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3D bioprinting of BM-MSCs-loaded ECM biomimetic hydrogels for in vitro neocartilage formation

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Abstract

In this work we demonstrate how to print 3D biomimetic hydrogel scaffolds for cartilage tissue engineering with high cell density (>10^7 cells ml^{-1}), high cell viability (85 ÷ 90%) and high printing resolution (≈100 μm) through a two coaxial-needles system. The scaffolds were composed of modified biopolymers present in the extracellular matrix (ECM) of cartilage, namely gelatin methacrylamide (GelMA), chondroitin sulfate amino ethyl methacrylate (CS-AEMA) and hyaluronic acid methacrylate (HAMA). The polymers were used to prepare three photocurable bioinks with increasing degree of biomimicry: (i) GelMA, (ii) GelMA + CS-AEMA and (iii) GelMA + CS-AEMA + HAMA. Alginate was added to the bioinks as templating agent to form stable fibers during 3D printing. In all cases, bioink solutions were loaded with bone marrow-derived human mesenchymal stem cells (BM-MSCs). After printing, the samples were cultured in expansion (negative control) and chondrogenic media to evaluate the possible differentiating effect exerted by the biomimetic matrix or the synergistic effect of the matrix and chondrogenic supplements. After 7, 14, and 21 days, gene expression of the chondrogenic markers (COL2A1 and aggrecan), marker of osteogenesis (COL1A1) and marker of hypertrophy (COL10A1) were evaluated qualitatively by means of quantitative fluorescence immunocytochemistry and quantitatively by means of RT-qPCR. The observed enhanced viability and chondrogenic differentiation of BM-MSCs, as well as high robustness and accuracy of the employed deposition method, make the presented approach a valid candidate for advanced engineering of cartilage tissue.

1. Introduction

Articular cartilage (AC) is a highly specialized connective tissue of diarthrodial joints. Its main function in the human body is to provide a smooth, lubricated surface for articulation and to bear loads with low frictional coefficient. AC is mainly composed of a dense extracellular matrix (ECM) made of proteoglycans, collagen type II and water in which a single type of cells—chondrocytes—are embedded [1]. Due to its avascular nature and low chondrocytes density, AC has a limited capacity of self-repairing, a condition that often leads to degenerative diseases upon injury. Currently, the surgical approaches to treat AC lesions are mainly four: microfracture, autologous chondrocyte implantation (ACI), mosaicplasty and osteochondral allograft [2, 3].

The size of the lesion, the physical demands of the patient, and treatment history all are important factors to consider when selecting a surgical approach instead of another. In most of the cases, these approaches provide a relief to patients after a rehabilitation period. However, they present some disadvantages as they are: (i) invasive, (ii) time consuming (multiple steps are involved), (iii) costly and (iv) in long term they generally fail in recreating a fully functional hyaline
cartilage that on the contrary in many cases resembles fibrocartilage [4].

For these reasons, there is a growing demand for developing new cell-based strategies for cartilage repair that can lead to better functional hyaline cartilage. So far, numerous studies have explored the fabrication of in vitro cartilage equivalents by using a combination of porous scaffolds or hydrogels with differentiated chondrocytes [5–10]. Although in some studies the quality of such engineered cartilage tissues was approximately similar to native one, the limited supply of chondrocytes and their tendency to de-differentiate during extensive monolayer cellular expansion limit the applications of the above mentioned approaches to clinical application [11, 12].

Recently, mesenchymal stem cells (MSCs) have been used in cartilage tissue engineering as an alternative cell source to chondrocytes due to ease of isolation, high expansion capacity in vitro and ability to differentiate towards chondrocytes [13–16]. Furthermore, they can be harvested from different tissues including bone marrow, adipose tissue, synovium, periosteum, umbilical cord vein or placenta [17–19].

3D bioprinting (3DBP) is a fast-emerging technique in TE, based on the controlled and simultaneous 3D deposition of living cells and supporting biomaterials [20, 21]. This technique combines the features of 3D printing such as the complete control over the design, fabrication and modelling of the scaffold being constructed with the possibility of precisely depositing living cells in the 3D space. The rationale for such significant control is to generate cell-biomaterial engineered constructs that, thanks to their internal and external spatial arrangement, may mature into functional tissue equivalents [22].

Given the stratified nature of AC, 3DBP represents an ideal fabrication method for cartilage tissue engineering as it makes possible to improve the functionality of tissue engineered constructs through the precise placement of cells—chondrocytes and/or MSCs—and ECM biomimetic polymers—gelatin, hyaluronic acid, chondroitin sulphate, etc.—in 3D space to recapitulate the structure and function of native tissue.

