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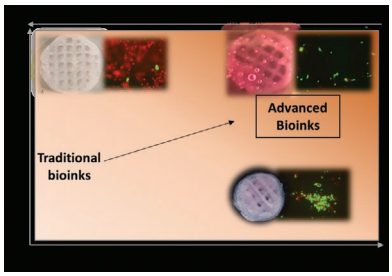

A bioink based on a self-assembled peptide and methylcellulose is developed. The addition of methylcellulose allows the bioprinting of the self-assembled peptide. The bioink exhibits good printability, print fidelity, and biocompatibility. The developed bioink maintains the differentiation potential of the encapsulated cells.

Macromolecular Materials and Engineering
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Development of a Self-Assembled Peptide/Methylcellulose-Based Bioink for 3D Bioprinting

Carla Cofiño, Soledad Perez-Amodio, Carlos E. Semino, Elisabeth Engel, and Miguel A. Mateos-Timoneda*

The introduction of 3D bioprinting to fabricate living constructs with tailored architecture has provided a new paradigm for biofabrication, with the potential to overcome several drawbacks of conventional scaffold-based tissue regeneration strategies. Hydrogel-based materials are suitable candidates regarding cell biocompatibility but often display poor mechanical properties. Self-assembling peptides are a promising source of biomaterials to be used as 3D scaffolds based on their similarity to extracellular matrices (structurally and mechanically). In this study, an advanced bioink for biofabrication is presented based on the optimization of a RAD16-I-based biomaterial. The strategy followed to build 3D predefined structures by 3D printing is based on an enhancement of bioink viscosity by adding methylcellulose (MC) to a RAD16-I solution. The resultant constructs display high shape fidelity and stability and embedded human mesenchymal stem cells present high viability after 7 days of culture. Moreover, cells are also able to differentiate to the adipogenic lineage, suggesting the suitability of this novel biomaterial for soft tissue engineering applications.

1. Introduction

The development of novel biomaterials whose properties closely match those needed for a particular application has become increasingly important in advanced tissue engineering.[1,2] Self-assembling peptides (SAPs) are a promising class of biomaterials in this field due to their unique properties. This class of 8–32 amino acid long oligopeptides are composed of hydrophilic and hydrophobic amino acid side chains forming a β-sheet structure that can self-assemble when exposed to physiological salt concentrations into a network of interweaving nanofibers of 10 nm diameter, forming hydrogel scaffolds with pores 5–200 nm in diameter and over 99% water content.[3,4] The weak interactions formed among peptides, their nanometer size (1000-fold smaller than synthetic polymer microfibers) and mechanical properties are similar to the natural extracellular matrix (ECM), better mimicking the 3D environment found in vivo.[5] This environment does not contain any specific peptide signaling motif, allowing the addition of specific signaling cues to specifically decorate the environment as desired.[6] Among different SAPs,[7–9] RAD16-I, commercially available as PuraMatrix, is one of the best candidates for tissue engineering applications.[10] RAD16-I consists of 16-amino acid peptide comprised of four repetitive units of Arginine (R), Alanine (A), Aspartic acid (D), and Alanine (A), forming the RADA motif.[3] The resultant hydrogel structure of this self-assembling peptide is permeable to small molecules such as gases, nutrients, and growth factors. Moreover, it has been demonstrated that RAD16-I is not immunogenic, does not cause inflammatory reactions nor cytotoxicity[2] and has been shown to support attachment, growth, maintenance, and differentiation of a variety of mammalian cells, such as hepatic stem cells, cardiac myocytes, neuronal stem cells, or endothelial cells.[3] Therefore, self-assembling peptides can be used for in vitro applications for cell 3D culture as well as in vivo for tissue regeneration.[7,11]

The construction of cell-laden structures able to recapitulate the complexity of native tissues is an engaging strategy to create functional tissue equivalents.[12,13] 3D bioprinting is an innovative technology that combines different biomaterials, cells and biological molecules to generate 3D constructs with well-defined architectures based on a layer-by-layer deposition

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DOI: 10.1002/mame.201900353

process.[14] Hydrogel matrices with embedded viable cells, named bioinks, are the preferred building materials in bioprinting due to their instructive and aqueous 3D environment properties, allowing proper conditions for cells.[14,15] Although the benefits of bioinks, they trigger some complications regarding their biofabrication, due to their relatively weak mechanical properties.[16] In order to build complex and tissue-like structures with high resolution, physical and biological properties must be rigorously adjusted. On the one hand, suitable bioinks should form 3D constructs with high shape fidelity and integrity, by remaining stable for the time of printing procedure and by bearing proper gelation characteristics after being extruded from a nozzle tip and forming solidified filaments mechanically strong enough to support the deposition of the upper layers.[17] On the other hand, cell viability is representative of the physiological properties of the bioinks, as they should offer the appropriate microenvironment to support cell survival and activity after printing.[17,18] These opposing requirements on the properties of hydrogel materials are reflected in the "biofabrication window" (**Figure 1**).[15,19]

While stiff hydrogels made of high polymer concentrations/high viscosity or high crosslinking densities allow obtaining optimal shape fidelity structures, these dense polymer networks restrict cell migration, growth, and differentiation resulting in low biocompatibilities. On the contrary, soft hydrogels promote adequate cell viability and function, but their low density/low viscosity leads to poor shape fidelity structures unable to maintain the construct designed. As a result, traditional structures are fabricated with a moderate degree of hydrogel crosslinking densities in order to maintain cell viability. It is desired to shift the bioprinting window and design advanced bioinks with tailored properties that enhance both printability and cell viability.[15] In this study, the self-assembling peptide RAD16-I has been proven as a novel biomaterial for 3D bioprinting due to its synthetic origin, properties and high similarity to the natural extracellular matrices. In order to obtain tissue engineering constructs by 3D bioprinting, a strategy to enhance printability has been applied resulting in an optimized RAD16-I/methylcellulose (MC) blend bioink.

