Biofabrication

PAPER

3D printed oxidized alginate-gelatin bioink provides guidance for C2C12 muscle precursor cell orientation and differentiation via shear stress during bioprinting

Thomas Distler¹,², Aditya A Solisito³, Dominik Schneidereit², Oliver Friedrich³, Rainer Detsch¹ and Aldo R Boccaccini¹

¹ Department of Materials Science and Engineering, Institute of Biomaterials, Erlangen 91058, Germany
² Department of Chemical and Biological Engineering, Institute of Medical Biotechnology, Erlangen 91052, Germany
³ These authors contributed equally to this work.

E-mail: aldo.boccaccini@fau.de and rainer.detsch@fau.de

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Abstract

Biofabrication can be a tool to three-dimensionally (3D) print muscle cells embedded inside hydrogel biomaterials, ultimately aiming to mimic the complexity of the native muscle tissue and to create in-vitro muscle analogues for advanced repair therapies and drug testing. However, to 3D print muscle analogues of high cell alignment and synchronous contraction, the effect of biofabrication process parameters on myoblast growth has to be understood. A suitable biomaterial matrix is required to provide 3D printability as well as matrix degradation to create space for cell proliferation, matrix remodelling capacity, and cell differentiation. We demonstrate that by the proper selection of nozzle size and extrusion pressure, the shear stress during extrusion-bioprinting of mouse myoblast cells (C2C12) can achieve cell orientation when using oxidized alginate-gelatin (ADA-GEL) hydrogel bioink. The cells grow in the direction of printing, migrate to the hydrogel surface over time, and differentiate into ordered myotube segments in areas of high cell density. Together, our results show that ADA-GEL hydrogel can be a simple and cost-efficient biodegradable bioink that allows the successful 3D bioprinting and cultivation of C2C12 cells in-vitro to study muscle engineering.

1. Introduction

Besides cardiac and smooth muscle, skeletal muscle is one of the three types of vertebrae muscles [1]. It accounts for approximately one third of the adult human body mass [2] and is crucial for body support and mobility. Small damage to skeletal muscle caused by crush or strain injury or even toxins can be self-repaired in a highly orchestrated manner [3], which is made possible through multipotent muscle satellite cells, or muscle stem cells (MuSCs) [3, 4]. However, skeletal muscle cannot restore significant tissue loss, such as ongoing degeneration caused by Muscular dystrophies (MD) [5] or volumetric muscle loss (VML) resulting from severe injuries, tumor ablations, or prolonged denervation [6–8]. Over time, intrinsic changes in cells cause a decrease in muscle cell regeneration capabilities with advanced age [5]. As demographics change towards an elderly population [9], the treatment of muscle atrophy and the related significant healthcare burden must be considered [4, 10]. Common clinical strategies to treat diseased and injured skeletal muscle are either free muscle transfer, the use of advanced braces [7, 11, 12], or myogenic stem cell transplantation, which involves the use of radiation and the administering of toxic myeloablative drugs [5]. Despite technological advances, those methods rarely allow to fully restore muscle function or involve clinical procedures of high risk for patient health [7, 13].

Thus, new methods to restore functional skeletal muscle are being developed to overcome the drawbacks of current therapies. Tissue engineering (TE) and regenerative medicine are promising approaches...
to tackle the need for alternative muscle-repair strategies [14, 15]. The aims to create functional tissue constructs by combining cells, biomaterials, and biologically active molecules [13, 16] as well as methods of seeding and growing muscle cells in 2D or on biomaterial scaffolds have been demonstrated [17–24]. It has been possible to improve the proliferation, alignment, and differentiation of muscle cells by matching biomaterial scaffolds of defined geometries, surface properties, porosities, and mechanical properties [25]. An alternative strategy is the encapsulation of cells inside three-dimensional (3D) hydrogels [19, 20]. As an attempt to create 3D muscle tissue constructs with similar complexity as native tissue, additive manufacturing-based biofabrication may be a key route for in-vitro muscle engineering [26–29]. As one particular biofabrication technique, bioprinting offers versatility in process parameters and allows to combine different materials with the ability to create 3D constructs by embedding cells inside the printed hydrogel constructs [29, 30]. Chung et al [31] used an alginate-gelatin bioink, and reported the improvement of the attachment and proliferation of primary myoblast cells by covalently crosslinking the GRGDS (apasep) peptide sequence to alginate [31]. However, no cell proliferation was observed within the alginate-gelatin matrix in-vivo using BL6 primary myoblast cells [31], a result which was assigned to relatively high matrix stiffness and the short length of the study. Kang et al [32] 3D printed gelatin, fibronogen, hyaluronic acid, and glycerol polymer embedding 3 × 10⁶ C2C12 cells per ml bioink [32], combined with a PCL pillar support and pluronic fugitive ink. The cells were able to differentiate into myotubes with high alignment [32]. Despite the promising results, muscle performance was identified by the authors to be lower than the positive control (normal gastrocnemius muscle), hence leaving room for improvement [32]. Bandopadhyay et al [33] demonstrated the 3D bioprinting of C2C12 cells embedded in gelatin, alginate, and type-I collagen hydrogel [33]. Printing constructs that were stable for 14 d in-vitro, the group showed that cells were able to proliferate and form a cellular network within the hydrogel [33]. Despite proliferation, cellular alignment and differentiation were not observed, while not providing a sufficiently biodegradable matrix by using pristine alginate. A biodegradable yet bioprintable hydrogel matrix allowing cell attachment, alignment, and differentiation of C2C12 cells is required for successful muscle engineering. The oxidation of alginate offers the possibility of achieving controlled degradation by hydrolysis [19, 34]. Oxidized alginate (or alginate di-aldehyde, ADA) based hydrogels have demonstrated their potential for vascular [35–37], bone [38, 39], cartilage [40, 41] and other TE approaches, as well as for bioprinting [39, 42–44]. Boontheekul et al [19] seeded C2C12 cells on ADA hydrogels, functionalized with the oligopeptide sequence G₄RGDSP, thus offering motifs for cell attachment as well as matrix degradability [19]. High cell attachment and proliferation of C2C12 myoblast cells were observed, indicating the potential of ADA hydrogels for muscle TE [19]. Dalheim et al [45] confirmed that periodate oxidized alginates modified using GRGDYP peptide sequences allowed increased C2C12 cell adhesion in comparison to pristine alginate [45]. As a means of introducing RGD cell adhesion motifs, gelatin has been used in combination with ADA hydrogels, allowing 3D printability and providing cell adhesion capability [34, 46].