Recent studies have shown the feasibility of 3DBP hydrogels made of hyaluronic acid grafted with poly (N-isopropylacrylamide) crosslinked by methacrylated hyaluronic acid (HA-pNIPAAM-HAMA) or hyaluronic acid grafted with poly(N-isopropylacrylamide) crosslinked by methacrylated chondroitin sulphate (HA-pNIPAAM-CSMA) [23], gelatin-methacrylde (GelMA) [24–26], alginate [27] and nanocellulose-alginete [28] and Pluronic F127 diacrylate (PF127DA) or Plastic F127 diacrylate crosslinked by methacrylated hyaluronic acid (PF127DA-HAMA) [29] loaded with chondrocytes or MSCs. Although notable proofs of concept, it is evident that some problems and limits still need to be addressed and overcome. Firstly, the printing quality was rather low in all cases. Fibers diameter ranged between 800 µm and 2 mm with spacing between adjacent fibers always >1 mm. Moreover, fibers from different layers generally collapsed one over each other creating an ‘unicum’ scaffold in which individual fibers are difficult to be recognized thus partially vanishing the 3DBP process. In the second instance, the formulation of employed bioinks was strongly influenced by the deposition step and just a partial biomimicry of cartilage tissue was achieved. Glycosaminoglycans (GAGs) solutions generally have either too low viscosity (CS) or not ideal shear thinning rheological behaviour (HA) to be printed alone. For these reasons, synthetic polymers such as pNIPAAM or Pluronic F127 were added to bioinks to impart thermostressive properties to their solutions while nano-fibrillated cellulose was employed to enhance bioink shear-thinning behaviour. However, due to biological inertness of pNIPAAM and PF127, cells embedded in these matrices might preferentially establish cell–cell interactions rather than cell-matrix interactions that, consequently, might lead to the formation of undesired cell clusters.

In this work we present a novel method to 3D bio-print highly ECM biomimetic hydrogels scaffolds for cartilage regeneration through an innovative deposition system based on a coaxial-needles extruder developed in-house.

We demonstrate that our method allows for (i) high printing resolution (>≈100 µm) of photocurable bioinks made of either GelMA, GelMA-CS-AEMA and GelMA+CS-AEMA+HAMA, (ii) high cell density printing (>10^7 BM-MSCs/ml) and (iii) high cell viability (>85 ± 90%). Furthermore, we discuss on the key role played by alginate in allowing the printing of low viscous bioinks through our coaxial-needles system and employing cell friendly cross-linking conditions.

Finally, we demonstrate by RT-qPCR and by fluorescence immunohistochemistry that bioprinted BM-MSCs cultured for 3 weeks in chondrogenic medium differentiated towards chondrocytes and started producing neocartilage.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Alginate (ALG) (M_w ≈ 33 kDa) was a kind gift from FMC Biopolymers. HA (M_w ≈ 200 kDa) was a kind gift from FIDIA Biopolymers (Abano Terme, Italy). Chondroitin sulfate (CS) (M_w = 5.9 kDa) was purchased from Sigma-Aldrich.

2.2. Synthesis of polymers

2.2.1. GelMA

Gelatin (type A3 from porcine skin) methacrylamide (GelMA) was prepared by reaction of gelatin with
methacrylic anhydride. After dissolution of gelatin (1 g) in phosphate buffer (pH 7.5) at 50 °C, 0.8 ml methacrylic anhydride was added dropwise under vigorous stirring. After 2 h of reaction, the reaction mixture was diluted and dialyzed (MWCO = 2 kDa) for 3 days against distilled water at 40 °C and finally freeze-dried.

2.2.2. HAMA

Methacrylated hyaluronic acid (HAMA) was synthesized by the addition of methacrylic anhydride (MA, ~20-fold molar excess with respect to disaccharide repeating unit of HA) to a solution of 1 wt% HA in deionized water following a procedure published elsewhere [30]. Briefly, MA was added dropwise to HA solution and pH was continuously adjusted to 8 with 5 N NaOH solution. After MA addition, mixture was left to react on ice for 24 h. Finally, the reaction mixture was dialyzed (MWCO = 2 kDa) against deionized water for 3 days and then freeze-dried.