2. Experimental Section

2.1. Bioink Preparation

RAD16-I peptide sterile working solution was prepared from the commercial 1 wt% solution (DB PuraMatrix, Corning). Puramatrix was placed into a conic tube, subsequently in liquid nitrogen until frozen and in the freeze dryer for 72 h. Sucrose 10 wt% (Sigma-Aldrich) was added to the freeze-dried sample to generate a RAD16-I solution at a stock concentration of 3 or 5.5 wt%. Sonication was done when preparing solutions to

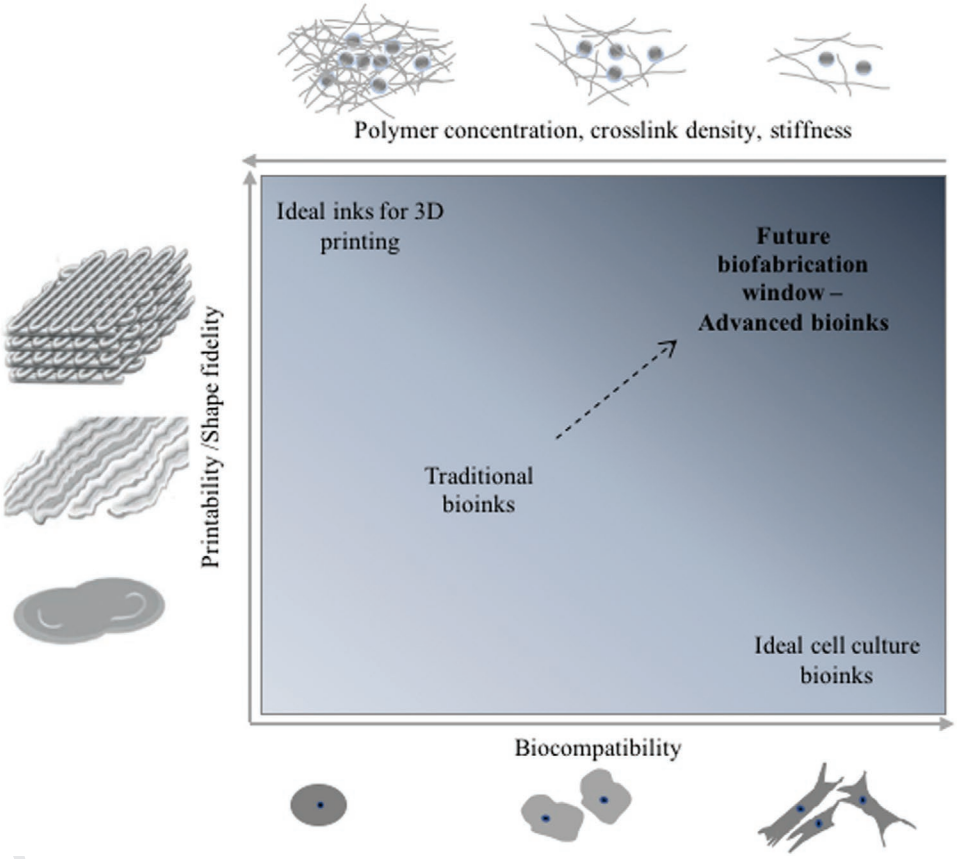

Figure 1. Biofabrication window for rational design of bioinks. Future biofabrication window requires advanced bioinks: medium crosslinked hydrogels that provide high shape fidelity with high biocompatibility. Reproduced with permission.[14,16]

resolubilize the material and facilitate easy handling. Methylcellulose (viscosity: 4000 cPs, MW: 88 kDa, Sigma-Aldrich) (MC) was sterilized by UV irradiation at 254 nm for 15 min. MC was prepared in sucrose 10% or PBS (from 1× to 10×) following the procedure indicated by the manufacturer. After 24 h at 4 °C to ensure complete dissolution, the final solutions were clear and transparent. Ratios of RAD:MC 1:2, 3:4 were prepared based on the dry mass of the compounds adding MC powder to the RAD16-I 3 wt% solution. For low MC concentrations (from 3% to 1.5%), the plotting material was prepared based on the volume of each compound. RAD16-I 5.5 wt% was blended with MC 6.88 wt% to reach a stock concentration of RAD 3.5 + MC 2.5 wt%. The mixtures were thoroughly stirred to homogenize the solution and stored at 4 °C overnight to ensure complete swelling of MC. The desired concentration of each compound was adjusted with the cell suspension or sucrose 10 wt% before printing. All reagents and materials were sterilized and manipulated under sterile conditions.

2.2. Viscosity Characterization

Viscosity of the peptide solution and RAD:MC blends was determined at room temperature using an AR 2000ex rheometer (TA Instruments, UK) and a conical plate 20 mm with a gap of 38 mm. Samples were sonicated for 1 h, one day before the assay.[4] Prior to measure, samples were incubated in a water bath at 37 °C for 30 min. Viscosity was measured at a shear rate of 1 s^{-1} for 1 min. Viscosity mean values were calculated at plateau viscosity range ($n = 3$).

