Published in final edited form as:

Bioprinting. 2018 June ; 10: . doi:10.1016/j.bprint.2018.e00038.

3D Printing Bioactive PLGA Scaffolds Using DMSO as a Removable Solvent

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Abstract

Present bioprinting techniques lack the methodology to print with bioactive materials that retain their biological functionalities. This constraint is due to the fact that extrusion-based printing of synthetic polymers is commonly performed at very high temperatures in order to achieve desired mechanical properties and printing resolutions. Consequently, current methodology prevents printing scaffolds embedded with bioactive molecules, such as growth factors. With the wide use of mesenchymal stem cells (MSCs) in regenerative medicine research, the integration of growth factors into 3D printed scaffolds is critical because it can allow for inducible MSC differentiation. We have successfully incorporated growth factors into extrusion printed poly (lactic-co-glycolic acid) (PLGA) scaffolds by introducing dimethyl sulfoxide (DMSO) for low temperature printing. Mechanical testing results demonstrated significantly different compressive and tensile properties for PLGA scaffold printed with or without DMSO. In particular, the PLGA-DMSO scaffold displayed a highly stretchable feature compared to the regular PLGA scaffold. The cellular response of growth factor introduction was evaluated *in vitro* using human mesenchymal stem cells (hMSCs). By evaporating the DMSO after printing, we ensured that there was no cytotoxic effect on seeded hMSCs. The addition of lineage specific growth factors led to increased expression of corresponding genetic markers for chondrogenesis, osteogenesis, and adipogenesis. We concluded that the use of DMSO for 3D printed scaffold fabrication with bioactive items is a revolutionary methodology in advancing regenerative medicine. The incorporation of bioactive molecules opens pathways to more therapeutic uses for 3D printing in treating damaged or deteriorating native tissue.

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Disclosure Statement

No competing financial interests exist.

Keywords

bioactive scaffold; low temperature 3D printing; mesenchymal stem cells; differentiation; poly (lactic-co-glycolic acid)

1. Introduction

The development of 3D printing biocompatible systems with extrusion-based printers has advanced the field of tissue engineering, in that this technology allows for precise layering of cells, biologic scaffolds, and biomolecules with the intention to emulate biologic tissues. This type of printing is a widely used method with customizable printing procedures (temperature, speed, and pressure) in order to adapt to a range of materials [1]. The two classes of biopolymers for printing are natural polymers and synthetic polymers [2]. In many cases, synthetic materials are favored over natural ones due to the higher precision in which printing conditions and mechanical properties can be controlled [3–5]. Although a direct melting approach for printing thermoplastics can achieve high-quality prints with ease, extrusion with a fine needle size (less than 0.4 mm), which is commonly used in printing polymer scaffolds with micro-patterns, often requires temperatures above 100°C to melt materials to an appropriate viscosity. In particular, polymers with high glass transition temperatures such as polylactic acid and polyvinyl alcohol are frequently printed at temperatures above 100°C [6, 7]. This elevated temperature prevents the incorporation of bioactive materials into the polymer because it can cause degradation of the biomolecules. To address this problem, we aimed to develop a low temperature printing method for thermoplastic polymer for the incorporation of bioactive molecules.

Poly lactic-co-glycolic acid (PLGA) is a synthetic polymer that is frequently used in tissue engineering scaffolds due to its biocompatibility and tunable mechanical properties [8–11]. Its use in medical devices and drug delivery systems been approved for clinical use in humans [10, 12]. In general, the temperature required for direct melting extrusion printing for PLGA is higher than 120 °C [13]. Previous approaches to lower the printing temperature usually require binder that are incompatible with bioactive molecules [14]. Dimethyl sulfoxide (DMSO) is a polar, aprotic solvent that is commonly utilized in cell culture. It is able to dissolve both polar and nonpolar compounds, including PLGA and proteins that are soluble in water [15]. Dissolution of these materials in DMSO allows for increased mobility (low effective viscosity) at lower temperatures due to reduced intermolecular interactions between PLGA molecules. Additionally, as a polar aprotic solvent for PLGA, DMSO has been demonstrated to potentially shield ester bonds of the polymer from hydrolysis for relatively minimal degradation compared to water [16]. It has apparent low toxicity at concentrations less than 10%, which has led to its frequent use in cryopreservation and other cell culture applications [15]. Applications of DMSO as a solvent for PLGA have been investigated for the manufacturing of micro/nanospheres allowing for physical entrapment of the drug for controlled release [17]. However, it has been demonstrated that similar concentrations are toxic to some cell lines *in vivo*, which highlights the importance of removing DMSO from the implantable scaffold and assessing cytotoxicity [15]. The use of DMSO as a solvent for PLGA, in conjunction with bioactive molecules, for the application