2.2.3. CS-AEMA

Vinyl moieties were introduced along chondroitin sulphate (CS-AEMA, MW ≈ 5.9 kDa) chains through the EDC/NHS coupling reaction with 2-aminoethyl methacrylate (AEMA). Briefly, 1 g of CS was dissolved in 100 ml MES buffer (pH = 6.5, 50 mM) containing 0.5 M of NaCl. N-hydroxy-succinimide (NHS, 0.27 g) and 1-ethyl-3-(3-dimethylaminopropyl)-carbo-dii-mide hydrochloride (EDC, 0.85 g) were added to the mixture to catalyze the coupling reaction between carboxylic and amino groups. After 5 min, AEMA (0.38 g) was added and the reaction was maintained at room temperature for 24 h. Finally, the reaction was dialyzed (MWCO = 2 kDa) against distilled water for 3 days and then freeze-dried.

2.2.4. Determination of degree of vinylic functionalization.

$^1$H-NMR (Bruker AVANCE AQS 600 MHz) operating at 600.13 MHz was used to determine the final functionality and purity of CS-AEMA and HAMA (D$_2$O). The degree of functionalization of HAMA (~30% with respect to disaccharide repeating unit) was obtained by the ratio of the integrals of the signals relative to the vinyl protons (5.7–6.2 ppm) and of the anomeric proton in position ~5 ppm (see figure S1). In the case of CS-AEMA, the degree of derivatization (~11% with respect to disaccharide repeating unit) was obtained by the ratio of the integrals of the peaks at 5.7–6.2 ppm and the proton at position 2 of the glucuronic residue at ~3.5 ppm (see figure S2). In the case of GelMA, the maximum theoretical functionalization degree is related to the content of ε-amino groups attributable to lysine and hydroxylysine residues (0.34 mmol per gram of Gelatine Type A).

2.2.5. Bioinks formulation

3D bioprinted scaffolds presented in this study were fabricated out of three different bioinks. Bioinks were formulated with the rationale of obtaining a high and increasing degree of biomimicry of ECM present in the cartilage tissue. The chemical composition of the three bioinks was: (i) 6% w/w GelMA, (ii) 6% w/w GelMA + 4% w/w CS-AEMA and (iii) 6% w/w GelMA + 4% w/w CS-AEMA + 0.5% w/w HAMA. Biopolymers were dissolved in HEPES buffer (25 mM) containing 10% v/v of fetal bovine serum (FBS). 0.05% w/w Irgacure 2959 was added to all bioinks as the radical photoinitiator. In order to process and 3D bioprint our bioinks with high resolution, 4% w/w alginate (ALG) was also added to all solutions. The addition of ALG to bioinks allows them to instantaneously undergo gelation in presence of Ca$^{2+}$ ions.

The concentrations of ALG and Ca$^{2+}$ ions were optimized through a systematic screening to guarantee both a cell-friendly environment and high accuracy and resolution of 3D-printed structures. As a general rule, the higher is the concentration of ALG employed, the lower is the concentration of CaCl$_2$ needed for an instantaneous gelation. We thus proceeded by increasing slowly the concentration of alginate (starting from 1% w/w) and decreasing the concentration of CaCl$_2$ (starting from 0.6 M) until getting an optimal printing resolution. We then conducted 3DPB experiments to confirmed that the two concentrations were not harmful for cells.

2.2.6. Fabrication of the dispensing coaxial system

The custom coaxial dispensing system was obtained by combining two parts. The first part was a microfluidic chip bearing a single microchannel (cross-section = 500 μm, length = 15 mm) in which the inner needle (25 G) of the coaxial system was inserted and glue watertightly. The microfluidic chip was fabricated by micromilling two polycarbonate (PC) sheets 5 mm in height. After milling, the two sheets were sonicated in isopropanol for 30 min and then sealed with a hot press at 130 °C for about 30 min [31]. The second part was a standard needle (19 G) in which we drilled a hole sideways in the plastic hub to fit a small tube. This needle was then glue to the microfluidic device to form the coaxial system, paying the highest attention to have the two needles perfectly centred. This custom dispensing system was finally screwed at the bottom of a low-temperature cartridge of a commercial 3D printer (3D-Bioplotter, Envision-TEC, GmbH).