In this study, we investigate the suitability of oxidized alginate-gelatin bioink for 3D printing C2C12 cells (figure 1), by combining the promising results of 2D adhesion of C2C12 cells on RGD functionalized ADA films [19] with the 3D printability and degradability of oxidized alginate-gelatin (ADA-GEL) hydrogels [34, 42, 46]. The influence of different printing parameters on proliferation and alignment of C2C12 cells was examined. Horse serum studies were performed to assess the influence of different concentrations on the differentiation of C2C12 using second harmonic generation (SHG) imaging. We demonstrate that it is possible to influence the alignment of C2C12 cells in 3D printed ADA-GEL hydrogel constructs by altering processing parameters, which has an effect on the proliferation of C2C12 over time in ADA-GEL hydrogels. A simple and affordable bioink based on alginate and gelatin is presented, combined with a process to obtain aligned microarchitectures by inducing shear forces at the extrusion nozzle.

2. Materials and methods

2.1. Materials

Sodium alginate (VIVAPHARM alginate PH176) was purchased from JRS PHARMA GmbH & Co. KG (Rosenberg, Germany). Dialysis tubings (MWCO: 6–8 kDa) were from Repligen (Waltham, USA). Calcein AM, Propidium Iodide (PI) Rhodamine phalloidin, and 4’,6-Diamidin-2-phenylindol (DAPI) were from Thermo Fisher Scientific™ (Waltham, USA). Glutaraldehyde was from Polysciences, (Warrington, USA), sucrose, and N’-bis(2-ethanesulfonic acid/PIPES were from Merck (Kenilworth, USA). Ethanol (99.5%), sodium metaperiodate (NaIO₄), and ethylene glycol were purchased from VWR (Radnor, USA). All other chemicals were obtained from Sigma Aldrich if not otherwise noted.

2.2. Synthesis of oxidized alginate

The synthesis process of ADA was performed as described elsewhere [34]. Sodium alginate (10 g) was dispersed in 50 ml of ethanol. Next, sodium metaperiodate (2.674 g) was dissolved in 50 ml ultrapure water (Direct-Q, Merck Millipore, Kenilworth, USA) in the absence of light. NaIO₄ solution was added
Figure 1. Schematic of 3D printing C2C12 cells embedded in oxidized alginate-gelatin bioink (ADA-GEL). (a) Bioink extrusion through a printing nozzle (velocity profile $\vec{v}$) causes shear forces ($\vec{\tau}$) acting on the bioink and cells inside the nozzle, which are highest in proximity of the nozzle inner wall. (b) Molecular structure of ADA-GEL bioink, forming imine bonds between the aldehyde groups of oxidized alginate (ADA) and the ε-amine groups of gelatin (GEL). (c) Cells embedded in bioink (i) without applied pressure or bioink flow ($p = 0$), followed by (ii) pressure application to trigger bioink extrusion, which leads to a shear force profile orienting bioink macromolecules and cells in extrusion direction; (iii) ionic crosslinking of oriented bioink using $\text{Ca}^{2+}$; (iv) hypothesis of cell growth along oriented and crosslinked bioink that possesses RGD cell adhesion motifs of GEL over time, leading to potential C2C12 cell orientation and directed myotube formation.