of 3D printing has not been conducted. In order to form a new technique of low temperature bioprinting with bioactive molecules, it was appropriate to investigate the potential of this method for use in tissue engineered scaffolds.

Mesenchymal stem cells (MSCs) possess a multilineage potential that can contribute to regeneration of mesenchymal tissues, including bone, cartilage, adipose, and others. This capability of MSCs has shown promising results in regenerative medicine [18]. As an attractive source for cell therapy, MSCs can be easily obtained from regenerative tissue, such as bone marrow or fat [19]. Multilineage differentiation can be induced using controlled *in vitro* conditions [20]. MSCs also exert therapeutic effects with the secretion of paracrine factors and stimulation of host cells; they have been utilized extensively in research the aims to treat myocardial injuries, and bone and cartilage defects, as well as pulmonary and liver diseases [18, 21–23].

The regenerative potential of MSCs is a result of several mechanisms involving migration towards injured tissues, immunomodulatory properties, differentiation, and/or secretion of regenerative factors, which promote cell survival and proliferation [24–27]. MSCs seeded on scaffolds together with bioactive molecules can provide therapeutic benefits to tissue damaged by trauma or disease [28, 29]. Tissue engineering techniques using scaffolds loaded with MSCs have promoted the healing process in multiple types of tissues [30–33]. Integrating biomolecules into scaffolds can facilitate lineage-specific differentiation. For tissue defects associated with mesodermal lineages, incorporating growth factor promotes adipogenesis, chondrogenesis, and osteogenesis [34]. Differentiation to each lineage is stimulated by specific growth factors. For example, chondrogenic differentiation is primarily promoted by transforming growth factor- β (TGF- β)[35] and insulin is required for differentiation to several pre-adipocyte cell lines[36, 37]. Bone morphogenic protein-2 (BMP-2) has been found to support osteogenic differentiation of MSCs for bone formation [8, 38].

The overall goal of this study is to develop a low temperature, 3D printing methodology that utilizes DMSO as a solvent to allow for lower printing temperatures and ultimately, the ability to incorporate bioactive molecules within polymer scaffolds. We hypothesize that our printing methodology will enhance differentiation of human mesenchymal stem cells by providing additional growth factors in the printed scaffold. There are three main objectives to comprehensively examine the proposed hypothesis: optimize and evaluate the PLGA/DMSO printing methodology for constructing scaffolds, investigate the release of growth factors within the scaffold, and assess the propensity of the hMSCs to differentiate to different lineages in response to growth factor loading.

2. Materials and Methods

2.1 Scaffold fabrication

Poly(lactic-co-glycolic acid) (PLGA, Lactel, Birmingham, AL) with inherent viscosity range of 0.55–0.75 dL/g and 85:15 lactic acid to glycolic acid ratio and dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO) were mixed with the desired proteins including fibronectin (Sigma-Aldrich, St. Louis, Missouri), TGF- β (R&D Systems, Minneapolis,

