Improved Quality of 3D-Printed Tissue Constructs Through Enhanced Mixing of Alginate Hydrogels

Daniel L. Cohen,[1] Andrew M. Tsavaris,[1,2] Winifred M. Lo,[1,3]
Lawrence J. Bonassar,[1] Hod Lipson[1]

[1]Cornell University, Ithaca, NY, [2]Princeton University, Princeton, NJ
[3]Northwestern University, Evanston, IL

ABSTRACT

While alginate hydrogel is a desirable material platform for Solid Freeform Fabrication (SFF) of cell-seeded tissue engineering scaffolds, achieving consistently high-quality results can be challenging. Local variations in the material properties cause inconsistent material deposition behavior and consequently decrease the resultant geometric fidelity of the construct. The effects of gel mixing on material property consistency, geometric fidelity, and cell viability were characterized in an attempt to improve the formulation's compatibility with SFF processing. Material homogeneity was quantified through a novel experimental setup composed of an EnduraTEC mechanical test-frame and custom syringe-extrusion jig. Cell viability and geometric fidelity were assessed using standard protocol. The baseline mechanical stiffness of the printed samples was 16 ± 3 kPa (n=6). We found that increasing mixing reduced material inconsistency and improved geometric fidelity, without adversely affecting cell viability: the printed construct quality was drastically improved by increasing mixing well beyond previously established limits.

Introduction

SFF is a powerful tissue engineering technique; compared with scaffolds produced through traditional tissue engineering techniques, SFF delivers scaffolds with increased geometric complexity, spatial heterogeneity, and patient-specificity. Many previous SFF efforts have been limited by high variations in print quality due to material property inconsistency.[1] To address this limitation, it is necessary to develop a platform for quantifying heterogeneity of SFF inks. As such, the objectives of our study were to (a) develop a framework for quantifying heterogeneity of the material properties of SFF inks by measuring temporal variation in load during the printing process, (b) determine the effect of mixing intensity on variations of mechanical properties and geometric fidelity of printed parts, and (c) verify maintenance of cell viability.

Methods

Alginate Gel Preparation

Gels were created by mixing a 2:1 ratio of 2% alginate and 1% calcium sulfate (a cross-linking agent), both in PBS, by pumping the reagents back and forth through a three-way stopcock

(Fig. 1). Gels were loaded into a syringe and allowed to cure for 30 minutes between the completion of mixing and the beginning of testing. Four different curing times were characterized: 15, 30 45 and 60 minutes. Thirty minutes was the minimum curing time that resulted in a gel with sufficiently high stiffness for SFF; curing times less than thirty minutes resulted in gels which did not hold their shapes upon deposition.

In some mixtures, the gel at the nozzle of the syringe had solidified more than the gel present in the barrel of the syringe; this first bit of gel was expelled from all syringes prior to starting all tests. This was performed because it was observed that the viscosity and the resistance exhibited by this particular portion of the gel was not representative of the majority of the material in the barrel of the syringe. A potential explanation for this phenomenon is that the gel closest to the nozzle was the portion most exposed to air and evaporation occurred. Thus, with less water present in the gel composition, the material extruded might have exhibited more solid-like behavior.

Mechanical Noise Testing

Gel was loaded into a syringe and mounted on an EnduraTEC mechanical test-frame (ELF 3200; Bose Corporation; Eden Prairie, MN) using a custom syringe-extrusion jig (Fig. 2). In each test, gel was extruded by depressing the syringe piston at a constant rate of 0.25 mm/s, resulting in a volumetric flow rate of about 0.043 mL/s. Throughout each test, the piston of the syringe was moved a total of 9.5 mm. Extrusion occurred through a tapered syringe tip with a diameter of 0.010 in. The resultant load, measured to within 0.01 N by a 50 lb load cell, was recorded at a frequency of approximately 200 Hz. Tests were run on gels which were mixed to varying extents, as defined by the number of times the gels were pushed through the stopcock: 8, 16, 32, 64, 128, and 200 times.
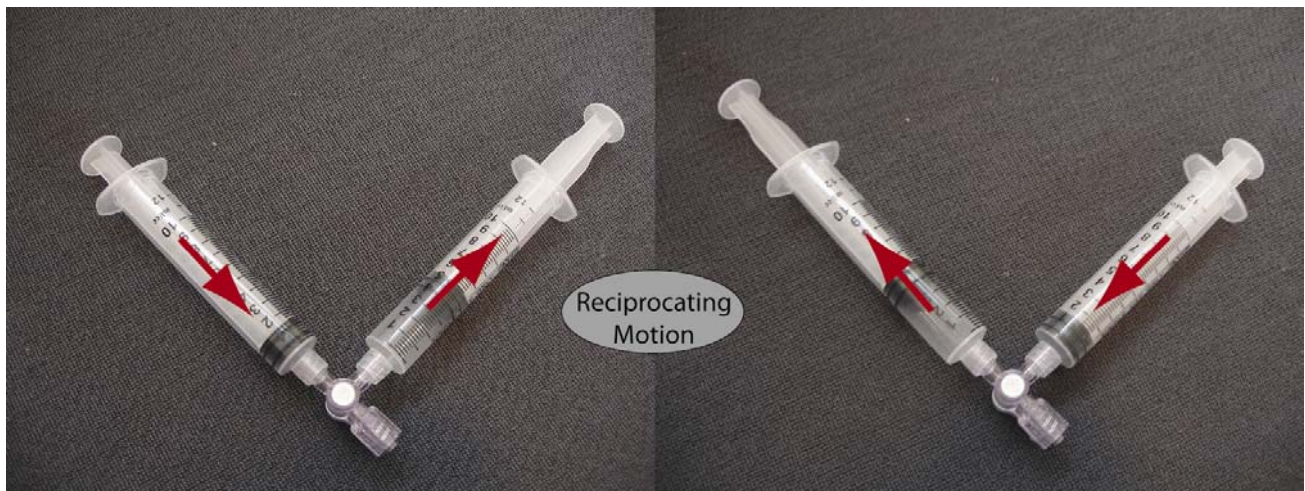