5 Due to the heterogeneity of alginates in terms of molecular weight and guluronic/mannuronic molar ratio, the concentrations of alginate and calcium chloride should be always tuned opportune-ly for each type of alginate to achieve high printing resolution and high cell viability.
2.2.7. Fabrication of 3D bioprinted scaffolds
Bioinks—loaded or not with BM-MSCs—and calcium chloride solution (0.3 M) were loaded in 2 ml sterile plastic syringes and supplied to the internal and external needles, respectively, of the dispensing coaxial syringes and fabricated with the same bioinks compositions and crosslinked for 30 s with a UV lamp (DYMAX, BlueWave® 75). The UV light intensity was measured at the exit of fiber lightguide with a UV radiometer (DYMAX, ACCU-CAL™ 50) and set at 800 mW cm⁻². Compression tests were performed with DMA Q800 Dynamic Mechanical Analyzer (TA Instruments, New Castle, DE, USA) in the strain rate mode. All tests were performed at 37 °C with the following parameters: 0.001 N preload force, 20% min⁻¹ strain rate and final deformation was set at 70%. Young’s moduli were calculated from the initial linear regions (0%–20% of strain) of the obtained stress-strain curves. Each measurement was performed in quintuplicate and results are reported as the mean ± standard deviation.

2.2.8. Bioinks and 3D bioprinted scaffolds coding system
For presentation clarity, we will use throughout the text a coding system to discriminate between the bioinks or the scaffolds fabricated with a specific bioink. In the case we refer to bioinks—i.e. to the precursor solutions of the scaffolds—we will use the following coding system: (i) AlgGel, (ii) AlgGelCs and (iii) AlgGelCsHa. When we will refer to 3D bioprinted scaffolds, we will add a prefix to the bioinks coding system: (i) 3D-AlgGel, (ii) 3D-AlgGelCs and (iii) 3D-AlgGelCsHa.

2.3. Materials characterization
2.3.1. Bioinks rheology
Rheological properties of the bioinks were analyzed using an Anton Paar RHEOPLUS-32 rheometer equipped with a cone-plate geometry (diameter = 40 mm). All measurements were performed at 25 °C, and the samples were allowed to reach equilibrium temperature for 60 s prior to each measurement. Shear viscosity behaviour of the bioinks was evaluated by applying a shear rate from 0.01 to 100 s⁻¹.

2.3.2. μCT
Micro-computed tomography (μCT) is a non-destructive, high-resolution technique that allows for studying 3D morphology of complex small objects. To produce CT images, multiple x-ray images are taken as the sample is rotated around a central rotation axis. This data set is then processed and 2D stacks of images—in any desired plane—or 3D reconstructions can be easily obtained.

Acquisition of 3D printed scaffolds morphologies was performed on acellularized samples. Prior to be scanned, samples were partially dried in a mixture of water/ethanol 50:50 for five minutes and then were dipped into sunflower oil to increase x-ray contrast. Scanning was performed using an Xradia MicroXCT-400 with the following parameters: 40 kV voltage, 10 W power, no filter material, 0.18° rotation step in an angle interval of 184°. The voxel size was 4.97 × 4.97 × 4.97 μm³. Image analysis and 3D reconstruction of printed samples were performed with ImageJ software.

2.3.3. Mechanical testing—Young’s modulus
The compressive stress–strain measurements were performed on water swollen cylindrical discs (6 mm in diameter, 2 mm in height) fabricated with the same bioinks compositions and crosslinked for 30 s with a UV lamp (DYMAX, BlueWave® 75). The UV light intensity was measured at the exit of fiber lightguide with a UV radiometer (DYMAX, ACCU-CAL™ 50) and set at 800 mW cm⁻². Compression tests were performed with DMA Q800 Dynamic Mechanical Analyzer (TA Instruments, New Castle, DE, USA) in the strain rate mode. All tests were performed at 37 °C with the following parameters: 0.001 N preload force, 20% min⁻¹ strain rate and final deformation was set at 70%. Young’s moduli were calculated from the initial linear regions (0%–20% of strain) of the obtained stress-strain curves. Each measurement was performed in quintuplicate and results are reported as the mean ± standard deviation.

2.4. BM-MSC culture
2.4.1. Ethics statement
The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, section A. Participants provided their written informed consent to participate in the study.

2.4.2. Isolation, characterization and culture of BM-MSCs
Approximately 50 ml of bone marrow was aspirated from the iliac crest of healthy voluntary donors. The mononuclear cells were isolated from the aspirate using density gradient centrifugation (Lymphoprep, Fresenius Kabi, Oslo, Norway). The cells were seeded in 175 cm² flasks (Nunc, Roskilde, Denmark) and cultured in Dulbecco’s modified eagle medium: nutrient mixture F-12 containing GlutaMAX (DMEM/F-12 GlutaMAX, Gibco, Paisley, UK), supplemented with 10% human AB serum (Sigma Aldrich, St. Louis, MO), 10 ng ml⁻¹ rh FGFb (R&D Systems, Minneapolis, MN, USA), 1% penicillin/streptomycin and 1.5 μg ml⁻¹ amphotericin B. After 48 h, non-adherent cells were removed by medium change and the remaining adhering cells were expanded in monolayer culture using the medium described above, until colonies reached 70% confluence. Cells isolated from donors were validated as MSCs following protocols developed in the past [32, 33].
After reaching 70%–80% confluence, BM-MSCs were trypsinized and subsequently washed with 25 mM HEPES buffer containing 10% FBS and 1% of antibiotic-antimycotic solution (10 000 units/ml penicillin, 10 mg ml⁻¹ streptomycin and 25 μg ml⁻¹ amphotericin B). After centrifugation, BM-MSCs were resuspended in bioinks polymeric solutions at a final density of approximately 1.0 ÷ 1.5 × 10⁷ cells ml⁻¹, loaded in 1 ml syringes and, finally 3D-bioprinted.