MN), insulin (R&D Systems,), and BMP-2 (R&D Systems,) for bioprinting. To ensure homogeneity, half of the DMSO amount was initially mixed and then the solution was continuously rotated in an oven for one hour at 50 °C. Next, pre-mixed protein (with the appropriate amount of DMSO to yield a final PLGA: DMSO weight to volume ratio of 2:1) was added to this mixture and allowed to rotate for another 30 minutes. The 3D printed scaffolds were fabricated with the 3D Bioplotter (EnvisionTEC, Gladbeck, Germany) (Figure 1A). In the software provided by EnvisionTEC, scaffolds were programmed for printing with dimensions of 5 mm (length) × 5 mm (width) × 2 mm (height) and a crosshatch inner pattern. After virtual sectioning, the scaffolds were printed layer by layer. The batch scaffolds were printed concurrently (e.g: 1 layer for all scaffolds then move to second layer) to ensure the cooling down of each layer. For printing, the material was allowed to reach approximately 45 °C. The extrusion was conducted at 4 bar with an average speed of 1 mm/s using a 0.4 mm inner diameter needle. The extruded material was solidified on the platform with a controlled temperature of 4°C to allow for sufficient solidification for each layer. The scaffolds were kept in −20°C freezer before evaporation or use to better preserve the shape. Regular PLGA scaffolds without DMSO used in mechanical testing were printed at 130°C onto a room temperature platform.

2.2 Mechanical testing

The Bluehill Instron mechanical testing system (33 R/44 65, Norwood, MA) was used to perform both compression and tensile mechanical testing of the scaffolds. For compression testing, a 5 kN load cell was applied with a displacement rate of 0.5 mm/min and pre-load of 0.05 N to 5mm × 5 mm × 2mm PLGA and PLGA/DMSO printed scaffolds [1]. The applied compression force was kept constant until the force observed dropped by 10% or if the compression risked exceeding the machine protection distance. Tensile testing was performed with a 50 N load cell applied to 20mm × 5mm × 2mm PLGA and PLGA/DMSO dog bone-shaped printed scaffolds. The testing was ended when the scaffolds reached a failure point, reached 25 mm in elongation distance, or when the force applied reached a value of 0 N.

2.3 Evaporation testing

The two methods of evaporation utilized in this setup were categorized as *not washed* and *washed*. In the *not washed* method, four PLGA/DMSO scaffolds were taken from the freezer and the initial mass was measured. In the *washed* method, four PLGA/DMSO scaffolds were taken from the freezer, the initial mass was measured, and each scaffold was rinsed with cold deionized water before transferring to the vacuum chamber. The scaffolds were placed into a vacuum chamber to allow for DMSO evaporation (Figure 1B). Mass of each scaffold was measured daily over the course of six days. Percent mass remaining was calculated for each scaffold in the two groups as a comparison.

2.4 Cell culture and seeding

Human mesenchymal stem cells (hMSCs, RoosterBio, Frederick, MD) were cultured at 37°C and 5% CO₂ in hMSC growth media made from High Glucose DMEM supplemented, 10% fetal bovine serum (FBS, Invitrogen), 1% v/v penicillin/streptomycin (Gibco), and 0.1 mM nonessential amino acids (Invitrogen). The cells were maintained and passaged

according to the manufacturer's recommendation. In preparation for seeding, scaffolds were placed in an uncoated 24-well plate. hMSCs were then seeded on the scaffolds at a density of approximately 1 million cells/scaffold. Scaffolds were kept at 37 °C for 4 hours to allow for cell adhesion before adding required amount of hMSC growth media. For differentiation studies, scaffolds were coated with fibronectin at a recommended concentration of 1 $\mu\text{g}/\text{cm}^2$ to ensure cell adhesion before seeding. For the tri-lineage differentiation, basal media supplemented only with 1% v/v penicillin/streptomycin and 0.1 mM nonessential amino acids was used to test the effect of growth factors in the printed scaffolds.