Fig. 1 Mixing process used to create alginate gel with two syringes and a three-way stopcock. The solutions were pumped back-and-forth through the stopcock. Each stroke counted as one time that the gel was mixed; a back-and-forth cycle would count as two mixings.

The output extrusion force vs. time data was run through a band-pass filter to eliminate irrelevant low and high-frequency noise. The high frequency noise was attributable to measurement and environmental noise, whereas the low frequency component was resulting from the one degree internal tapering of the syringe barrel which is a manufacturing artifact of the syringe.

The standard deviation of the linear region of this force vs. time data was named "mechanical noise." In order to characterize the sample-to-sample variation in the levels of mechanical noise, the standard deviation of the mechanical noise was measured.
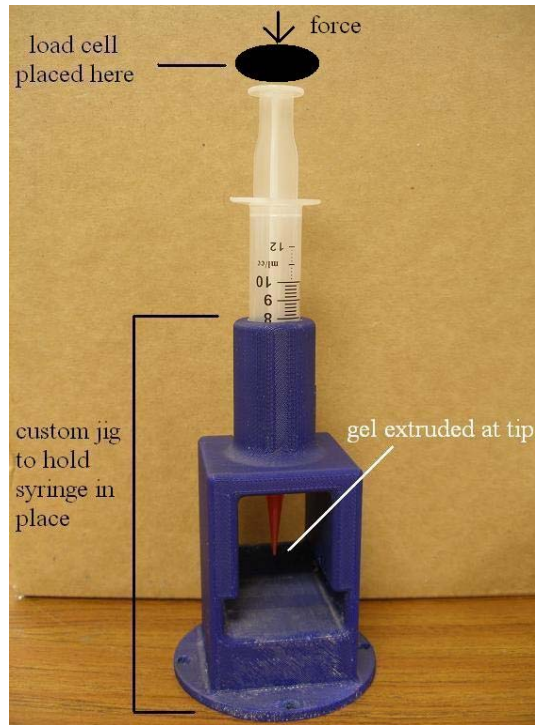

Fig. 2 Custom syringe-extrusion jig used in mechanical testing.

Cell Viability Testing

For viability tests, articular chondrocytes were harvested from the condyles and patellofemoral groove of bovine joints collected 1-3 days prior. Cells were isolated via digestion using a 0.25% collagenase solution in media over 18 hours and seeded in a 2% alginate gel in PBS the following day. Gels were cross-linked in a 2:1 volume ratio with 1% $CaSO_4$, allowed to cure for periods of time ranging from 15-45 minutes, and then extruded through a syringe at a rate of 0.024 mL/s. The effect of tip diameters was also examined by extruding the gel through syringe tips including 0.010 inch, 0.023 inch, and 0.200 inch diameters. Printed samples were incubated in media overnight at 37° C. Cell viability was assessed using fluorescent microscopy and the LIVE/DEAD cell viability assay the following day.