2.3. Circular Dichroism

Circular dichroism (CD) studies were performed on a JASCO J-810 spectropolarimeter at room temperature in a quartz cuvette (Hellma Standard Cuvette 110 QS) with a pathlength of 1 mm and in the wavelength range 190–260 nm at a band width of 1 nm and using three times scans for average. RAD16-I and MC samples were diluted to get a final concentration in the RAD+MC blend of 25 and 85 μM, respectively. Solutions were allowed to equilibrate one day at 4 °C prior to analysis. Spectra for RAD16-I and MC were also analyzed separately.

2.4. 3D Printing

Hydrogel scaffolds were 3D printed using the 3D Discovery bioprinter (RegenHU, CH) by dispensing the bioink placed in a 3 mL syringe through a dosing metal needle of an inner diameter of 250 or 330 µm (Nordson EFD: precision dispensing tips, 6.35 mm length). Extrusion parameters for printing were adjusted by observing the flow of the material out of the needle in a fixed position. The initial pressure was set when a steady fiber flow was produced. The feed rate was adjusted based on the quality and accuracy of the fiber deposition. If the corners of the crosshatch pattern were not maintained, the speed was lowered. On the contrary, if too much material was being deposited, non-circular fibers were observed and the pressure and/or the feed rate were lowered. Hence, pressures applied ranged from 1.2 to 7 bar and feed rates from 1 to 8 mm s^{-1}. The initial height of the needle above the substrate plate was adjusted by raising the zero position 2/3 of the needle diameter and it was further optimized observing the attachment and consistency of the printed fibers. Note that if air bubbles in the bioink were formed, samples were centrifuged before printing for 2 min at 25 °C and 1000 rpm. 3D scaffolds were built up layer-by-layer in a circular crosshatch pattern and after printing the hydrogel scaffolds were allowed to gel in the flow cabinet with warm cell culture media for 20 min. At this point, constructs were manipulated carefully since improper handling before complete crosslinking could break the structure. After this time, media was replaced with fresh one to eliminate sucrose from media. At 24 h of incubation, medium was replaced again and then, every 2–3 days. Vacuum aspiration was not used when removing the media to avoid direct contact with the sample and material disruption.

2.5. Morphological Scaffold Characterization

Scanning electron microscopy (SEM) of the 3D bioprinted scaffolds was performed. Samples were washed twice with PBS and dehydrated by a gradation series of ethanol/distilled water solutions. After critical point drying a carbon coating was performed. Morphological characterization was done with an ultra-high resolution field SEM Nova NanoSEM 450 FEI microscope. Image J was used to evaluate fiber diameter and pore size.[20] At least, 20 measurements were taken from ten different images. The values are reported as value ± SD.

2.6. 3D Bioprinting with Human Mesenchymal Stem Cells

Human mesenchymal stem cells (hMSCs) derived from adipose tissue were used. Cells were expanded in monolayer culture in Advanced Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich), 1% L-Glutamine 100X (Invitrogen), 1% Penicillin/Streptomycin 100X (Invitrogen) and 0,1% Fibroblast Growth Factor basic (bFGF). Storage conditions were 37 °C in a 5% CO_2 humidified environment. hMSCs at 80–90% of confluence were cultured over night with media containing 2% FBS before trypsinization for bioink preparation, adapting cells to a more stringent environment. Then, a cell suspension in sucrose 10 wt% was prepared to get a concentration which corresponds to a final concentration in the bioink solution of 4–1.5 × 10^6 cells mL^{-1} (taking into account the volume of cell suspension to be added to the bioink to get the final concentration of all compounds). Sucrose 10 wt% was used as it is an isotonic and non-ionic medium, protecting cells from the acidic pH of the hydrogel while avoiding peptide gelation during the mixing process.[21] Because cells are in a quite hostile environment due to the peptide low pH before medium starts buffering the suspension, the mixture of cells with the bioink must be done carefully but quickly at the same time. Hence, the blending step was performed directly in the 3 mL syringes. After, bioinks were 3D bioprinted. Printing parameters were set

following the same premises for 3D printing without cells. For all the bioprinting assays, a positive control of cells embedded in the hydrogel solution but not printed was done following the same procedure except for printing. Negative controls were produced as cell-laden scaffolds but replacing cell suspension for sucrose 10 wt%. A heating plate was used at 42 °C during the printing process in order to induce the gelation of the MC when prepared in PBS 4X. Treatment of the cell-laden scaffolds and controls and their 3D culture was conducted in standard conditions (37 °C, 5% CO_2) and following the media changes indicated in Section 2.4.

2.7. Cell Viability Studies

hMSCs viability in the hydrogel was evaluated with the Live/Dead assay. Samples were gently washed in DPBS 1X (Gibco) 3 times. Then, a solution containing 2 µM Calcein-AM (Life Technologies) and 2 µM Propidium Iodide (Fluka) was prepared in DPBS 1X. The staining solution was added to the samples and incubated for 30 min avoiding light. After that time, samples were extensively rinsed with DPBS (three washes). Thereafter, images of the constructs were taken using an inverted fluorescence microscope (Leica DM IL Led). Four images per scaffold were taken and viability was estimated using Fiji-ImageJ (National Institutes of Health, NIH). For cell-laden scaffolds, the Live/Dead assay was performed at 0/4 h of printing, 1, 3, and 7 days.