2.5 Live/Dead staining and confocal imaging

A live/dead assay was performed to assess the cell attachment and viability promoted by the scaffolds. First, the seeded scaffolds were briefly washed with Hanks Balanced Salt Solution (HBSS, Lonza, Walkersville, MD). After the excess media had been diluted by washing, the scaffolds were incubated in live/dead solution-composed of 2 mM Ethdium Homodimer-1 (EH; ThermoFisher Scientific), 4 mM Calcein AM (CAM; ThermoFisher Scientific), and 1mL HBSS per scaffold for 30 minutes in the dark. Scaffolds were contained in PBS until observation via confocal microscopy [39].

2.6 Release profile testing using ELISA

The scaffolds printed with growth factors were individually placed in a sealed glass vial with 5 ml PBS. All samples were kept at 37 °C on shaker for the course of 25-day study. At each time point, a 100 μL sample was taken and frozen at -20 °C until tested. ELISA kit (Sigma-Aldrich) with pre-coated plates were prepared according to the manufacturer's Sandwich Assay Procedure. Briefly, standards and samples were loaded as 100 μL triplicates into the well plate and incubated at room temperature for 2.5 hours. The solution was removed and the wells were washed 4 times. Afterwards, 100 μL of detection antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking. The solution was removed and the wells were washed 4 times again. The wash solution was discarded and 100 μL of Streptavidin solution was loaded into each well; the plate was covered and incubated for 45 minutes at room temperature with gentle shaking. After washes, wells were coated with 100 μL of TMP One-Step Substrate Reagent then the plate was covered and incubated for 30 minutes at room temperature with gentle shaking. Finally, 50 μL of stop solution was added to each well and the plate was immediately read at an absorbance of 450 nm.

2.7 Cell culture in chondrogenic media

In the study using chondrogenic media, the hMSCs were first expanded as previously stated in hMSCs growth media to reach the appropriate amount. After seeding the printed scaffolds with hMSCs, the seeded scaffolds were cultured in chondrogenic media for 21 days. In addition, chondrogenic media was made of high glucose DMEM supplemented with 0.1% penicillin/streptomycin (Life Technologies), 40 mg/mL proline (Sigma-Aldrich), 0.1% sodium pyruvate (Life Technologies), 1% ITS+ premix (BD Biosciences, Bedford, MA), 50 mg/mL ascorbate 2-phosphate (Sigma-Aldrich), and 0.1 mM dexamethasone.

2.8 qPCR for gene expression of hMSCs differentiation

With the RNeasy Plus Mini Kit (Qiagen, Frederick, MD), RNA extraction and isolation was performed on the hMSCs attached to the scaffolds at the selected time points. The manufacturer's protocols were followed to reverse transcribe the RNA to complementary DNA (cDNA) with the High Capacity cDNA Archive Kit (Life Technologies). We performed quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) by mixing cDNA samples with Universal Master Mix (Life Technologies), oligonucleotide primers, endogenous gene control glyceraldehyde 3 phosphate dehydrogenase (GAPDH; Life Technologies), and Taqman probes for Sox 9, type II collagen, aggrecan, SP7, PPARgamma, OPN, and adiporin. A 7900HT real-time PCR system (Applied Biosystems) was used to perform the reaction at thermal conditions of 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The mean gene expression of GAPDH in each group was used to normalize the relative expression level of each target gene; the fold change determination relative to day 1 gene expression followed. Using the 2^{-CT} relative comparative method, fold change calculations were completed and mean $\pm$ standard deviation was reported [40].

2.9 Statistical analysis

The analysis of relative expression of genes from the PCR results was performed using variance analysis and Turkey's multiple-comparison test. With a 95% significance level, a p-value less than 0.05 indicated a significant difference between samples. The mechanical and evaporation testing results were analyzed by the student t-test to determine a significant difference.

3. Results

3.1 Solvent evaporation and scaffold mechanical properties

With the developed mixing method, we were able to achieve a visually homogeneous mixture of PLGA and DMSO. The scaffold printed using 0.4 mm needle showed clean fibers with consistent thickness (Figure 1A). In the evaporation assay, a plateau was reached for the percent mass remaining within scaffolds at approximately 65%. Based on the original percent mass of DMSO (~33–34% of total scaffold mass), this result demonstrated that by the end of day 2, almost all of the DMSO in the scaffold was evaporated. The first two days of evaporation indicated a trend of faster mass loss for the scaffold with additional wash of deionized water compared to the not washed method. No discernable difference was retrieved with percent mass remaining from scaffolds of both the not washed method (65.78 % average mass loss) and the washed method (65.74 % average mass loss) by day 6, as the trend in percent mass remaining reached equilibrium (Figure 1C).