At desired time points, the 3D bioprinted constructs loaded with BM-MSCs were washed twice with Dulbecco’s PBS (Sigma) and incubated with live-dead fluorescent dyes (Live Dead Cell Imaging Kit, R37601, Molecular Probes, USA) at 37 °C for 20 min. Then, the constructs were rinsed with PBS and analyzed using fluorescent microscope (Leica TCS SP8) at wavelengths corresponding to fluorophores of interest. For each analyzed sample, cells were counted from high magnification (10×) fluorescence images by means of Image J software by counting the number of green-fluorescent (live) and red-fluorescent (dead) cells, in separate analysis. Cell viability was quantified by averaging five different regions for each scaffold. One-way ANOVA analysis was carried out within each group of samples, followed by multiple pairwise comparison. Assumptions of normality were checked and met. Significance was at the 0.05 or 0.02 level.

3D Bioprinted hydrogels were washed with HEPES buffer (25 mM) and incubated in high-glucose DMEM supplemented with 100 μM ascorbic acid-2-phosphate; 1 mM sodium pyruvate; 40 μg ml⁻¹ insulin; 20 μg ml⁻¹ apo-transferrin; 25 ng ml⁻¹ sodium selenite; 1.25 mg ml⁻¹ BSA; 5.35 μg ml⁻¹ linoelic acid; 10 ng ml⁻¹ TGF β3; 100 μM dexamethasone and 1% PSN. The chondrogenic and expansion media were exchanged 3 times per week. At pre-determined time points, the constructs were washed with PBS and either snap-freeze (for RT-PCR analysis) or fixed (for immunocytochemistry).

Total RNA were purified from MSC in alginate hydrogels using the RNeasy kit following the manufacturer’s protocol (Qiagen, Valencia, CA). The hydrogels were first grinded in Eppendorf tubes with corresponding pestles while frozen in liquid N₂ and further grinded into a fine powder using QiaShredder columns (Qiagen). RNA was treated with DNase I (Ambion, Austin, TX) and quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). 100ng of the purified RNA was reverse transcribed into cDNA in a total volume of 20 μl using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer’s protocol (Applied Biosystems, Abingdon, UK). Polymerase chain reaction was used to probe cDNA samples for relevant genes, using Taqman gene expression assays (supplementary table 1) and Taqman universal PCR master mix with the 7300 Real-Time RT PCR system (Applied Biosystems). All samples were run in technical triplicates. The thermocycling parameters were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After verifying the stable expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), this gene was selected as endogenous control. Relative mRNA levels were calculated by the comparative CT method [34].

Bioinks characterization
3DDBP technologies drastically revolutionizing all manufacturing fields—including TE—as they offer a multitude of advantages compared to conventional approaches. 3DDBP technologies hold great promise for advances in the field of regenerative medicine as they allow, in a single step, for the generation of spatially controlled cell patterns through the deposition of a bioink. Bioinks are aqueous solutions of polymers in...
which cells are suspended and deposited in the form of hydrogel fibers to fabricate a 3D scaffold. Formulating a proper bioink is challenging as from one side it has to provide an ideal environment for cells growth, spreading and differentiation and from the other it deeply influences the quality of printing. To address all of these challenges, we have developed recently an innovative deposition system based on a coaxial-nozzles printing head [35]. This method relies on the property of solutions containing ALG to undergo an instantaneous gelation when exposed to divalent calcium ions. The internal nozzle of the dispensing system is fed with a bioink containing ALG while the external one is supplied with a solution of calcium chloride. When the two flows come into contact at the tip of the inner nozzle, the bioink undergoes instantaneous gelation and hydrogel fibers can be precisely laid down with high resolution in 3D. A schematic representation of the coaxial nozzles system and of scaffolds production is reported in figure 1. The possibility of printing with high-resolution solutions containing ALG is a great advantage of our technique and opens new vistas in the formulation of bioinks. In fact, most of commonly used biopolymers in TE such as GAGs or gelatin do not have ideal rheological behaviour to be printed alone and up-to-date have found only limited applications in 3DBP.