to the sodium alginate dispersion and stirred continuously at 22 °C (room temperature, RT) in the absence of light for 6 h. The oxidation reaction was quenched by adding 10 ml of ethylene glycol followed by additional stirring for 30 min. The oxidized alginate product was filled into dialysis tubings and dialyzed against ultrapure water for 5 d with water change daily. After purification, the resulting ADA solution was frozen for at least 24 h and lyophilized using a freeze dryer (Alpha 1–2 LD plus, Martin Christ, Osterode am Harz, Germany). The degree of oxidation of the product was approx. 25% and it was determined as reported elsewhere [34, 47]. In brief, the residual amount of IO$_4^-$ ions was determined after ADA synthesis by combining 1 ml of the reaction solution (ADA) and 1 ml of indicator solution (potassium iodide 20% (w/v), starch solution 1% (w/v), in phosphate buffer pH = 7). A blue triiodine-starch complex resulted, which was measured at 486 nm by a UV–Vis spectrometer (Specord 40, Analytik Jena, Germany).

2.3. Bioink formulation

Pretreated gelatin (GEL) solution was prepared by dissolving 15% (w/v) of GEL (from porcine skin, Bloom 300, Type A) in ultrapure water for 3 h at 80 °C. ADA solution was prepared by dissolving 7.5% (w/v) of ADA in Phosphate Buffered Saline (PBS) at RT. Both ADA and GEL were passed through 0.45 µm and 0.22 µm filters (Carl Roth, Karlsruhe, Germany), preheated to 60 °C, to disinfect and sterile filter, respectively. Equal volumes of ADA and GEL solution were mixed at 37 °C for 10 min, resulting in ADA-GEL with final concentrations of 3.75% (w/v) ADA and 7.5% (w/v) GEL. Cell pellets of 8 mio.ml$^{-1}$ C2C12 cells were added to the ADA-GEL and dispersed by gentle stirring prior to 3D printing.

2.4. Bioprinting of C2C12 containing scaffolds

Scaffold fabrication was performed in a sterile environment using a 3D extrusion-bioprinter Bioscaffold 2.1 (Gesim, Germany). In order to stabilize the viscosity of the bioink, the 3D printer cartridge holder temperature was set at 30 °C. The bioink cartridge was inserted in the holder and left for 30 min before printing in order to allow a homogenous temperature distribution and gelation in the hydrogel. The bioink was then extruded through micro-sized nozzles of different diameters into tissue culture-treated polystyrene (PS) 6-well plates (Sarsted, Nümbrecht, Germany) (figure S6 (https://stacks.iop.org/BF/12/045005/ mmedia)). After printing, the well plate was placed inside an incubator to immediately return the cells to physiological environment. All samples were printed at a tip velocity of 10 mm.s$^{-1}$, strand length of 15 mm, the number of struts was 10, and samples were formed by three layers with a layer height set to 100 µm. The samples were crosslinked using 0.5 M CaCl$_2$ solution for 10 min.
2.5. Shear force model
To assess the influence of shear forces during bioprinting on C2C12 cell growth, C2C12 cell containing hydrogel scaffolds were fabricated using Nordson extrusion needles with different diameters and extrusion pressures. Needles of diameters d = 330 µm (O) and d = 250 µm (R) were used, corresponding to orange and red color of the Nordson precision (Nordson EFD, USA) extrusion tips (supplementary table S1). The needles were combined with extrusion pressures of 30, 40 and 60 kPa, resulting in the printing setups O30 (d = 330 µm, p = 30 kPa), O40 (d = 330 µm, p = 40 kPa), and R60 (d = 250 µm, p = 60 kPa). From a power law region of hydrogel viscosity over shear rate (figure S2), an approximation of the maximum shear forces of the ADA-GEL bioink during extrusion was obtained. Derived from the Hagen-Poiseuille law combined with the power-law equation (Ostwal-de Waele relationship) to account for non-Newtonian fluid behavior, an equation (E1) describing the wall shear stress is given as:

\[
\tau_{\text{wall}} = k \sqrt[1.2]{\frac{Q}{\pi R^3}}
\]

as discussed in literature [48, 49], where Q is the volumetric flow rate (m³·s⁻¹), R is the needle radius (m), and \( \tau_{\text{wall}} \) describes the wall shear stress (Pa). A detailed derivation of the equation can be found in the supplementary data to the manuscript (supplementary information D1).