The addition of DMSO incorporated in the scaffold was found to significantly alter the mechanical properties of PLGA. The compressive Young's modulus of the PLGA/DMSO scaffold was 0.68 ± 0.27 MPa, while the PLGA scaffold exhibited a much higher value of 131.19 ± 24.13 MPa. Further tensile testing with dog bone scaffolds confirmed that the PLGA scaffolds also had a much higher tensile modulus (66.09 ± 19.95 MPa) than the PLGA/DMSO scaffolds (0.52 ± 0.18 MPa) (Figure 2B). The strain at failure for the PLGA

scaffolds and the PLGA/DMSO scaffolds were 150% and 350%, respectively, which indicates that the material became tougher after the solvent treatment (Figure 2A). In the Live/Dead assay, without evaporation or washing, almost all of the hMSCs seeded directly onto the scaffolds were found to be dead during culture. In contrast, the cells seeded on scaffolds evaporated for 2 days demonstrated good viability (Figure 2D). Notably, the extra wash step resulted in slightly higher cell adhesion density, when compared to the scaffolds treated with evaporation only (Figure 2C). As an initial step, we dissolved fibronectin in DMSO and mixed with regular PLGA to test if the bioactivity of the molecule is preserved under the mixing and printing conditions. The Live/Dead results demonstrated a large improvement in cell attachment efficiency due to the presence of fibronectin in the scaffold. The wash step before evaporation also seemed to help maintain the elongated morphology of hMSCs (Figure 2D).

3.2 Evaluation of protein release from the printed scaffold

The protein release profile was assessed using a sandwich ELISA. For all three proteins, the molecules were continuously released from the scaffold over the 25-day course. As a general trend, the release rate started to increase after 10 days as shown in the graphs. In particular, by day 20, TGF- β 3 reached a concentration of 1.92 ng/ml while the BMP-2 concentration was 0.55 ng/ml. The insulin released from the printed scaffold yielded a concentration of 1.72 ng/ml (Figure 3). Based on the loading amount, the calculated remaining protein mass percentile were 83.2% for TGF- β 3, 97.6% for BMP-2, and 96.9% for insulin.

3.3 Evaluation of the biological function of printed scaffold

In the gene expression assay, the hMSC differentiation was compared between scaffolds with and without the incorporation of the growth factors. As early markers, the transcription factors Sox9, sp7, PPARGAMMA for chondrogenesis, osteogenesis, and adipogenesis, respectively, were all up regulated at early time point (day 7). The major positive chondrogenic marker type II collagen showed increased expression at both time points. As a late osteogenic marker, OPN expression was up regulated in the BMP-2 loaded scaffold at day 21, with approximately 3 times more than the control group. The scaffold containing insulin as the growth factor for adipogenesis induced a significantly higher expression of positive marker adiporin by day 21 (Figure 4).

We further evaluated the differentiation potential of hMSCs seeded on the scaffolds with and without the growth factor in the conditioned media (in this case, chondrogenic media). Since the polymer used in this study has similar mechanical properties compared to cartilage tissue, cell differentiation in chondrogenic media was chosen as an example. As a transcription factor and an early marker, Sox9 was found to peak on day 7 for both PLGA/DMSO and PLGA/DMSO/TGF- β 3 (Figure 5A). With TGF- β 3 loaded in the scaffold, the Sox9 expression was upregulated significantly. At later time points (day 21), the positive chondrogenic markers, type II collagen and aggrecan, also showed increased expression with the bioactive scaffold (Figure 5A). The gene expression of type II collagen was further confirmed by the protein deposition. After 21 days, immunofluorescent staining revealed a visually stronger type II collagen signal (stained in green) from the hMSCs seeded on scaffold printed with TGF β -3 compared to the control PLGA/DMSO scaffold (Figure 5B).