For these reasons, we decided to formulate ECM biomimetic inks for cartilage regeneration by blending ALG with photocurable gelatin (GelMA), chondroitin sulphate (CS-AEMA) and hyaluronic acid (HAMA) (See paragraph 2.2.4 for more information about bioinks composition.) The concentration of all biopolymers—with the exception of ALG that is used only as templating agent for fibers deposition—was tuned to match their amount in the ECM of healthy AC [1]. The employment of photocurable polymers in tandem with ALG allows for the formation of stable hydrogels after UV exposure that can undergo long culturing in vitro. A typical printing process consists of the following steps. Firstly, ALG based bioinks are deposited in forms of Ca2+-ionically crosslinked hydrogel fibers to obtain a 3D multi-layered constructs. After printing, photocurable polymers contained in the microfibers can be covalently crosslinked by exposition to UV light. This process guarantees a good bonding among fibers belonging to adjacent layers and determines the overall mechanical properties of the scaffolds. After few days, ALG ionically crosslinked by Ca2+ dissolves in culture media leaving a 3D microfibers mesh composed of UV covalently crosslinked polymers [36].

To demonstrate the possibility of 3D printing of the aforementioned bioinks and the robustness of our deposition system, we printed our bioinks without cells to form different geometrical solids. In figure 2 optical images of the printed objects are reported (see also figure S3 in which a 3D bioprinted neonatal-size ear is shown). As it can be seen, the shape fidelity was high in all cases: all solids were 50 layers tall with a layer thickness of 100 μm (htot = 5 mm) and a distance between fibers in the X–Y plane of 300 μm. To gain further insights into the morphology of 3D printed structures, we performed micro computed tomography (μCT) scan of a printed scaffold 6 × 6 × 1 mm3 with 0°–90° fibers orientation. As layer thickness and distance between fibers in X–Y plane we used the same parameters employed for printing the solids. The 3D reconstructions obtained from μCT scan are reported in figure 3. Fibers upon printing preserve a rod-like shape and they can easily be stacked without signs of collapse along z-direction: this is particularly evident in figure 3(b) in which lateral pores—i.e. pores generated from fibers belonging to different z-planes—can be appreciated throughout the sample.

Prior to starting 3DBP experiments, we studied the rheological behaviour of our three bioinks. These investigations are critical, as cells during printing will be exposed to shear stress fields that should not be harmful to them. For instance, at levels in the order of 1 Pa the morphology and metabolic activity of articular chondrocytes is significantly changed [37]. In figure 4, the dynamic viscosity of the bioinks, η, plotted as a function of shear rate, γ, is reported. In the range of shear rate studied (0.01–100 s−1), the three bioinks present different rheological behaviours.
AlgGelCs and AlgGelCsHa have a slightly shear thinning behaviour with the dynamic viscosity decreasing as the shear rate increases—from around 1 Pa s to 300 mPa s—while AlgGel exhibits essentially a Newtonian behaviour with a constant viscosity of around 100 mPa s. By applying the Poiseuille’s law for the case of a fluid flowing through a long cylindrical pipe of constant cross section:

\[ \tau_{\text{max}} = \left( \frac{4\eta Q_{\text{bioink}}}{\pi R^4} \right), \]

where \( Q_{\text{bioink}} \) is the volumetric flow rate of the bioink, \( \eta \) is the dynamic viscosity and \( R \) is the radius of the pipe (in our case a 25 G nozzle—inner diameter 260 \( \mu \)m), we estimated the maximum shear stress \( \tau_{\text{max}} \) to which cells are exposed during 3DBP experiments. As \( Q_{\text{bioink}} (5 \mu l \text{ min}^{-1}) \) and \( R (130 \mu m) \) were constant for all experiments, the only parameter that changed among different 3DBP experiments was the dynamic viscosity of bioinks. The estimated value of \( \tau_{\text{max}} \) ranged between 4.8 Pa—for AlgGel—to around 50 Pa—for AlgGelCsHa. It is worth to mention that a potential bias in the evaluation of \( \tau_{\text{max}} \) may arise from the fact that we did not take into consideration the presence of cells within the bioinks. However, these values can be interesting for comparisons with the
bioinks were loaded with $10^7$ BM-MSCs. In all cases suggesting that the cells were not negatively affected by such shear stress values.

