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3D cell bioprinting of self-assembling peptide-based hydrogels

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Abstract

Bioprinting of 3D cell-laden constructs with well-defined architectures and controlled spatial distribution of cells is gaining importance in the field of Tissue Engineering. New 3D tissue models are being developed to study the complex cellular interactions that take place during both tissue development and in the regeneration of damaged and/or diseased tissues. Despite advances in 3D printing technologies, suitable hydrogels or 'bioinks' with enhanced printability and cell viability are lacking. Here we report a study on the 3D bioprinting of a novel group of self-assembling peptide-based hydrogels. Our results demonstrate the ability of the system to print well-defined 3D cell laden constructs with variable stiffness and improved structural integrity, whilst providing a cell-friendly extracellular matrix "like" microenvironment. Biological assays reveal that mammary epithelial cells remain viable after 7 days of in vitro culture, independent of the hydrogel stiffness.

Keywords: Tissue Engineering, 3D Printing, Cell encapsulation, Hydrogels, Epithelial cells.

1. Introduction

The introduction of automated systems, in particular Additive Manufacturing (AM) techniques in the field of Tissue Engineering (TE) has promoted the establishment of a new area of research called Biofabrication [1]. As recently reported by Groll *et al*, Biofabrication can be defined as “the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through Bioprinting or Bioassembly and subsequent tissue maturation processes” [1]. Therefore the term Bioprinting can be employed to classify processes that combine different biomaterials, cells and biological molecules in order to generate 3D constructs/scaffolds with well-defined internal/external geometries and optimized biomechanical properties [2]. The methodology by which 3D porous scaffolds are seeded with cells and cultured in vitro prior to implantation, also known as a “top-down” approach, has improved our ability to design tissue analogues [3]. They have enhanced cell-cell and cell-ECM (extracellular-matrix) interactions, 3D cell spatial distributions, higher cell densities and fully interconnected pore networks for vascularization and media diffusion [4]. The benefits, when compared to cell-based strategies, are clear and extensively reported in the literature [5-6]. However this approach is still hindered by the inability of the scaffold to promote homogeneous cell distributions, or to mimic the native ECM microenvironment [6]. To address these issues, a “bottom-up” approach, combining Bioprinting and highly hydrated polymers with encapsulated cells have been investigated [4]. The range of

manufacturing systems available is wide and can be classified into extrusion-based; binder jetting (or Ink Jet); and vat photo-polymerization (or laser-based) categories [7-9]. Different natural and synthetic hydrogels are used, for example Alginate, Gelatine, Hyaluronic Acid (HA), Polyethylene Glycol (PEG), Polyvinyl Alcohol (PVA), etc. [10-15]. Despite a possible release of toxic products during degradation and the low biocompatibility, synthetic hydrogels are still preferred for Bioprinting, as they allow for fine tuning of both chemical and physical properties while degrading at a controlled rate. The material requirements for Bioprinting are complex and often antagonistic. Low viscosity, low stiffness and low crosslinking densities enable cell migration, nutrient diffusion and neo tissue formation. High viscosity, high yield stress and rapid gelation ensure both the geometrical/dimensional accuracy of the constructs as well the post printing mechanical stability [14]. Ideally a compromise between the physicochemical (i.e. rheological) and biological properties of the hydrogel should be achieved to ensure the printing of constructs with high cell viability. For that purpose, different strategies have been proposed encompassing blending of the hydrogel material with gelatines, printing hybrid scaffolds using a thermoplastic as a structural reinforcement, photocrosslinking as a post-curing mechanism, physical crosslinking of spider silk recombinant proteins or dual stage crosslinking [10, 16-20]. While effective in increasing the mechanical stability of the constructs, these approaches generally require long fabrication times as well as the use of high temperatures and/or UV radiation, which can have detrimental effects on cell viability [21]. Here we describe a new methodology for 3D Bioprinting of cell-laden constructs with high geometrical definition, mechanical stability and cell viability. This is based on the use of a novel commercial self-assembling peptide based hydrogel (PeptiGelDesign.Ltd), that utilizes specific synthetic peptides that self-assemble from fluid precursors into a nanofibrous hydrogel when presented with physiological level ionic strengths. This 3-dimensional hydrogel matrix promotes cell growth and migration. Through optimization of the printing parameters, we show the ability of the system to print both soft and stiff hydrogels without compromising the viability of encapsulated cells. Furthermore, we demonstrate the potential of the hydrogel system for direct printing of viable biological constructs. These have tuneable mechanical properties, and do not need physical or chemical post processing.

2. Experimental Section

2.1. Materials

Peptide hydrogels were purchased from PeptiGelDesign (Cheshire, UK): Alpha1, AlphaProB. Alpha1 has G' (oscillatory shear modulus) values in the range of ~10 kPa and AlphaProB has G' values of ~1 kPa after media addition (Further information on the hydrogels can be obtained upon request from PeptiGelDesign

www.peptigeldesign.com). LIVE/DEAD assay (Invitrogen), DMEM-F12, PBS and Trypsin were purchased from Sigma (Sigma Aldrich, UK).