2.8. Adipogenic Differentiation

Rat MSCs (rMSCs) at passage 8 and at 90% of confluence were used. Bioink final concentration was: RAD16-I 2.7% + MC 1.5% + rMSCs 2×10^6 cells mL^{-1}. After 3D bioprinting the cell-laden scaffolds, they were incubated over a period of 8 days in culture medium as follows: for 72 h scaffolds were incubated with adipogenic induction medium containing Dubelcco's Modified Eagle Medium (DMEM, Sigma-Aldrich), 10% FBS, 1% L-Glutamine 100X and 1% Penicillin/Streptomycin 100X, 1 µM dexamethasone (Sigma), 0.2 mM indomethacin (Sigma), 5 µg mL^{-1} insulin (Sigma), 1 mM 3-Isobutyl-1-methylxanthin (IBMX, Sigma); then, for 24 h, they were cultured with adipogenic maintenance medium containing DMEM base medium, 10% FBS, 1% L-Glutamine 100X, 1% Penicillin/Streptomycin 100X and 0.1 mg mL^{-1} insulin; this cycle was repeated three times. For adipogenic control, non-printed cell-laden hydrogels were cultivated under the same conditions of the printed scaffolds. Negative controls consisted on cell-laden scaffolds cultured with basal medium (DMEM, 10% FBS, 1% L-Glutamine 100X and 1% Penicillin/Streptomycin 100X). Samples were analyzed for adipogenic differentiation at day 8 of culture for light microscopy study. Moreover, viability of cell-laden scaffolds was checked by Live/Dead staining at day 1, 3, and 7 of culture. For lipid droplets staining, Oil-red O 0.5 wt% reagent was used (Sigma-Aldrich). Samples were washed three times with PBS and fixed for 30 min with 4% paraformaldehyde. Afterward, hydrogels were washed twice with PBS and a staining with Oil-red O (6:4 in water) for 30 min was done. Then, two more washes with PBS were done. Stained samples were imaged with confocal laser scanning microscopy (LSM 800, Zeiss) using excitation wavelength of 543 nm (619 nm emission) for Oil-red O. If crystals of the dye were observed, an extra rinse with isopropanol was performed.

2.9. Statistical Analysis

Each experiment was performed in three or five replicates (n = 3 or 5). Data are represented as mean and standard deviation of the replicates. Statistical significance was assessed performing Student's t-test using Origin 8.0 Software (OriginLab, USA).

3. Results and Discussion

3.1. Bioink Characterization for 3D Bioprinting

3.1.1. Improvement of RAD16-I Printability

A bioink formulation should be able to be deposited in successive layers without collapsing when printed in air.[22] In order to produce biological constructs with well-defined geometry and integrity, RAD16-I (3% w/v) was 3D printed and the resultant structure was unable to maintain the designed pattern due to the fluxion of the material because of the low viscosity of the SAP despite increasing its concentration (**Table 1**).

Then, to improve the shape fidelity of RAD16-I, and consequently the viscosity of the bioink,[23] methylcellulose (MC) was used as a reinforcer material and thickening agent. This biopolymer was chosen as it is biocompatible and biodegradable and its gelling capacities can be tailored by its concentration and molecular weight and influenced by the presence of salts.[24–26] In order to set which concentration of methylcellulose was the most appropriated to blend with the peptide solution, three bioinks were formulated and 3D-printed. Ratios of RAD:MC (RAD16-1:MC) were tested (1:1, 1:2, 3:4) (**Figure 2**, **Table 2**).

MC allows the precise deposition of the bioink material (Figure 2a,b) and a concentration of 4 wt% mixed homogeneously with a 3 wt% peptide solution allows plotting scaffolds of high shape fidelity, showing a strand width of 396 ± 37 µm, closer to the theoretical one (330 µm) (Figure 2d). Rheological studies of the chosen bioink confirmed that MC improved significantly the viscosity of the solution. At low shear rates (1 s^{-1}) viscosity of the pregel solution of RAD16-I 3% + MC

Table 1. Viscosities of different bioink solutions before gelation.

Material [wt%]	Viscosity [Pa s][a)]
RAD16-I 1%	4
RAD16-I 3%	20
RAD16-I 3% + MC 2%	256
RAD16-I 3% + MC 4%	290

[a)]Viscosity calculated at 1 s^{-1} shear rate.