Rapid Prototyping

For rapid prototyping, gels were mixed and transferred to syringes compatible with the Fab@Home rapid prototyper (Fig. 3). Prior to printing, gels were allowed to cure for periods of time between 15 and 60 minutes. Desired geometries for printing were uploaded to the Fab@Home software in the form of STL files. Gels were then deposited along prescribed paths using the Fab@Home machine.
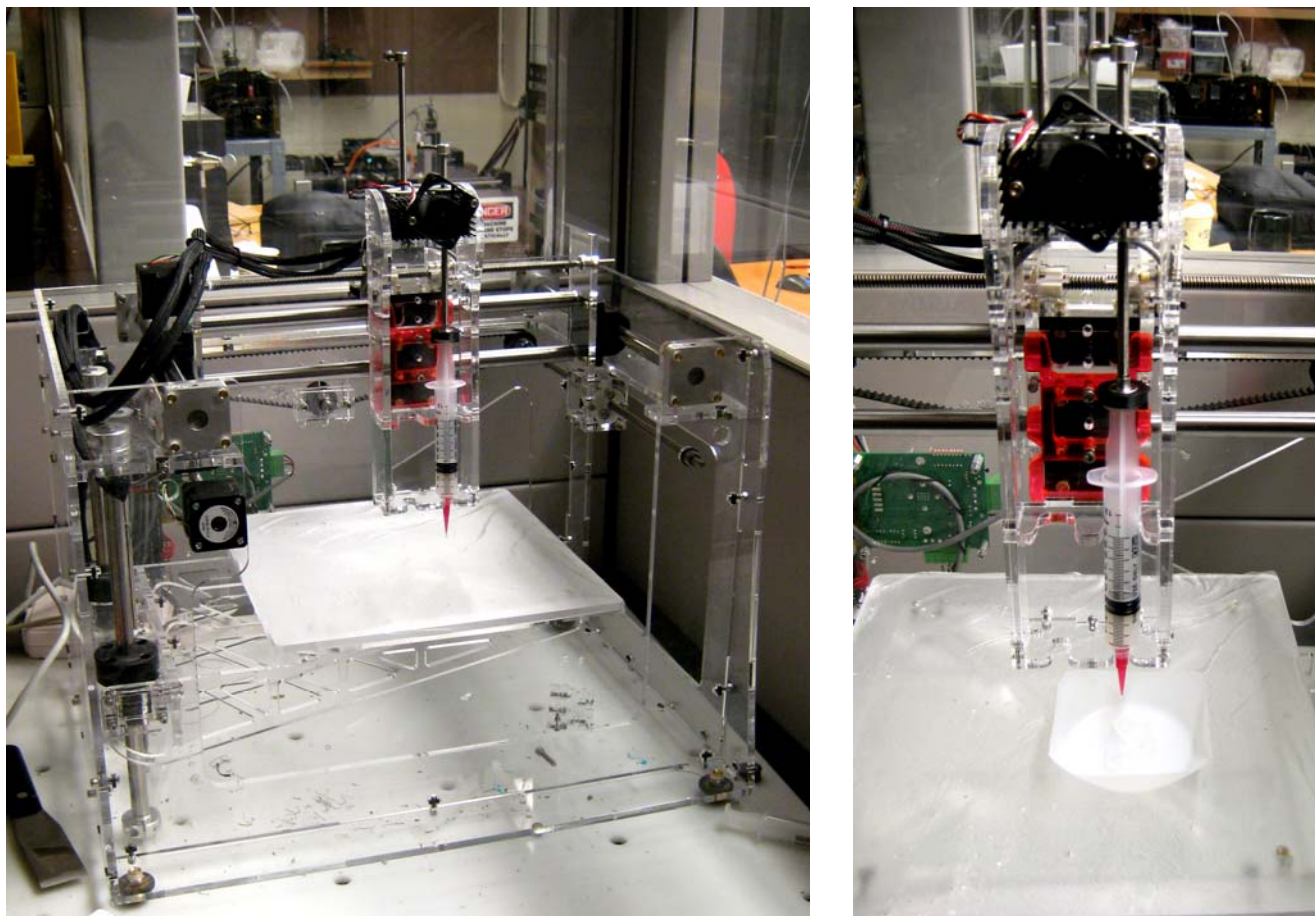
Fig. 3 (left) The Fab@Home rapid prototyper; (right) the custom deposition head built for use of sterile syringes.

