, 35]. The SEM images of the printed ALG constructs demonstrate variance in their interior structures (figure 2), and the

polymer networks gradually become denser in 2%, 5% and 10% ALG hydrogels due to the existing of relatively larger amount of ALG molecular chains.

No significant differences of cell viability among the 3 experimental groups over 2 weeks' culture when they are seeded on 2%, 5% and 10% ALG hydrogels due to its excellent biocompatibility. The cell morphology and attachment on the hydrogel surfaces are significantly regulated by the hydrogel stiffness (figure 3 b, c d and e). The cells are in spherical shapes at 2% ALG surface due to the weaker attachments, while the stronger cell attachments on the high stiffness hydrogel surface are discovered. This can be proved by the development of cell morphology on the surfaces of 2%, 5% and 10% ALG hydrogels, and native culture plates (figure 3). The cells propagated on the relatively softer surface with smaller and dynamic adhesions, on the contrary cells show more larger and stable adhesions on high stiffness surfaces, for example matrix-coated glass and plastic[36]. Thus the cell will show weak adhesions on soft gel surface while form relatively stable adhesions on high stiffness gel surfaces.

The live/dead assay is performed 24 hours after the 3D bio-printing (figure 4), and most of the cells are viable, and almost no dead cells are observed (red color). The ALG polymer solution can protect the cells during printing, and the ALG hydrogel has similar property just like ECM that ensures high viability of bio- printed cells [22, 23]. The MTT assay is used to evaluate the cell viability on the ALG hydrogels, and demonstrates that the increased concentrations of ALG will not affect the cells viability. It is a different scenario to investigate the cell viability in 3D environments especially in 3D hydrogels. The cells are encapsulated by the ALG polymer chains that slow down interactions of cells and reagents, meanwhile the hydrogel may also absorb and block the reagents that may mislead the experimental data. Thus only live/dead assay is performed, and the viable cells are stained green while dead cells with damaged membranes are in red color. The biocompatibility of ALG has been proved for decades, and it has been applied to almost all tissue engineering

research fields, for example cartilage, bone, skin and eye tissue regenerations etc.[23]. Moreover, the bio-printing process is streamlined, thus the cells get benefits from all the advantages to form the viable and robust 3D constructs.

The cell morphology is further explored by actin staining. The cells in 2% printed ALG constructs may move freely due to enough space for cell migration, thus they may start to aggregate in 2% printed ALG construct at day1 (figure 5a), while the cells remain separately in 5% and 10% printed ALG constructs (figure 5 b and c). In day7 and 14, the cells massively reproduced in 2% and 5% bio-printed ALG hydrogels, and the cell migration is observed clearly in both groups. Interestingly the cells form spheroids in the 10% 3D bio-printed constructs (figure 5 c1 and c2), and the 10% ALG polymer chains in the hydrogel may form very dense mesh to trap the cells, therefore the cell migration is probably significantly restrained, and then reproduced cells aggregate to share favorite ECM. In order to observe the cell proliferation, migration and morphology more clearly, the samples are processed for SEM analysis. In low magnification images (figure 6 a, b and c), the cells are dispersed evenly in the hydrogel at day 1, and they are apparently surrounded by the ALG polymers. In day7, more cells are identified in the ALG hydrogel due to cell proliferation (figure 6 a1, b1 and c1). In day 14, large amount of cells occupied the 3D bio-printed constructs as can be seen in figure 6 a2 and b2, the cells are in the blebbing conditions that may indicate weak adhesions to facilitate cell migration in 3D environment [37]. It is worth to mention that the cell spheroids break the ALG hydrogel polymer networks due to their size expansion (figure 6 c3). The morphology of cells can be clearly observed over 14 days in figure 7 a and b (microscopic views), the cells in 2% and 5% ALG constructs starts to aggregate in one days, due to a relax polymer networks that may allow cell migration. Meanwhile the ALG is bio-inert, that may encourage cells to aggregate to form their own ECM. The cells in the 10% ALG remain singular. Subsequently, some of cells are showing spindle shapes in 2% ALG constructs, and this may the cells can stretch themselves after their own ECM production and interactions among cells. The aggregation of cells in 5% ALG show spherical shapes due to

the restrictions from the surrounding polymer networks (figure 7 b1). Moreover, the spheroids can be clearly observed after 7 days' culture in 10% ALG constructs, and the cells are tightly packed with very distinguished morphological changes. In day 14, the cells in 2% and 5% ALG hydrogels massively proliferate (figure 7 a2 and b2), and lots of spheroids in 10% ALG constructs are discovered (figure 7 c2). The mechanism of cellular behavior in bio-printed 3D constructs of several stiffness can be further explained in figure 8. It is obvious that the density of polymer networks are magnificently enhanced from 2% to 10%, and this can be proved by SEM analysis and values of stiffness, therefore the cells in 2% bio-printed ALG hydrogel will have more space that allows free cell proliferation and movements. In 5% bio-printed ALG hydrogel, the cells can move within the hydrogel with the blebbing shape although they are moderately restrained. At last, the proliferated cells cannot move freely within the bio-printed 10% ALG hydrogel construct thus the reproduced cells aggregate and form spheroids.