2.6. Cell culture and maintenance
C2C12 murine myoblast cells (passage 10–20) were cultured in cell culture flasks (Sarstedt, Germany) using growth medium (GM) containing RPMI 1640 medium (Thermo Fisher Scientific, USA), supplemented with 2 g l⁻¹ glucose, 0.3 g l⁻¹ glutamine, 5 mg l⁻¹ phenol red, 10% Fetal Bovine Serum (FBS) (Corning, USA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, USA). The cells were cultured in an incubator at humidified atmosphere of 5% CO₂, 95% humidity and 37 °C. 3D bioprinted constructs were cultured in differentiation medium (DM) containing Dulbecco’s Modified Eagle Medium (DMEM) (11 885–084, Thermo Fisher ScientificTM, Waltham, USA), supplemented with 1 g l⁻¹ D-Glucose, 0.58 g l⁻¹ L-Glutamine, 110 mg l⁻¹ sodium pyruvate, 15 mg l⁻¹ phenol red, 1% penicillin-streptomycin, 2% horse serum (HS) (Thermo Fisher Scientific, US) (5% HS in differentiation study) and 0.1% insulin (Thermo Fisher Scientific, USA). In order to create cell pellets, the cells were washed using PBS, removed from the culture flasks using 0.25% Trypsin-EDTA (Thermo Fisher Scientific, USA) and cell number was determined using Neubauer chambers and the trypan blue exclusion method. Next, the cells were diluted to the desired amount and centrifuged at 500 rpm using an Eppendorf 5810 R (Eppendorf, Germany) centrifuge.

2.7. Viability study
Bioprinted ADA-GEL constructs containing C2C12 cells fabricated with the O40 setup were cultured for 1, 7 and 14 d and stained using calcein AM/propidium iodide (PI) LIVE/DEAD staining to assess the viability of C2C12 cells directly after 3D printing (24 h) and over the incubation time (7, 14 d). Cell containing hydrogels were washed and incubated in Hank’s Balanced Salt Solutions (HBSS) containing 4 µl⁻¹ Calcein AM and 1 µl ml⁻¹ PI for 45 min at 37 °C, 5% CO₂, in humidified atmosphere. Fluorescence microscopy (FM) images were taken using an AxioScope A1 (Carl Zeiss, Jena, Germany) fluorescence microscope, visualising live (green) and dead (red) cells in the scaffolds. Water soluble tetrazolium salt (WST-8) calorimetric viability assays were performed after the same time points to examine metabolic activity of 3D bioprinted C2C12 inside ADA-GEL. By conversion of WST into insoluble formazan via dehydrogenase processes of the cells, an absorbance of 450 nm is detected. Samples (n = 3) were incubated with 1% WST-8 solution in cell culture medium for 3 h at 37 °C, according to the manufacturer’s instructions. Supernatant was removed, transferred as technical duplicates into 96-well plates, and the absorbance at 450 nm was read using a well plate reader (type Phomo, Anthos Mikrosysteme GmbH, Krefeld, Germany).

2.8. Cell orientation analysis
To assess the local orientation of myotubes, five images for each sample (n = 3) at 10x magnification stained for F-actin (Rhodamine Phalloidin F-Actin, Thermo Fisher Scientific, USA) were examined. The cells were fixed using a fixing solution containing 1 mM ethylene glycol tetraacetic acid (EGTA), 4% (w/v) polyethylene glycol (PEG), 100 mM piperazine-N, N′-bis(2-ethanesulfonyl acid)PIPES, and 3.7% (w/v) paraformaldehyde in HBSS [39] prior to staining. DAPI was used as counter staining for cell nuclei. The FM images were rotated based on the angle θ measured between the horizontal axis of the image and the direction of the printed hydrogel struts (n = 3). Images were processed using the Orientation1 distribution plugin of ImageJ [70]. Cubic spline interpolation representing the myotube orientation distribution data was obtained. F-Actin orientation distribution was plotted as polar graphs via Origin 9.0 software and orientation factors were calculated with a method similar to the work done earlier by Yano et al [50]. At first, the azimuthal profile of the myotubes was calculated from the Lorentz peak fitting function via Origin 9.0 and the full width at half maximum (FWHM) was determined. Subsequently, the orientation factors were calculated by the following equation:

\[
\hat{f} = \frac{\text{FWHM} - 180}{180}
\]
2.9. Scanning electron microscopy (SEM)
To assess the porosity and microstructure of 3D printed ADA-GEL scaffolds, SEM images were recorded (Auriga CrossBeam, Carl Zeiss AG, Germany). Samples were washed using HBSS and immersed in fixing solutions containing glutaraldehyde (GA), sucrose, and sodium cacodylate trihydrate for 60 min at RT. Samples were immersed in another fixing solution containing GA and sodium cacodylate for another 60 min. Samples were washed with HBSS and dehydrated by the use of a graded ethanol series in steps of 30%, 50%, 70%, 80%, 85%, 90%, 95%, and 99.8% ethanol. Each immersion step was performed for 20 min to allow for ethanol diffusion into the gels. Following this, the samples were dried using a critical point dryer (EM CPD300, Leica, Germany). After drying, the samples were immersed in liquid nitrogen (LN2) and manually broken using metal tweezers prior to SEM examination.

2.10. Cell differentiation study
The potential to enhance the differentiation of C2C12 cells by using DM containing different concentrations of HS was investigated. C2C12 cells were cultured in GM medium. Next, the cells were detached and seeded in 35 mm petri dishes at high cell densities (2.3 × 10⁵ cells) and cultured in 0%, 0.1%, 1%, 2%, 5% and 10% HS supplemented DM. The cells were incubated over 14 d, washed using HBSS, and fixed using a fixing solution as performed in the orientation study. Cell differentiation was assessed using a multiphoton microscope.