2.2. Cell Culture and hydrogel encapsulation

EpH4 (mammary epithelial cells) were cultured in DMEM-F12 supplemented with 5% FCS, 250 μ l insulin and 1% Pencillin/Streptomycin. Cells at passage 4 were suspended at a concentration of 4×10^6 cells ml^{-1} in 2 ml of hydrogel solution. After complete homogenization the cell-gel solution was transferred to the bioprinting system cartridge and immediately printed into 6-well culture plates.

2.2. 3D cell bioprinting of self-assembling peptide based hydrogels

The 3D printing of cell-laden hydrogel constructs was performed using a commercial extrusion-based AM system called 3D Discovery (regenHU, Villaz-St-Pierre, Switzerland). Initially, Disc-shape constructs with an outer diameter of 10 mm, lay-down pattern of $0/90^\circ$, filament distance (FD) of 550 μ m (horizontal plane), layer thickness (LT) of 500 μ m and height of 1 mm were directly designed in the BioCAD software (regenHU, Villaz-St-Pierre, Switzerland). Subsequently, using an extrusion nozzle of 300 μ m, constant deposition velocity (DV) of 10 mm/s, dosing distance of 0.1 mm, and varying the extrusion pressure (EP) between 0.2 – 1 Pa, multiple constructs were printed in order to find the optimal processing parameters for both soft (AlphaProB) and stiff (Alpha 1) hydrogels. After printing, cell constructs were supplied with culture media and incubated for 7 days at 37°C and 5% CO₂ in a humidified incubator.

2.3. Cell viability

Cell viability was determined by LIVE/DEAD assay (Sigma) consisting of calcein (2 μ M) as a marker for viable cells and propidium iodide (1.5 μ M) as a marker for dead cells. Observations were made by fluorescence microscopy (Zeiss LSM 700) with 494 nm (green, calcein) and 535 nm (red, propidium iodide) emission filters.

2.4. Atomic Force Microscopy (AFM)

Scanning probe microscopy was performed on a MFP-3D Atomic Force Microscope (Asylum Research, High Wycombe, UK), with an AC240TS probe ($k = 2.04 \text{ Nm}^{-1}$, Olympus, Japan). AMFM nanomechanical mapping [22] and loss-tangent imaging [23] were applied, with measurements based on the shift in the probe's resonant frequencies dependent on the strength of the interaction, or tip-sample contact force [24]. A tip correction factor was calculated based on the known compressive Young's modulus of a polycaprolactone calibration sample (300 MPa). Three 5 μ m x 5 μ m areas were then randomly selected and measured from each material. 512 x 512 pixel maps of height, Young's modulus and loss tangent were recorded for each image.

3. Results and Discussion

3.1. 3D cell bioprinting of hydrogel constructs

One of the major challenges in 3D cell printing is the ability to produce biological constructs with well-defined geometries and structural integrity, which both avoids the collapse of printed pores and guarantees cell viability. This becomes particularly challenging when the stiffness of the hydrogels is reduced, in order to increase cell viability. Initially we optimized the printing process parameters, namely EP and valve opening time (Table 1). We then examined the ability of the system to print 3D cell-laden constructs of mammary epithelial cells using both soft and stiff hydrogels (Fig 1). Our results revealed a high structural integrity and geometrical/dimensional accuracy of the printed cell-laden constructs.

Process Parameters					
	EP (Pa)	DV (mm/s)	Dosing distance (mm)	Valve opening time (μ s)	Temperature ($^{\circ}$ C)
PGD-AlphaProB	0.2	10	1	400	37
PGD-Alpha 1	1	10	1	300	37

Table 1: Optimal process parameters for 3D printing of soft and stiff cell-laden hydrogel constructs.

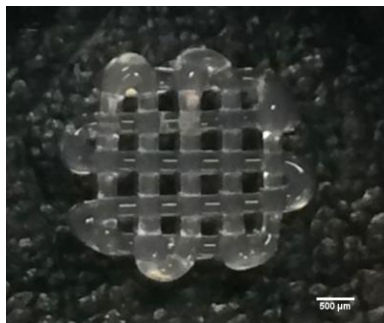


Figure 1: Top view of 3D printed mammary epithelial cell-laden construct (Alpha 1 hydrogel) with two layers of square interconnected pores of 250 μ m and filament diameter of 300 μ m (Scale bar: 500 μ m)

3.2. Cell viability

The viability of printed cells can be influenced by a detrimental effect of shear stresses induced by the extrusion-based process, namely nozzle diameter and extrusion pressure. However our gel-printing methods

revealed large number of viable cells, independent of the hydrogel stiffness, after 7 days of culture. These cells had proliferated and started to form clusters in AlphaProB (Fig 2). The observed high level of cell viability and evidence of proliferation confirms that the printing parameters were not detrimental to cell health. The formation of aggregates, and the differences seen in the morphology of the cells encapsulated in stiff or soft gels, may be related to the rheological properties of the gel; further work to characterize this effect is currently underway. Our data reiterate previous findings that this family of self-assembling hydrogels is conducive to cell survival and growth [25-27]