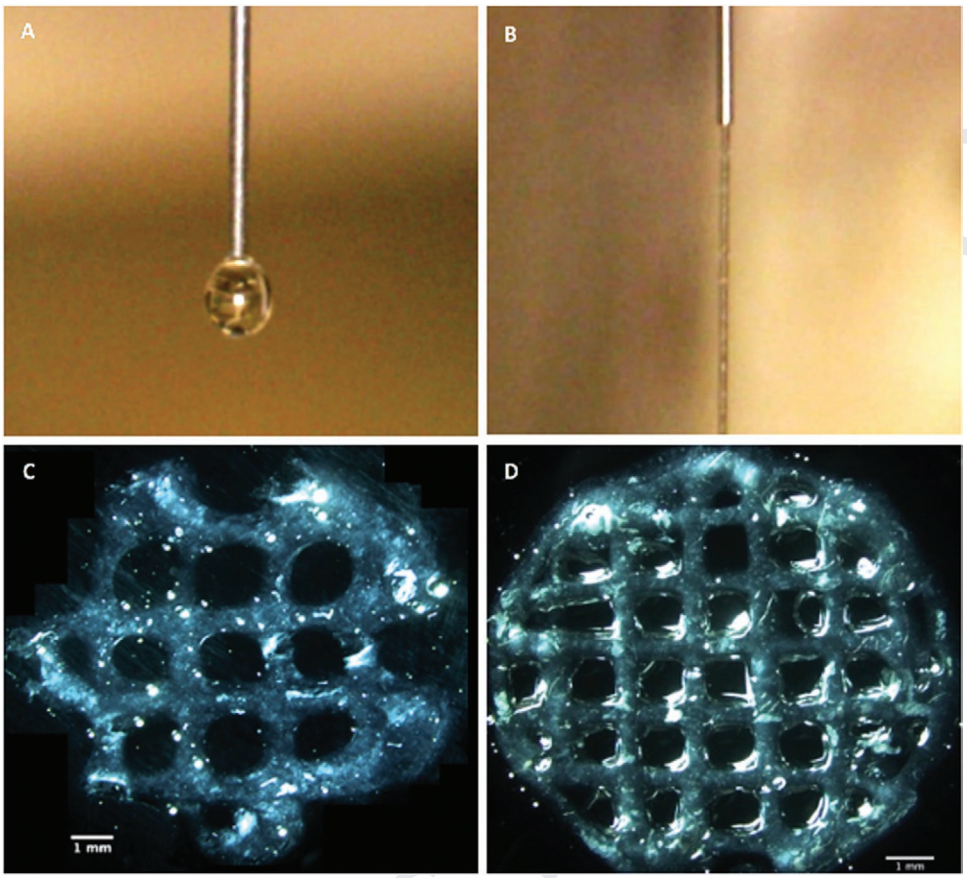

Figure 2. Printability studies to set the adequate methylcellulose (MC) concentration to blend with RAD16-I 3 wt%. A) Air extrusion of RAD16-I 3%. B) Air extrusion of RAD16-I 3% + MC 6% solution. C,D) Stereomicroscopy images of scaffolds fabricated with RAD16-I 3% + MC 3% and RAD16-I 3% + MC 4%, respectively (scale bar 1 mm).

4% was 14-fold higher than the 3% peptide solution (Table 1). Therefore, the viscosity of the bioink solution mainly determines the overall performance of the fiber deposition during the printing process. Viscoelastic properties were indicative of the relative mechanical stiffness of the peptides in the preassembled versus the assembled peptides. The linear viscoelastic region, where both G' and G'' moduli were constant,[27] was 0.01 (dimensionless) (Figure S1, Supporting Information). Despite the fact that both RAD16-I and RAD16-I + MC solutions presented a gel-like behavior with $G' > G''$ constant along the range of frequencies tested, the RAD16-I+MC solution showed a higher modulus (G' and G'') than the peptide solution (Figure S2, Supporting Information). After crosslinking the solutions, the modulus showed an enhancement of 100-fold for RAD16-I solution and 20-fold for the composite solution, indicating a significant increase of gel strength (Figure S2, Supporting Information). Moreover, each gel reached equilibrium with the buffer solution, maintained stable rheological properties with time and no significant differences were observed between RAD16-I and with the addition of MC, indicating that the crosslinking of the peptide was not altered (Figure S3, Supporting Information). Additionally, circular dichroism (**Figure 3**) studies proved that the addition of MC did not interfere in the β-sheet structure of the peptide and thus it could self-assembled to form nanofibers.

Moreover, SEM of the hydrogel scaffolds revealed that the structure was presented as a flat membrane at low magnifications (**Figure 4**a), but the membrane appears formed of interwoven individual filaments when observed at higher magnifications (Figure 4b). The mean fiber diameter of the nanofibers was 34 ± 7.25 nm and the pore size of the interwoven structure 158 ± 59.76 nm, similar to theoretical values of RAD16-I nanofibers (nanofibers of 10–20 nm in diameter and pore size between 5 and 200 nm).[4] Therefore, SEM analysis confirmed that the resulting scaffolds presented a porous surface, combining both micro and nanoporosity, allowing biomolecular diffusion and mimicry to the ECM.[27]

Table 2. Printing parameters used for 3D-print the different blended hydrogel scaffolds.

Printing parameters[a)]	Bioink solution		
	RAD 3% + MC 6%	RAD3% + MC 3%	RAD3% + MC 4%
Pressure [bar]	6–7	1.2–1.4	4
Feed rate [mm s^{-1}]	1	6–8	5

[a)]Needle inner diameter: 0.25 mm.

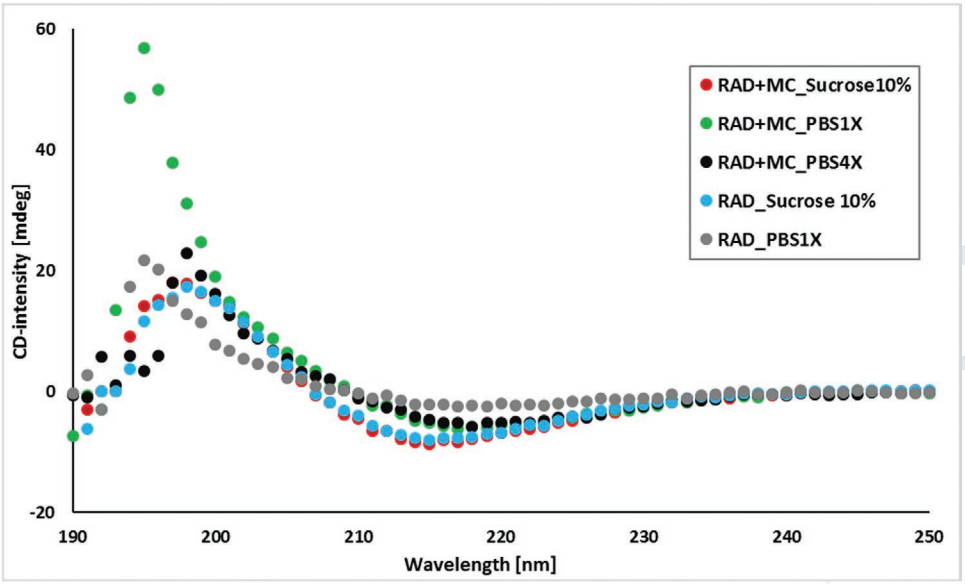

Figure 3. Circular dichroism spectra of RAD16-I (RAD) dissolved in sucrose 10 wt% or PBS 1X and RAD + MC dissolved in sucrose 10 wt%, PBS 1X, or PBS 4X.