4. Discussion

In this study, we have developed a low temperature, 3D printing methodology using DMSO as a solvent to allow for the incorporation of bioactive molecules in polymer scaffolds. As a polar aprotic solvent, DMSO is capable of dissolving polar and nonpolar compounds [41, 42]. Previous studies have demonstrated that proteins can be dissolved in DMSO and loaded in PLGA fibers through a wet spinning process [43]. Similarly, our printing methodology resulted in visually homogenous mixture and PLGA/DMSO/protein scaffolds with fibers of consistent thickness. A temperature hold at 4°C was chosen in order to allow for rapid solidification of each layer prior to the next layer being printed while minimizing the water deposition on the printing platform. As DMSO has a relatively high freezing point just below room temperature, the low temperature stage allows for solidification of each of the layers as they are printed. The following freezing at -20°C allows for further solidification of the scaffold without crystal formation due to the nature of DMSO as a cryoprotectant. The step stabilizes and hardens the scaffold to prevent it from losing structure during initial vacuum. Due to the high boiling point of DMSO, it evaporates slowly at room temperature [44]. With a vacuum chamber, our evaporation testing results indicated that DMSO can be fully evaporated from the PLGA/DMSO scaffolds. The initially faster evaporation rate of DMSO with the washed method can be attributed to the higher affinity of DMSO for water than for PLGA [17].

Addition of DMSO resulted in changes in compressive and tensile strength of the resulting PLGA/DMSO scaffolds when compared to printed PLGA scaffolds. Mechanical strength of PLGA has been well documented to vary due to many factors including molecular weight, molar ratio of LA to GA, and polydispersity index [7, 12]. High molecular weight and LA content generally leads to higher strength and higher viscosity due to entanglement of elongated chains. However, as reflected in the lower glass transition temperature of PLGA/DMSO, the dissolution of PLGA by DMSO may lead to reduced compressive strength due to reduced entanglement of chains in the polymer [45]. On the other hand, the higher tensile strength of PLGA/DMSO scaffolds reflects potentially improved flexibility through reduced crystallinity when compared to PLGA scaffolds.

DMSO possesses relatively low toxicity in cell storage procedures when compared to other commonly used solvents like dioxane [46]. Results of the cell adhesion tests demonstrated good viability for hMSCs seeded on DMSO evaporated scaffolds. Other studies have similarly showed that 0.1% DMSO solvent does not affect cell viability [41]. Improved cell morphology in the washed method may be attributed to DMSO removal on the surface of the scaffold [17]. As exhibited by cytotoxicity results, fibronectin activity was maintained post-print. This result reinforces previous studies where proteins hydrated in lower DMSO concentrations maintain their native state [47].

Previously, PLGA has been used as delivery vehicles by encapsulating proteins for prolonged protein release [48]. The release profile of TGF- β 3, BMP-2, and insulin in those cases from the 3D printed scaffold demonstrated continuously slow release, favorable for induction of differentiation. Here we demonstrated a continuous and stable protein release profile using our 3D printing approach. The final concentrations (on day 21) of TGF- β 3 and

BMP-2 from our scaffolds were found within previously demonstrated working concentrations [49, 50]. The slow release of insulin may be due to polymer and solvent interactions and heterogeneous distribution of protein in the printing resin [48, 51]. Similar to the use of PLGA as a microparticles, the controlled release of proteins from PLGA relies on the degradation of the molecule, primarily through hydrolysis of its ester linkages, which allow for release of the proteins which had previously been physically entrapped. As such, many factors contribute to the overall protein release rate such as molecular weight, surface area, and protein type [12, 52].