2.11. Second harmonic generation imaging (SHG)
To examine the differentiation of C2C12 cells into myotubes alongside with the formation of myosin-II containing ordered myofibrillar sarcomeres, second harmonic generation imaging (SHG) was performed using a multiphoton microscope (TriMScope II, LaVision BioTec, Bielefeld, Germany). The presence of ultrastructural myofibrillar architecture in fixed hydrogel samples was analysed via multiphoton SHG. Microscope specifications as well as image analysis capability of ultrastructural myofibrillar architectures were described elsewhere [51]. SHG signal was excited using a modelocked ps-pulsed Ti:Sa laser (Chameleon Vision II, Coherent, Santa Clara, USA) at 810 nm wavelength. The transmitted SHG signal was detected using a 405/20 nm single band-pass filter (Chroma Technology group, Acal BFi Germany GmbH, Germany) and an ultrasensitive, non-descanned transmission photomultiplier tube (PMT) (H 7422–40 LV 5 M, Hamamatsu Photonics, Japan). The sample’s two-photon autofluorescence was recorded using a 525/50 nm (Chroma Technology group, Acal BFi Germany GmbH, Germany) single bandpass filter and a non-descanned PMT in backscattered configuration. A 25x HC FLUOTAR L (Leica Microsystems GmbH, Germany) objective was used on the excitation side of the setup and SHG light was collected in transmission side through a U-AAC condenser lens (OLYMPUS, Japan). The recorded images for the horse serum variation experiment and the differentiation assessments of 3D printed C2C12 cells containing ADA-GEL had a voxel size of 0.4 × 0.4 × 2 µm³ in images with a 440 µm² field of view and a voxel size of 0.2 × 0.2 × 1 µm³ in images with 100 µm² field of view, maintaining a pixel count of 1024 × 1024 pixels in all images.

2.12. Statistical analysis
Testing for statistically significant differences among multiple groups was performed via pairwise multiple comparison using analysis of variance (ANOVA) tests, followed by post hoc Bonferroni’s mean comparison using Origin 9.0. Pairwise comparisons of groups were performed using Welch’s t-test. Number of samples per group were n = 3 for bioink density and mass flow examination, n = 3 for orientation analysis, and n = 3 for WST-8. The data were expressed as mean ± standard deviation (SD). Differences were considered significant with *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3. Results
3.1. Cell viability
To investigate the influence of the 3D printing process and the ADA-GEL biomaterial on cell viability, C2C12 cells were processed and cultured for 14 d in-vitro. LIVE/DEAD stainings of C2C12 cells 24 h after printing confirmed a high cell viability inside the ADA-GEL hydrogels (figure 2(a)). Dead cells were sparsely visible in the constructs (figure 2(a), red PI staining). The results confirmed that the 3D printing process and the accompanied shear forces did not impair cell viability. The cells were cultured for subsequent 7 and 14 d to assess cell proliferation and growth inside the hydrogels. FM images confirmed that predominantly alive live cells were present in the hydrogel constructs after 7 and 14 d of incubation (figure 2(a), green), preferably proliferating at the interface between the struts and the surrounding culture medium. Interestingly, cell clusters orienting in the direction of the printed hydrogel struts were identified after 14 d of incubation (2% HS) (figure 2(a), right image). The results indicated high cytocompatibility of the oxidized alginate gelatin formulation, allowing 3D C2C12 cell culture inside the hydrogel. Indirect viability assessments using watersoluble tetrazolium salt assays (WST-8) performed
Figure 2. In-vitro cytocompatibility of bioprinted (O40 setup) ADA-GEL hydrogel containing C2C12 cells. (a) Fluorescence microscopy images of Calcein AM (green)/PI (red) LIVE/DEAD stained C2C12 cells in 3D printed ADA-GEL after 1, 7 and 14 d of incubation (2% HS DM) (scale bars: 200 μm). (b) Indirect viability WST-8 data of 3D ADA-GEL constructs containing C2C12 cells (n ≥ 3). All data are displayed as mean ± SD. **** indicates p < 0.0001, analysed via Welch’s t-test.

Figure 3. Orientation analysis of C2C12 cells grown in 2D on tissue culture polystyrene substrates. (a) Fluorescence microscopy image of F-Actin (red)/DAPI (blue) stained C2C12 (scale bar: 100 μm). (b) Corresponding frequency of F-Actin filament orientations in the images (n = 5).

3.2. 2D cell orientation
As a reference to the F-actin orientation of 3D printed C2C12 cells containing ADA-GEL samples, cell orientation was assessed on 2D-cultured C2C12 cells. Figure 3 depicts FM images of F-Actin stained C2C12 cells cultured on PS (n = 3), and the corresponding quantification of F-actin fibre orientation. The results show that for 2D-cultured C2C12 cells, no trend in cell orientation can be observed, as F-actin orientation is expressed with similar frequencies in all x-y directions of the images.