3.2. 3DBP of BM-MSCs

After checking the feasibility of 3D printing our bioinks, we performed the 3DBP experiments. In all cases, bioinks were loaded with $10^7$ BM-MSCs/ml. One of the advantages of using low viscous bioinks in conjunction with our dispensing system is the possibility of dispersing homogeneously a high cell concentration. To investigate whether the printing process and UV crosslinking affected cell viability, a live/dead assay was performed 3 h after printing (figures 5(a)–(f)). As shown in figure 5(g), the as printed cell viability was always higher than 85 ± 90% for all the samples. These results demonstrate that ionic crosslinking of ALG and the following crosslinking induced by UV light are cell friendly processes and do not cause significant cells death. In support to this experimental evidence, in a recent work [38] it was demonstrated that hMSCs can tolerate well UV dose as high as 5.25 J cm$^{-2}$ without significant effects on gene expression, a value much higher than the one employed in this work (∼0.180 J cm$^{-2}$). By analysing the results of live/dead assays obtained soon after deposition, after one week and three weeks of culture, we found that cell viability slightly decreased over time in all cases. Interestingly, we found that the most consistent decrease in cell viability was observed in the case of 3D-AlgGelCsHa. The different sets of samples prepared from the three bioinks were then cultured in expansion and chondrogenic media to evaluate the possible stimulation towards chondrogenesis exerted solely by the biomimetic matrix or the combined effect of the biomimetic matrix and chondrogenic supplements. However, since we did not observe a significant differentiation of BM-MSCs embedded in hydrogels and cultured in expansion media, the data will not be presented. One factor that plays an important role in regulating TGFβ-induced MSC differentiation is cell shape. It has been proved that when MSCs flatten and spread on stiff substrates, with high cytoskeletal tension, they express high levels of bone cell markers [39, 40] while when they preserve a round shape, a chondrogenic differentiation is favoured [41]. For this reason, we stained the cytoskeleton and nuclei of 3D bioprinted scaffolds cultured for 21 days in chondrogenic medium to analyze the morphology of cells embedded within the printed hydrogel fibers. As shown in figures 6(a)–(c), BM-MSCs preserved a round shape in all scaffolds during their culture. These findings indicate that the formulated bioinks provide an ideal chemical and mechanical microenvironment for the chondrogenic differentiation of BM-MSCs. Interestingly, cells that escaped out of the hydrogel fibers remained attached on the outer surface of the fibers and appear well spread (figures 6(b) and (c)). Due to cytoskeleton tensions, these cells might undertake an undesired differentiation pathway. However, elongated cells appear to be a restricted minority compared to round ones.

Chondrogenic differentiation of BM-MSCs in 3D bioprinted hydrogels was verified by RT-qPCR and by immunocytochemistry (ICC) analysis. In both cases, we decided to study the gene expression as well as the structural protein production and localization of collagen type I, type II, type X and aggrecan. These analyses give an idea of the differentiation status of BM-MSCs and provide quantitative (by using RT-qPCR) and qualitative (by using ICC staining) data relative to chondrogenic (collagen type II) and aggrecan, osteogenic (collagen type I) and hypertrophic (collagen type X) markers. In figure 7 a panel with the obtained ICC fluorescence images after 21 days of chondrogenic induction is reported. A good aggrecan production
can be observed in all samples with an even distribution within printed fibers (figures 7(a)–(c)). Regarding collagen type I and type II, they were both secreted by cells and started cumulating in the pericellular matrix region. On the contrary, the intercellular space appears devoid of fluorescence signal. This phenomenon may be detrimental for the in vitro fabrication of an engineered neocartilage. However, as reported in the literature better results can be obtained by increasing cell density during 3DBP [14], by 3DBP a co-culture of MSCs and chondrocytes [42] or by tailoring the transport and diffusion properties of the hydrogels [43–45]. Interestingly, in the case of collagen type X, we observed a modest homogeneous fluorescence signal throughout the scaffolds fibers. This may be related to the low molecular weight of collagen type X that favours its diffusion within and outside the hydrogels network.