As exhibited by increased expression of type II collagen, chondrocyte differentiation is triggered by the TGF- β signaling pathways in MSCs. After binding, TGF- β allows for gene expression that maintains the chondrocyte phenotype in MSCs and induces the production of extracellular matrix [53–55]. Compared to the positive control in conditioned media, there was a significantly higher expression of markers of chondrogenesis in the PLGA/DMSO/TGF- β scaffolds. The OPN marker for osteogenesis exhibited upregulation by day 21, coincided with increased BMP-2 release by day 21. BMP-2 allows for osteogenic differentiation of MSCs by binding to surface receptors and activating intracellular Smad proteins, which promote expression of osteoblastic genes [56–58]. A previous study assessing BMP-2 incorporation into various bone tissue engineering composites found retention of bioactivity associated with sustained release of the protein [50]. To overcome localized action and rapid local clearance, incorporation of growth factors into biomaterials ensures sustained delivery of components to the target site and subsequent cell differentiation [50, 59, 60]. Three basic components determining differentiation of MSCs to preadipocytes are insulin, dexamethasone, and isobutyl-methylxanthine [37]. Studies using isolated adipocytes have shown that pre-adipocyte cell lines respond exquisitely to hormones like insulin, which promotes lipogenesis [37, 61, 62]. Significantly high expression of the marker adiponin occurred by day 21. Expression is further supported by the ELISA results, which indicated an increased release rate of growth factors by day 20. Appropriate hormonal stimulation and cell density can allow hMSCs to differentiate into adipocytes within 18–25 days; morphologically, adipogenic potential is indicated by lipid droplet accumulation [37]. Overall, the incorporation of lineage-specific growth factors led to an increased expression of corresponding genetic markers for chondrogenesis, osteogenesis, and adipogenesis, which suggests that the activity of growth factors was retained with the novel printing methodology.

Since the particular PLGA we used in this study yielded a compressive strength similar to that of native cartilage [63], we decided to extend the potential applications using conditioned chondrogenic media. Compared to the positive control in conditioned media, there was a significantly higher expression of markers of chondrogenesis in the PLGA/DMSO/TGF- β . These observations reinforce previous findings that incorporating growth factors in our extrusion printing allows for retention of bioactivity. Cumulatively, our results suggest that the use of DMSO for 3D printing allows for enhanced bioactivity through the incorporation of bioactive proteins. By developing a methodology that allows for fabrication of bioactive 3D printed scaffolds, our work opens new doors to possible applications of 3D printing in therapeutic and regenerative medicine.

5. Conclusion

In vitro studies involving PLGA/DMSO scaffolds with embedded growth factors demonstrated significantly higher expression of multilineage cell differentiation when compared to that of control PLGA/DMSO scaffolds without embedded growth factors. The novel methodology using DMSO as a solvent to reduce printing temperature expands possibilities for growth factor incorporation into 3D printing material as a tool for tissue engineering applications. Different combinations of growth factors incorporated into this PLGA/DMSO platform can be printed for therapeutic uses employing the demonstrated concept. The feasibility of 3D printing growth factors within synthetic polymer with fine structures combines the benefits of both tunable mechanical properties and biological functions. To fill the large clinical need, the 3D printing technology developed in this study will facilitate and broaden the utilization of bioprinting by providing bioactive therapeutic implants with desired and tunable mechanical properties to treat native tissue damaged by trauma or overuse in the future.

Acknowledgement

This work was supported by National Science Foundation grant CBET 1264517, CBET 1604742, and National Institute of Biomedical Imaging and Bioengineering, Center for Engineering Complex Tissues (CECT, P41 EB023833).

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Figure 1. Scaffold fabrication procedure and evaporation testing

(A) PLGA/DMSO scaffold fabrication methodology. PLGA and half of the final amount of DMSO were added to a printing cartridge. The mixture was attached to a rotating machine and mixed in oven at 50°C for an hour. The second half of DMSO mixed with protein was added to the cartridge and rotated for 30 minutes. The mixture was then printed into a 5mm × 5 mm × 2mm scaffold. (B) Scaffold washing procedure. In the washed method, the scaffold was rinsed with cold deionized water and placed into a vacuum chamber to allow for DMSO evaporation. (C) Mass loss profile of washed and not washed evaporated scaffolds. Over the course of 6 days a plateau of approximately 65% mass remained starting at day 2. Based on the original composition of the scaffold (DMSO composing ~33–34% of original scaffold), most of the DMSO had evaporated in both washed and not washed conditions by day 2.