3.1.2. Cell Viability within the Bioink

It is known that SAPs are characterized by exhibiting a low pH, which implies working quickly when encapsulating cells within the prehydrogel solution in order to minimize the time that cells are in this hostile environment. Therefore, the encapsulation step is the main critical point of the whole process and where cell viability is more affected.[29] The RAD16-I 3% solution exhibits a pH of 1, and the addition of MC did not produce an increase of pH. Thus, when the RAD16-I 3% + MC 4% was first used to encapsulate quickly the human mesenchymal stem cells (hMSCs) at a final concentration of 4×10^6 cells mL^{-1} and bioprinted, the scaffold maintained high shape fidelity but the resultant cell viability was significantly low (**Figure 5**).

This low cell viability could be explained either by the increment of the printing pressure (5 bar) or because of the time cells were in contact with the low pH of the peptide solution before reaching physiological pH. MC was dissolved in different PBS concentrations in order to allow the diffusion of PBS in the MC to the RAD16-I solution once blended and stabilized. Here, two main factors were considered. On the one hand, if PBS could increase the pH of the pregel solution, this would imply that the peptide solution would partially cross-link. Then, the PBS concentration should increase the pH but to a point where the structure of the peptide was maintained enough to be 3D printed and not broken (for the weak interactions that form the hydrogel). On the other hand, due to the increment in the pressure for extrusion observed during

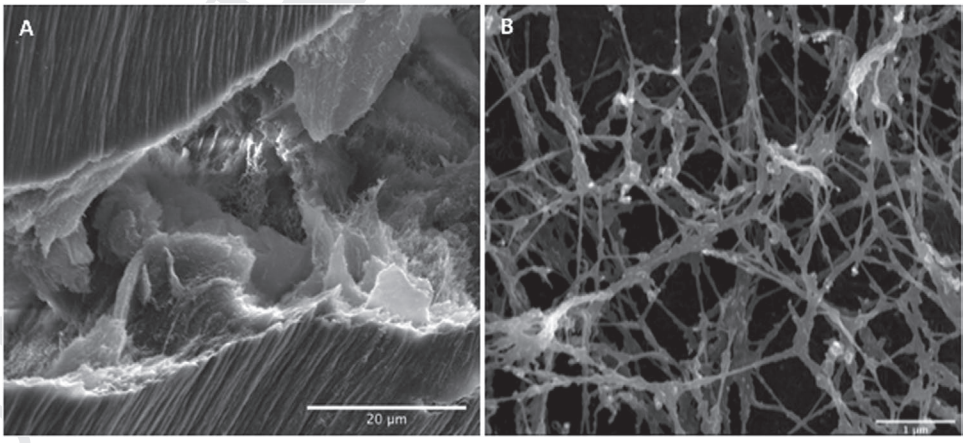

Figure 4. SEM images of RAD16-I 3% + MC 4% hydrogel scaffolds at different magnifications: A) 5000× (scale bar 20 µm) and B) 50 000× (scale bar 1 µm).

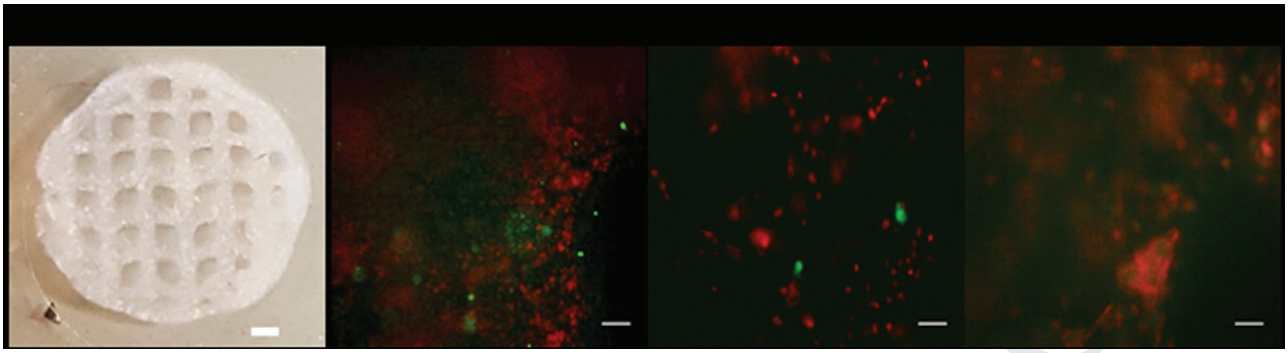

Figure 5. Assessment of RAD16-I 3% + MC 4% hydrogel scaffolds biofabrication. A) Scaffold 3D printed: 5 mm radius, 1.5 strand distance, 4 layers printed (Scale bar: 1 mm). B) Viability of cells embedded in the printed scaffolds at 0 h, 1, and 3 days after printing (scale bar: 500 µm). Green: live cells; red: dead cells.

the first bioprinting experiment and considered that the addition of PBS to the MC could reinforce the pregel solution, it was decided to reduce to 2 wt% the concentration of MC. Thereafter, different concentrations of PBS (from 1X to 10X) were used to dissolve MC and then MC was blended with RAD16-I to obtain a final concentration of RAD16-I 3% + MC 2% (wt). The pH of each solution was measured (Figure S4, Supporting Information) and it was observed that PBS 4X was the most appropriate to be used since it increased the pH of the solution to 2.5, as the pH of a typical working concentration of RAD16-I (0.5% w/v). Cell viability assays confirmed that RAD16-I 3% + MC 2% (MC in PBS 4X) could maintain a moderate cell viability in the pregel solution for 10 min before reaching neutrality, compared to the low cell viability obtained without the MC dissolved in PBS (Figure S5, Supporting Information).