3.3. 3D cell orientation
Since cellular orientation was observed in the viability studies (figure 2), C2C12 cells inside ADA-GEL were 3D printed using different nozzles and extrusion pressures to assess the influence of different extrusion shear forces on C2C12 cell alignment. Fluorescence images showed that with increasing shear forces during printing (figure 4(a), left-to-right), an increase in cell growth in the orientation of the 3D printed hydrogel struts was present. Quantification of the orientation confirmed the increase in frequency of F-actin orientation in direction of the 3D printed hydrogel by using more narrow extrusion nozzles (d = 250 μm) and increased extrusion pressure (p = 60 kPa) (figure 4(c)). Figure 4(c)) depicts SEM micrographs of C2C12 cells inside 3D printed ADA-GEL using the different printing settings. The images indicate an orientation of hydrogel pores and microstructure in the longitudinal direction of the hydrogel strut, when 3D printing was performed using the most narrow extrusion nozzle and highest pressure (figure 4(c), bottom right, red dashed line). Wall shear stress calculations indicate an increase in shear stress from τwall = 50.5 Pa to 62.5 Pa when changing the extrusion needle setup from O30 to R60 (figure S3). The orientation factor f of O40 and R60 extruding C2C12 cells containing ADA-GEL significantly increased from ~0.8 to 0.95 in comparison to the O30 setup, suggesting increased extent of C2C12 cell orientation for the O40 and R60 configurations (figure S4). In summary, the data show that with the highest shear forces applied (d = 250 μm nozzle, p = 60 kPa), the highest cell orientation and hydrogel microstructure orientation is present.

3.4. Differentiation study
Figure 5(a) depicts SHG images of C2C12 cells cultured for 7 d in 0.1%, 2%, 5% and 10% HS on 2D tissue culture substrates. SHG positive regions are
Figure 4. Orientation analysis of C2C12 cells in 3D, extrusion bioprinted ADA-GEL bioink. (a) Fluorescence microscopy images of F-Actin (red)/DAPI (blue) stained C2C12 cells, 3D printed using extrusion nozzles O30 (d = 330 µm, p = 30 kPa), O40 (d = 330 µm, p = 40 kPa) and R60 (d = 250 µm, p = 60 kPa) (scale bars: 200 µm). (b) Corresponding frequency of F-Actin filament orientations dependent on extrusion nozzles O30, O40 and R60. A narrowing of the orientation profile of F-Actin of C2C12 cells is observed from O30 and O40 to R60. (c) SEM micrographs of C2C12 cells inside ADA-GEL hydrogel, with indicated orientation of hydrogel microstructure for increased shear forces using R60 extrusion parameters (bottom right, red dashed lined) (scale bars: 10 µm).

Figure 5. Differentiation analysis of C2C12 cells. (a) 2P microscopy of 2D cultured C2C12 cells and corresponding orientation plots of SHG positive regions (teal) and cellular autofluorescence at 525 nm wavelength (red). (b) 3D printed C2C12 cells inside ADA-GEL structure cultured in 5% HS for 14 d, depicting an SHG positive differentiated area (teal) on the hydrogel scaffold surface, indicating myofibrillar architecture formation as a direct sign of myotube maturation.
shown in teal. Red color depicts tissue autofluorescence during imaging. The images indicate that for 5% and 10% horse serum, C2C12 cells differentiate into parallelly ordered myotube segments, not visible when incubated for lower horse serum concentrations. Using 5% HS medium, 3D printed C2C12-ADA-GEL constructs were incubated for 14 d to assess the potential of differentiation inside ADA-GEL. We observed that C2C12 cells migrated from within the hydrogels to the hydrogel surface (figure S5), finding the highest cell densities on the hydrogel surface (figure 5(b)), with C2C12 cells bridging hydrogel struts (supplementary video 1). In those regions, multiphoton microscopy images indicated SHG-positive regions, confirming the differentiation of C2C12 cells cultured in ADA-GEL into myofibris containing myotubes (supplementary video 2, figure 5(b)). The results show that for high cell density regions, differentiation was possible with ordered segments of C2C12 cells forming myotubes and promoted sarcomergenesis (figure 5(b)).