5 Conclusions

The influences of bio-printed hydrogel stiffness on cell migration, proliferation and development of cell morphology were investigated in this article. The cells showed great viability and robust reproduction in the bio-printed constructs. The blebbing cells existed in all the constructs, and cells in 2% ALG hydrogel may have more space to accommodate and reproduce, while the same cells in 5% bio-printed construct is moderately restrained but may migrate in blebbing shapes. The cells in 10% hydrogel might be significantly trapped and finally formed spheroids. Thus the development of cell morphology, cell migration and proliferation could be tuned by controlling hydrogel stiffness during bio-printing that provides valuable information for future bio-printing research.

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Figure Legends

Figure 1. Elastic and viscous moduli of alginate solutions at different concentrations as a function of frequency: 2% (a), 5% (b) and 10% (c); compressive stiffness of alginate hydrogel at 2%, 5% and 10% after calcium crosslinking (d, ** $P < 0.01$).

Figure 2. SEM images of the 2% (a and a1), 5% (b and b1) and 10% (c and c1) 3D bio-printed alginate constructs respectively: a, b and c (magnification: x1000); and a1, b1 and c1 (magnification: x10000).

Figure 3. Cell viability of cell seeding on alginate hydrogels at different concentrations (a, $P > 0.05$); cell morphology on 2% (b), 5% (c) and 10% (d) alginate hydrogels and on original plate as control (e) after 24 hours; scale bar: 50 μm .

Figure 4. Photo image of the 3D bio-printed alginate hydrogel scaffolds (a); live/dead staining of cells in the 2% (b), 5% (c) and 10% (d) 3D bio-printed alginate constructs at 24 hours after bio-printing; scale bar: 200 μm .

Figure 5. Fluorescent images of DAPI (blue) and actin (green) staining of cells in the 3D bio-printed alginate constructs: 2% (a, a1 and a2), 5% (b, b1 and b2) and 10% (c, c1 and c2) at day1, 7 and 14; scale bar: 50 μm .

Figure 6. SEM images of cell proliferation and migration in the 2% (a, a1 and a2), 5% (b, b1 and b2) and 10% (c, c1 and c2) 3D bio-printed alginate constructs at day1, day 7 and day 14 (magnification: x500).

Figure 7. SEM images of cell morphology in the 2% (a, a1 and a2), 5% (b, b1 and b2) and 10% (c, c1 and c2) 3D bio-printed alginate constructs at day1, day 7 and day 14 (magnification: x2000).

Figure 8. Diagram of cellular responses in the 2%, 5% and 10% bio-printed alginate constructs.

Supplementary figure legend:

Figure S1. Representative images of bio-printed cells in 10% alginate constructs at 1, 3 and 5 layers (a, b and c); scale bar: 3mm.

Figure 1. Elastic and viscous moduli of alginate solutions at different concentrations as a function of frequency: 2% (a), 5% (b) and 10% (c); compressive stiffness of alginate hydrogel at 2%, 5% and 10% after calcium crosslinking (d, ** $P < 0.01$).

72x52mm (600 x 600 DPI)

Accepted

Figure 2. SEM images of the 2% (a and a1), 5% (b and b1) and 10% (c and c1) 3D bio-printed alginate constructs respectively: a, b and c (magnification: x1000); and a1, b1 and c1 (magnification: x10000).

50x25mm (600 x 600 DPI)

Accepted

Figure 3. Cell viability of cell seeding on alginate hydrogels at different concentrations (a, $P>0.05$); cell morphology on 2% (b), 5% (c) and 10% (d) alginate hydrogels and on original plate as control (e) after 24 hours; scale bar: 50µm.

40x16mm (600 x 600 DPI)

Accepted

Figure 4. Photo image of the 3D bio-printed alginate hydrogel scaffolds (a); live/dead staining of cells in the 2% (b), 5% (c) and 10% (d) 3D bio-printed alginate constructs at 24 hours after bio-printing; scale bar: 200μm.

76x57mm (600 x 600 DPI)

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Figure 5. Fluorescent images of DAPI (blue) and actin (green) staining of cells in the 3D bio-printed alginate constructs: 2% (a, a1 and a2), 5% (b, b1 and b2) and 10% (c, c1 and c2) at day1, 7 and 14; scale bar: 50um.

73x52mm (600 x 600 DPI)

Accepted

Figure 6. SEM images of cell proliferation and migration in the 2% (a, a1 and a2), 5% (b, b1 and b2) and 10% (c, c1 and c2) 3D bio-printed alginate constructs at day1, day 7 and day 14 (magnification: x500).

73x52mm (600 x 600 DPI)

Accept

Figure 7. SEM images of cell morphology in the 2% (a, a1 and a2), 5% (b, b1 and b2) and 10% (c, c1 and c2) 3D bio-printed alginate constructs at day1, day 7 and day 14 (magnification: x2000).

72x51mm (300 x 300 DPI)

Accept

Figure 8. Diagram of cellular responses in the 2%, 5% and 10% bio-printed alginate constructs.

89x78mm (300 x 300 DPI)

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