3.2. Bioink Optimization for 3D Bioprinting

Rheological analysis confirmed that despite reducing the MC concentration, it was still able to increase the viscosity of the pregel solution up to 13-fold higher than the peptide solution viscosity (Table 1). After some 3D-bioprinting analysis, it was found that the best cell-laden scaffolds showing both high shape fidelity and high cell viability were produced when the bioink formulation was made of RAD16-I 2.7% + MC 1.5% (MC in PBS 4X) with hMSCs at a final concentration of 1.5×10^6 cells mL^{-1}. This reduction of concentrations might allow higher permeability of nutrients and oxygen within the printed scaffolds while the partially low crosslinking favored the maintenance of the pregel viscosity. The cell-laden scaffolds were printed with an extrusion pressure of 0.6-1 bar, a feed rate of 8–9 mm s^{-1} and with a needle of 330 µm inner diameter. Additionally, the surface of the wells where the strands were being deposited was previously equilibrated with cell culture media to induce quickly a physiological environment. Moreover, since it has been reported that the addition of PBS decreases the gelling temperature of MC,[24] a heater plate at 42 °C was used during printing in order to induce its gelation and reinforce the scaffold structure once deposited on the well plate. The resulting cell-laden scaffolds exhibited good printability (**Figure 6**a) with a strand width of 430 µm ± 66 µm, closer to the theoretical one (330 µm). At 4 h, 1, 3, and 7 days of culture after printing, cell viability was evaluated and quantified (Figure 6b,c). It can be observed that cells were also homogeneously distributed within the scaffold and that at day 3 of culture they exhibited both round and rudimentary filopodia morphologies. Immunostaining of embedded cells at day 2 of culture confirmed this morphology (Figure S6, Supporting Information).

Viability of cells embedded in the hydrogel but not printed was also monitored as a positive control. On the one hand, right after printing (4 h), a great number of living but also of dead cells were observed, leading to a viability of around 55%. Cells not being printed exhibited a cell viability about 70%. Then, the reduction of viability at 4 h could be associated with the printing process. At day 1 of culture, cells recovered from the shear forces applied to them during printing and showed a viability of approximately 65%, corresponding with the viability at day 1 of non-printed cells. Therefore, at day 1, cells were likely being adapted to the new 3D environment and recovering from the stresses applied during bioink manipulation. On the other hand, at days 3 and 7 of culture, cell viability started to increase in the non-printed embedded cells but not in the case of the cells embedded in the scaffolds. In this case, cell viability at day 3 was still similar to day 1 but after 7 days of culture, it increased to ≈80%, similar to the viability of the non-printed cells. For example, it was useful to compare these results with other cell viability studies of cell-laden hydrogels printed. It was observed that after 7 days of culture similar results were achieved with a cell viability of around 80%.[30] Other studies obtained opposite results, meaning that the viability of cells embedded and printed was higher the first day after printed than after 7 days of culture.[22] Then, this could indicate that the RAD16-I+MC hydrogel used is suitable for long-term cell studies since it allows maintaining both scaffold structure and cell viability during time, confirming the stability of the SAP. Since RAD16-I presents a low biodegradation[29] in contrast to MC, cells would be able to proliferate and generate their own ECM, replacing first the MC and then the RAD being degraded. Nevertheless, more studies should be done in order to assess for how long the scaffold structure can be maintained. As a result, when MC was dissolved