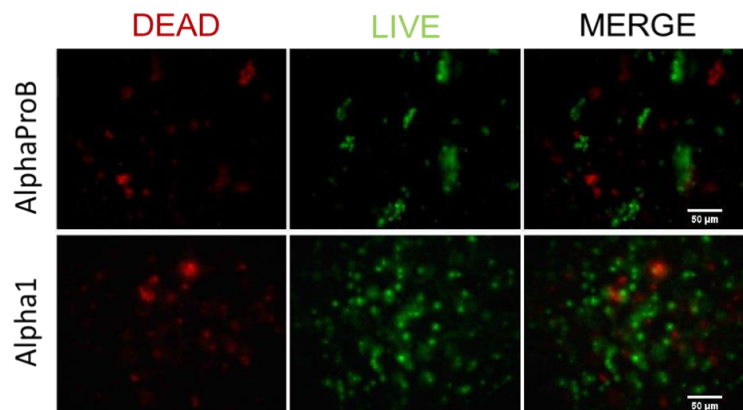


Figure 2: Fluorescence microscopy images of 3D printed mammary epithelial cell-laden constructs showing viable (green, calcein) and dead cells (red, propidium iodide) on both soft (AlphaProB) and stiff (Alpha1) hydrogels.

3.3. AFM analysis

Nano- to micro-scale structural and mechanical cues are associated with biological responses, in both native ECMs and synthetic constructs [28-31] Although the mechanistic understanding of this association is in its infancy, this level of structure in terms of assembly, control, and communication within the biological environment, are all key for biomaterials development [32-34]. We found that the nano-scale topographical appearance of unseeded samples were qualitatively similar, showing surface features on the order of 50-100 nm (Fig 3a,b). “Stiff” hydrogels however, consistently displayed large topographical features on the micro-scale, due to increased structural integrity maintaining the printed morphology (Fig 3b). In contrast, the “soft” hydrogel material flattened under surface tension to produce a smooth micro-scale topography. Calculated over $5\ \mu\text{m} \times 5\ \mu\text{m}$, roughness (R_q) was 0.7 and 11 nm for soft and stiff hydrogels, with low-level roughness of R_q 0.6 and 2 nm, respectively (calculated over $500\ \text{nm} \times 500\ \text{nm}$). Nano-mechanical properties were similarly varied between sample groups (Fig 3c,d). The self-assembled peptide components themselves showed opposite trends to the macro-scale properties. Nano-scale Young’s moduli of Alpha1 and AlphaProB were $330 \pm 55\ \text{MPa}$ vs

430±240 MPa ($p \rightarrow 0$, two-sided Wilcoxon rank sum test), compared to their macroscopic moduli on the order of 10 kPa and 1 kPa respectively. Loss tangent values were similarly distributed ($p \rightarrow 0$), with peptide components from macroscopically “soft” hydrogels more effectively damping, or dissipating, mechanical contact (Fig 3d). While bulk elastic [35] and viscous [36] properties have been shown to modulate cell response, the effects of local fibre properties within gels and matrices are less understood. It is not clear whether these properties can be decoupled in the current hydrogel system, however the range of mechanical properties observed at the different scales indicates strong potential for modulation of cell response via control of hierarchical viscoelasticity.

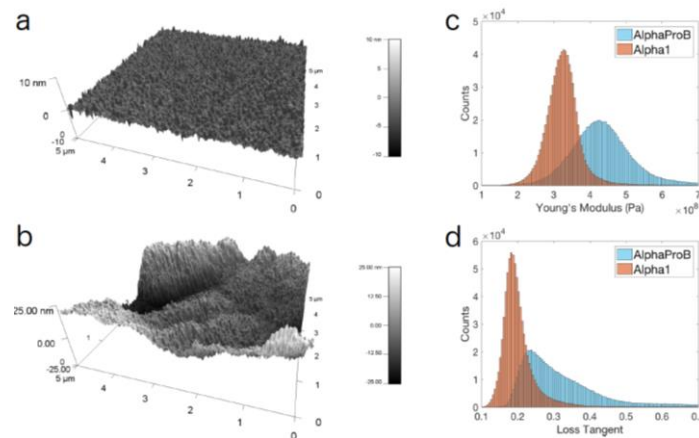


Figure 3: (a,b) Scanning probe microscopy shows representative 5 μm x 5 μm height images of soft and stiff unseeded hydrogels respectively. (c) Distribution of Young’s moduli. (d) Distribution of Loss tangent.

3. Conclusions

The successful application of 3D Bioprinting in the production of cell-laden constructs is required for the ability to develop suitable hydrogels or ‘bioinks’ with enhanced printability and cell viability. Here we report a novel methodology for the 3D printing of self-assembling peptide-based hydrogel constructs with encapsulated epithelial cells. Based on the optimization of the process parameters, we demonstrate the feasibility of the system to print 3D matrices with well-defined architectures and improved structural integrity. After 7 days of culture, biological results confirm that encapsulated mammary epithelial cells remain viable and start to proliferate, independently of the hydrogel stiffness. The potential of this new methodology relies on the possibility to manipulate, in a simple manner, the ECM microenvironment through 3D printing of hydrogels with adjustable hierarchical stiffness, thereby modulating cellular responses.

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