To gain a quantitative overview of the real differentiation status of BM-MSCs towards chondrogenic phenotype, we analysed the gene expression of structural proteins by RT-qPCR. The gene expression of analysed genes is reported relative to GAPDH. As it can be seen in figure 8, the level of aggregan gene expression was similar for all samples confirming the results obtained from ICC images. 3D-AlgGelCs outperformed the other scaffolds as the production of collagens type I and type X was limited while the production of collagen type II was favoured (figures 8(b)–(d)). This difference is presented in

Figure 5. Live/dead assays performed 3 h after printing BM-hMSCs with the formulated bioinks: (a), (b) 3D-AlgGel, (c), (d) 3D-AlgGelCs and (e), (f) 3D-AlgGelCsHa. Cell viability was quantified by means of image analysis of selected regions of scaffolds. Viability data as a function of time reported in (g) were obtained by averaging five different regions for each scaffold at desired time points. ANOVA analysis was carried out within each group of samples and significant differences at 95% (*) and 98% (**) confidence level are shown.
figures 8(e) and (f) where the ratio collagen type II/collagen type I and collagen type II/collagen type X as a function of days of culture are plotted. From these graphs it is evident that 3D-AlgGelCs scaffolds represent the best candidate for cartilage regeneration among the scaffolds fabricated with a gene expression of collagen type II comparable with collagen type I and a gene expression of collagen type II six times higher than collagen type X. Surprisingly, 3D-AlgGelCsHa scaffolds turned out to be the worst candidate with the highest expression of collagen type X and type I. This is supported by the cell viability results (figure 5(g)) which show that BM-MSCs embedded in AlgGelCsHa suffered the most pronounced decline with time. This might be explained in terms of crosslinking density and macromolecular permeability of the matrix in which MSCs are embedded. In previous studies [43–45], it was demonstrated that differentiation of MSCs embedded in HA-based hydrogels is deeply influenced by network crosslinking density. In fact, by either increasing HA concentration [43, 44] or increasing the amount of crosslinking agent [45], it has been found
that the quality of ECM deposited and its distribution within the hydrogels were progressively worsened with a clear shift towards hypertrophic differentiation of the chondrogenically induced MSCs.

In our case, the addition of a low concentration of highly derivatized HAMA (DS ~ 30%, see paragraph 2.2.3) is expected to enhance significantly the hydrogel network crosslinking density. An indirect proof of this speculation comes from the comparison of the compression moduli of hydrogels discs fabricated with AlgGel, AlgGelCs and AlgGelCsHa bioinks.

As reported in table 1, the addition of 4% w/w of CS-AEMA to AlgGel led to a 24% increase of the compression modulus while the addition of only 0.5% w/w of HAMA (4.8% w/w of total polymers weight) brought about to a 68% increase of compression modulus.

This can be explained only on the basis of both the high molecular weight and the high degree of functionalization of HAMA chains that acted as effective enhancers of hydrogel crosslinking density and stiffness, a factor that may promote hypertrophic differentiation of BM-MSCs [43–45]. This problem might be partially overcome by tuning the degree of substitution of HAMA. However, it is evident that the network density of the matrix in which MSCs are embedded is not the only parameter influencing MSCs chondrogenic differentiation since also the chemical composition of the 3D microenvironment plays a role. In fact, despite the addition of CS-AEMA to the AlgGel bioink increased the network density and stiffness of the final matrix, the embedded BM-MSCs underwent a more pronounced chondrogenesis. These results reveal that

**Figure 8.** mRNA expression levels relative to GAPDH of selected genes relevant to chondrogenic differentiation measured by RT-qPCR: (a)–(d) structural ECM proteins, (e) collagen type II/collagen type I and (f) collagen type II/collagen type X ratios plotted as a function of days of culture in chondrogenic medium.
additional studies must be conducted in the future to better clarify the connection between 3D micro-environment properties of the hydrogels and MSCs differentiation performances.

4. Conclusions

In this work we have presented an innovative method for 3D printing and bioprinting alginate and ECM analogues-based bioinks. We have demonstrated that this method allows for high-resolution 3D printing (>100 µm) of intricate solids geometries with exceptional shape fidelity. Moreover, we showed that by blending alginate with photocurable polymers such as GelMA, CS-AEMA and HAMA it is possible to formulate ECM biomimetic inks that can be used for cartilage tissue engineering.

All the employed hydrogels exhibited an enhanced chondrogenic differentiation of BM-MSCs after 3 weeks of culture in chondrogenic medium. Among the formulated bioinks, the one composed of alginate, GelMA and CS-AEMA turned out to be the best candidate in neocartilage formation with the highest collagen type II/collagen type I and collagen type II/collagen type X ratios. Surprisingly, the addition of HAMA to the bioink favoured the differentiation of BM-MSCs towards hypertrophic cartilage, supporting the hypothesis that a too crosslinked matrix does not allow for proper macromolecular diffusivity and hinders cartilage development. Our findings demonstrate that the presented 3D deposition method is highly robust and accurate making it a valid candidate for advanced 3DBP applications.

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