4. Discussion

The enhancement of C2C12 cell differentiation and alignment by means of patterned hydrogel substrates [52], micro-patterned surfaces in microfluidics [23], instructively aligned electrosprun fibres [53, 54], 3D printed substrates [55] and electrical stimulation [56] has been previously shown in 2D [14]. We report here the alignment of C2C12 cells in 3D hydrogels and confirm cell growth with high viability inside ADA-GEL hydrogels. Different hydrogel systems and approaches for 3D bioprinting of skeletal muscle have been recently reviewed [57]. Among other material combinations, alginate-based bioinks account for approx. 25% of biomaterials used for skeletal muscle bioprinting [57, 58]. Besides promising results in oxidized alginate hydrogels for 2D culture of C2C12 cells [19, 45], no study of 3D bioprinting of C2C12 cells inside oxidized alginate-gelatin hydrogel has been previously reported, to our knowledge, highlighting the novelty of this study. Baniasad et al characterized oxidized alginate-gelatin hydrogels for use in muscle TE applications [59]. The authors identified a 30/70 ratio of oxidized alginate to gelatin as the most promising material combination similar to the high-gelatin content (7.5%) in the 3.75%/7.5% (w/v) ADA/GEL used in our study. Costantini et al used alginate as a template in combination with photocurable PEG-fibrinogen hydrogels to print C2C12 cells [24]. Utilising alginate as a fugitive template to simultaneously increase printability, the authors showed a packed formation of aligned myotubes after 8–12 d of incubation [24]. A high myotube alignment was observed after 15 d of C2C12 incubation, similar to our results using ADA-GEL (figure 4). As the authors used a double nozzle system and a two component bioink with an additional leaching step required [24], the use of oxidized alginate-gelatin bioink alone, as introduced in this study, provides a rather simple and affordable hydrogel system to achieve alignment of 3D printed C2C12 myoblasts in comparison to that study [24]. We have observed orientation of fibroblast cells in ADA-GEL in a previous study when extruding ADA-GEL directly into Ba2+ crosslinker [37]. Based on the results from the present study, we hypothesize that 3D printing leads to molecular orientation of the ADA-GEL, which is ionically crosslinked via CaCl2 and thermally crosslinked via gelatin cooling at RT (figure 1(c)). The latter solidifies the printed hydrogel, fixing the oriented hydrogel microstructure and RGD motifs of gelatin, which may allow the observed oriented growth of embedded cells. Rowley et al identified the importance of RGD cell adhesion motifs in alginate hydrogels for C2C12 cell culture, showing increased myoblast adhesion and higher extent of myoblast fusion on high RGD-ligand density alginites [60]. As similar performance of ADA-GEL in comparison to RGD-modified-ALG was reported earlier [38], an ADA-GEL with > 60% GEL was chosen in the present study, to ensure an abundance of RGD cell adhesion motifs accounting for high RGD density. Seyedmamoud et al bioprinted C2C12-laden GelMA/alginate bioink [61]. The authors observed high cell viability and myotube formation. However, the results showed no defined orientation of C2C12 cells in contrast to the present study [61]. It is possible that the GelMA photocrosslinking used in their study may counteract the ionic crosslinking of the oriented alginate hydrogel. As a result, no oriented gelatin microstructure is present, and thus no promotion of cell orientation occurs. In contrast, the imine bond in ADA-GEL features reversible characteristics and can dynamically break and form, which has been utilized to achieve self-healing hydrogel properties [62, 63]. The imine bond may orient besides the ADA and GEL chains or break when shear forces are applied (figure 1). After finished extrusion, the imine bond may reconstitute while ADA and GEL are in oriented state (figure 1). In addition, crosslinking of ADA occurs via Ca2+, solidifying the oriented ADA-GEL microstructure and leading to the observed oriented growth in the present study. The mechanism preserving the underlying orientation of the structure might be the final ionic crosslinking of the oriented ADA chains via divalent cations, as gelatin may be the preferable hydrogel component prone to degradation [34]. The influence of the imine bond in ADA-GEL on the orientation of the biopotted hydrogel will be investigated in further studies. Sriphutkiat et al showed cell alignment and accumulation of printed C2C12 cells inside GelMA bioink using acoustic nozzles as a potential external trigger to increase cell density [64]. We were able to demonstrate similar alignment and cell densities by printing 8 mio.ml−1 C2C12 cells in ADA-GEL hydrogel, without the use of an external trigger. By the
showed successful differentiation and functionality using 5% HS for cultivating myoblasts cells using insulin supplemented 2% HS medium (~1/R). Results have potential implications for future studies. The steric hindrance of HS into cells embedded inside hydrogels. The increased availability of nutrients and oxygen on the hydrogel surface without the need to diffuse into the hydrogel, as well as by porosity formed through hydrogel degradation [37]. Thus, hydrogel degradation of the ADA-GEL system may be a key contributor to the observed proliferation, density, and resulting differentiation of C2C12 cells. However, differentiation of C2C12 cells was observed less inside the hydrogels in comparison to the densely populated hydrogel surface (figure S5), requiring further improvement to achieve increased cell differentiation in 3D. Hydrogels with higher diffusibility of nutrients and oxygen, or increased formation of porosity, e.g., by incorporating leachable components, may counteract cell migration to the surface and allow to achieve improved 3D cell growth inside the hydrogels. As a result, such approach would potentially allow high cell densities inside the hydrogels to improve cell differentiation in 3D. Sriphutkiat et al. used 10% HS to differentiate C2C12 cells [64]. Important literature exists suggesting the differentiation of C2C12 cells using insulin supplemented 2% HS medium [66–68]. However, Yaffe et al. showed a considerably higher formation of multinucleated myotubes with fibre-like morphologies when culture medium was supplemented with 10% horse serum (HS) instead of 20% fetal bovine serum (FBS) [69]. Carosio et al. used 5% HS for cultivating myoblasts in vitro and showed successful differentiation and functionality [18]. Our results suggest that high HS concentrations increase differentiation, as judged by increasing SHG signal formation related to myofibrillar protein expression and sarcomere arrangement that is not seen in C2C12 myoblast stages. Higher HS concentrations might be of relevance to increase the diffusion of HS into cells embedded inside hydrogels. The results have potential implications for future muscle engineering experiments utilizing cells embedded in bioprinted hydrogels.