Figure 2. Evaluation of mechanical properties and cellular response of PLGA and PLGA/DMSO scaffolds

(A) Tensile testing visualization of PLGA and PLGA/DMSO scaffolds. After testing (top row right), the PLGA scaffold did not show a discernable difference in length from initial (top row). PLGA/DMSO scaffolds showed an elongated morphology after applied strain (bottom row). (B) Mechanical testing of PLGA and PLGA/DMSO scaffolds. Both the Young's modulus for compression and tensile modulus were higher for the PLGA scaffolds compared to the PLGA/DMSO scaffolds. Overall the PLGA/DMSO scaffolds are tougher and have a higher elasticity compared to the PLGA scaffolds. (C) Comparison of cell adhesion between washed and not washed scaffolds. Washing scaffolds resulted in slightly higher cell adhesion density of human mesenchymal stem cells (hMSCs). Both washed and not washed scaffolds demonstrated good viability. (D) Confocal imaging of Live/Dead Assay. In the Live/Dead assay, evaporation of DMSO from the scaffolds resulted in good viability compared to not evaporated scaffolds (control). The addition of fibronectin improved cell attachment efficiency. Also, the washing methodology better maintained elongated morphology of hMSCs.

Figure 3. Protein release profiles of scaffolds made with TGF-β3, BMP-2, and insulin

A sandwich ELISA of protein release over 25 days showed an increase in release rate after 10 days. By day 20, concentration of protein of TGF-β3, BMP-2, and insulin reached 1.92 ng/ml, 0.55 ng/ml, and 1.72 ng/ml respectively. The percent mass remaining of each protein were 83.2% for TGF-β3, 97.6% for BMP-2, and 96.9% for insulin. Overall, PLGA/DMSO scaffolds present slow release of proteins over the course of 25 days with appropriate release of growth factors for induction of differentiation.

Figure 4. Expression of differentiation markers for hMSCs seeded on PLGA/DMSO scaffolds

The gene expression assay displayed an upregulation of early markers of chondrogenesis (Sox 9), osteogenesis (sp7) and adipogenesis (PPARGAMMA) at day 7 with the addition of TGF- β 3, BMP-2, and insulin, respectively. Increased expression of the late chondrogenic marker, type II collagen, was observed at days 7 and 21 with addition of TGF- β 3. At day 21, increased expression of the late osteogenic marker was observed in BMP-2 scaffolds. Higher expression was also observed in the adipogenic marker, adiporin, at day 21 in insulin scaffolds. Overall there is a demonstrated increase in differentiation markers for protein incorporated in PLGA/DMSO scaffolds when compared to PLGA/DMSO scaffolds without protein (control) in basal media.

Figure 5. Differentiation marker expression and immunofluorescent staining of PLGA/DMSO scaffolds in conditioned media

(A) Gene expression assay of scaffolds grown in conditioned media. An increased expression of early marker of chondrogenesis, Sox9, was displayed on scaffolds made with TGF-β3 at day 7. Upregulation of the expression of the late chondrogenic markers, aggrecan and type II collagen, in TGF-β3 scaffolds was observed at day 21. Overall there was a demonstrated increase in chondrogenic markers with the addition of TGF-β3 in the scaffold material in chondrogenic media. * indicates significant difference in each group ($p < 0.5$). (B) Confocal imaging of immunofluorescently stained scaffolds grown in conditioned media. Immunofluorescent staining of PLGA/DMSO/TGF-β3 scaffolds (right) displayed a higher visual expression of type II collagen signal (stained green) as compared to control PLGA/DMSO scaffolds (left).