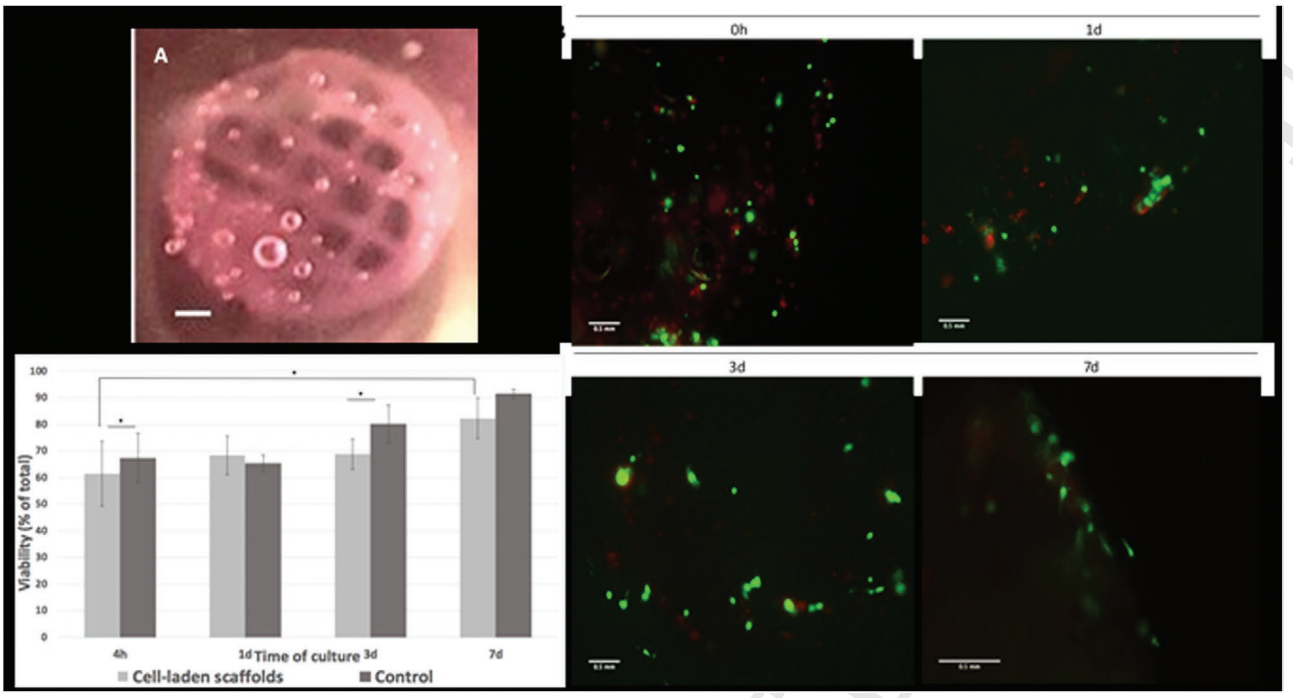

Figure 6. Optimized bioink for 3D bioprinting consisting on RAD16-I 2.7 wt% + MC 1.5 wt% + hMSC $1.5{\cdot}10^6$ cells mL^{-1}. A) 3D bioprinted scaffold in cell culture media with 4 mm radius, strand distance of 1.5 mm, and 2 layers height (scale bar 1 mm). B) Viability images of cells embedded within the scaffolds at 4 h, 1, 3, and 7 days (d) of culture (scale bar 0.5 mm). C) Viability quantification of embedded cells in the 3D bioprinted scaffold compared with the embedded cells non printed (control). Green: live cells; Red: dead cells.

in PBS 4X and the concentrations of both components of the bioink were slightly reduced, cell-laden scaffolds could be printed with both good shape fidelity and relatively high percentage of cell viability.

3.3. rMSCs Adipogenic Differentiation

Adipogenic differentiation of rat mesenchymal stem cells (rMSCs) embedded within the RAD 2.7% + MC 1.5% 3D-bioprinted scaffolds was evaluated by Oil-red O staining of lipid vesicles at day 8 of culture with or without adipogenic media.[31] rMSC were used in this assay as they are easier to handle and differentiate more quickly to the adipogenic linage than hMSCs. Results obtained from the Oil-red O staining indicated that rMSCs were able to differentiate to adipogenic linage (**Figure 7**) since lipid droplets inside the adipogenically induced cells were found after 8 days of culture.[32]

4. Conclusions

In this study, a novel bioink of RAD16-I and methylcellulose has been suggested as a candidate biomaterial for 3D bioprinting as a strategy to overcome the main bottleneck that biofabrication must face: the development of advanced bioinks that combine both high printability and biocompatibility. The optimization of the bioink formulation and the printing process lead to the construction of stable 3D scaffolds of well-defined geometry, high shape fidelity and cell viability after 7 days of culture. Adipogenic differentiation was a proof of concept to show the potential of this bioink for soft tissue engineering systems. The bioink presented here is thought to be a potential

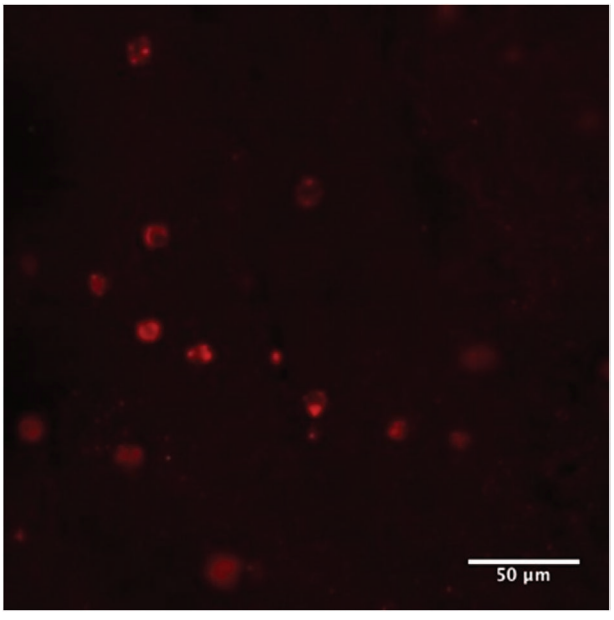

Figure 7. Oil-red O staining of adipogenically induced rMSCs at day 8 of culture. Red: lipid droplets in fat vacuoles (scale bar 50 µm).

biomaterial for advanced tissue engineering applications considering the advantages that offer the RAD16-I peptide and its versatility to be easily blended with CM.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Part of this work was supported by the Spanish Ministry of Economy and Competitiveness through the project [MAT2015-68906-R] (MINECO/FEDER) and CERCA Programme/Generalitat de Catalunya. This work has been developed in the context of QuirofAM project (COMRDI16-1-0011) with the support of ACCIÓ (Catalonia Trade & Investment; Generalitat de Catalunya) and the European Community under the Catalonian ERDF operational program (European Regional Development Fund) 2014–2020. Part of the work has been performed at the ICTS "NANBIOSIS," Unit 5 of CIBER in Bioengineering, Biomaterials & Nanomedicine (CIBER-BBN) at IBEC.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

3D bioprinting, biofabrication, bioink, self-assembling peptides, tissue engineering

Received: May 31, 2019
Revised: July 1, 2019
Published online:

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