From the shear force model, the nozzle radius (~1/R) is the key parameter to achieve increased nozzle inner shear forces, leading to aligned structures and cellular orientation. The extrusion pressure mainly influences the volumetric flow rate (Q), which contributes to the shear force model linearly (E1) and is dependent on the used extrusion nozzle. For different extrusion setups not covered in this study, Q should be measured for the specific needle and pressure combination. By the use of the shear force model (E1) and information on the viscosity-shear rate behaviour of the bioink (figure S2), the wall shear stress τwall could be calculated as an approximation if the utilized needle-pressure combination yields sufficient shear stress for potential cell-orientation (figure S3). We found power law parameters of k = 19.9 and n = 0.225 for the ADA-GEL bioink used in this study, which is close to power law parameters k of well-printable Nivea® Crème (k = 26.1 [48]) and alginate-gelatin hydrogel (k = 13.3 [48]) previously reported [48]. However, one limitation of this study is the influence of cell density in the bioink on the resulting viscosity and shear forces inside the extrusion nozzle, which should be taken into account. Still, the here presented model represents an approach to approximate the nozzle inner wall shear stress as a key contributor to achieve aligned structures. In addition, further research should be done to assess the working-limit of the approach, assessing at which conditions cell viability may be compromised due to increased shear stress during extrusion. The appearance of cells spreading between 3D printed struts (figure S5) may be the result from large cell clusters on the hydrogel reaching a size to finally touch and bridge the area between the hydrogel struts, which should be further investigated via live-cell imaging techniques.

We hypothesize that through alignment, the formation of multinucleated myotubes is favored due to an increase in cell density induced by the aligned growth. Indeed, the aligned growth of C2C12 cells may be an important contributor to achieve ordered and synchronous contractile muscle superstructures, as it has been shown to enhance the maturation of 3D C2C12 cell constructs into skeletal muscle [23]. Denes et al. cultured C2C12 cells on patterned and unpatterned gelatin hydrogels, accelerating differentiation and sarcomere formation on patterned gelatin hydrogels [52]. Highlighting the importance of oriented growth and cell density for C2C12 cell differentiation, which were both enhanced by patterning [52], we present here another means of enhancing C2C12 cell alignment by choosing 3D printing parameters that yield increased shear forces (>60 Pa) as a stimulus for cell orientation. The influence of alignment on the potential enhancement of C2C12 cell differentiation should be investigated in further detail. Electrical stimulation of C2C12 cells has been used to enhance myogenesis and differentiation of C2C12 cells, yielding more contractile and functional cell cultures [56]. The application of electrical stimulation or other external triggers in combination with increased HS concentrations (>2%) may enhance the observed growth and differentiation of C2C12 cells in ADA-GEL hydrogel even further, giving promising implications for future studies.

5. Conclusions

We demonstrated that ADA-GEL hydrogel is a promising biomaterial for 3D cell growth of C2C12 myoblasts. Using an extrusion-bioprinting approach, we
showed that cell orientation of C2C12 cells can be achieved inside the hydrogel, providing a cost-efficient and simple method for cell alignment. Cell orientation in printing direction increased with higher shear forces in the extrusion nozzle during 3D printing. The results indicate that the shear forces during bioprinting are capable of orient the hydrogel microstructure, subsequently locked by hydrogel crosslinking, which may cause oriented cell growth. We identified that by using biodegradable oxidized alginate-gelatin, C2C12 cells migrated to the hydrogel surface, where cells preferably differentiated in areas of high cell density. Verified by second harmonic generation imaging, SHG was compatible with differentiation of C2C12 cells. In addition, we demonstrated that using increased amounts of horse serum (>5%) during C2C12 cell culture led to enhanced cell differentiation into ordered myotube clusters. In summary, oxidized alginate-gelatin was shown to be a hydrogel matrix of high potential for 3D printing of C2C12 cells. The results may provide the foundation for further muscle engineering investigations using the developed hydrogel system inducing myogenesis.

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ORCID iDs

Thomas Distler https://orcid.org/0000-0002-0319-3763
Oliver Friedrich https://orcid.org/0000-0003-2238-2049
Aldo R Boccaccini https://orcid.org/0000-0002-7377-2955

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