Results

Mechanical Stiffness

The baseline mechanical properties of the printed gels, after the standard post-crosslinking treatment, was 16±3 kPa (n=6). This stiffness was determined using a standard confined compression test on the EnduraTEC mechanical test frame.
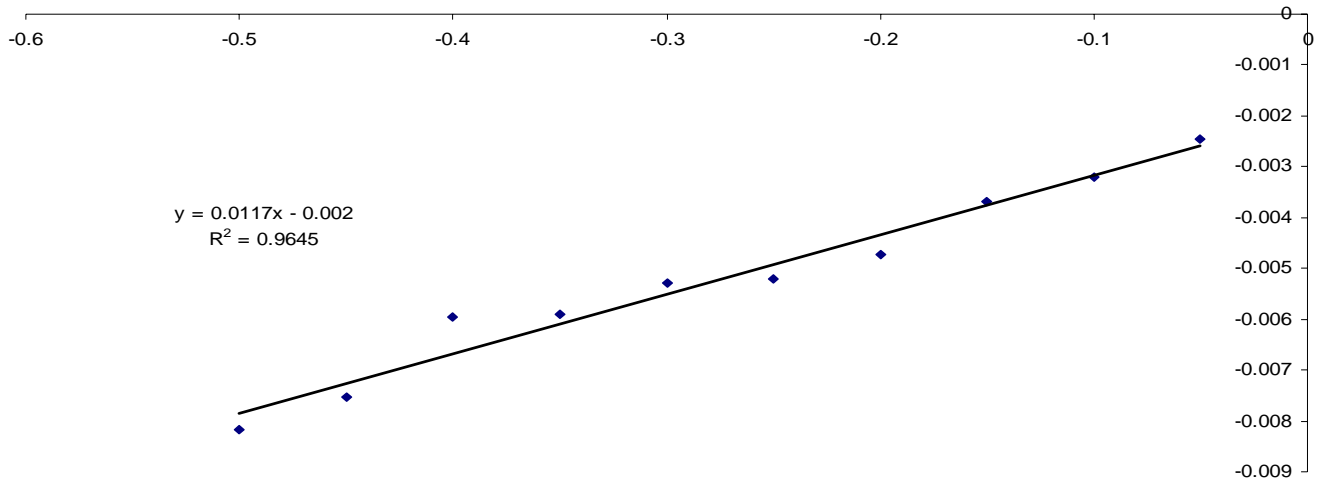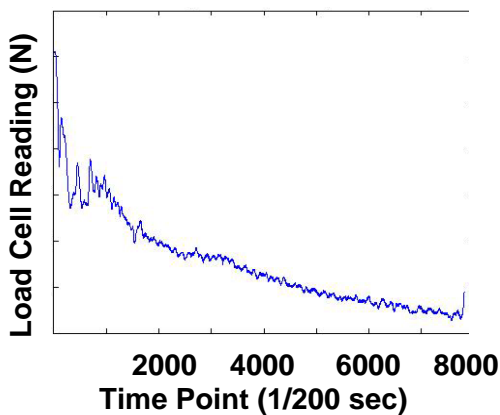
Fig. 4 Representative plot of stress-strain curve generated by EnduraTEC stress testing. The linearly-fit function is also displayed along with the correlation coefficient.

Mechanical Noise

Load vs. time data generated during the extrusion tests on the EnduraTEC mechanical test frame reflected material heterogeneity in addition to added noise from the machine and the syringe's changing inner diameter (Fig. 5, left). For example, while material heterogeneity was discernible between tests, a downward sloping trend reflecting a gradual increase in the amount of load required to maintain the same volumetric flow of material was observed. This trend was likely resultant from the one degree internal slope of the syringe barrel, i.e. manufacturing-related draft. The draft obscured the component of the data which resulted from material heterogeneity; consequently, quantifying noise via standard deviation of an average load cell response would not produce meaningful results. As such, it was decided that a band-pass filter, eliminating both high-frequency noise from the load cell and low-frequency noise from the syringe, was necessary to process the raw data prior to further analysis.
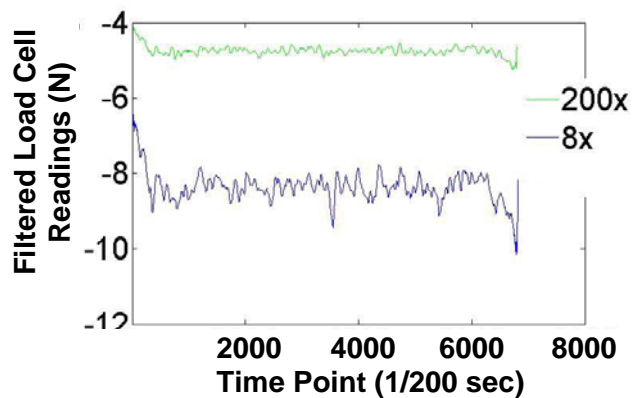

Fig. 5 (left) Representative *raw* load vs. time data for gel mixed 200 times; (right) Representative band-pass filtered data for gels mixed 200 times [green] and 8 times [black].

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While it is visually apparent that there is less temporal variation in the mechanical properties of gels that were mixed more as evidenced by narrower load reading bands (Fig. 5, right), this "mechanical noise" was quantitatively defined as the standard deviation of the load values over time (Fig. 6).
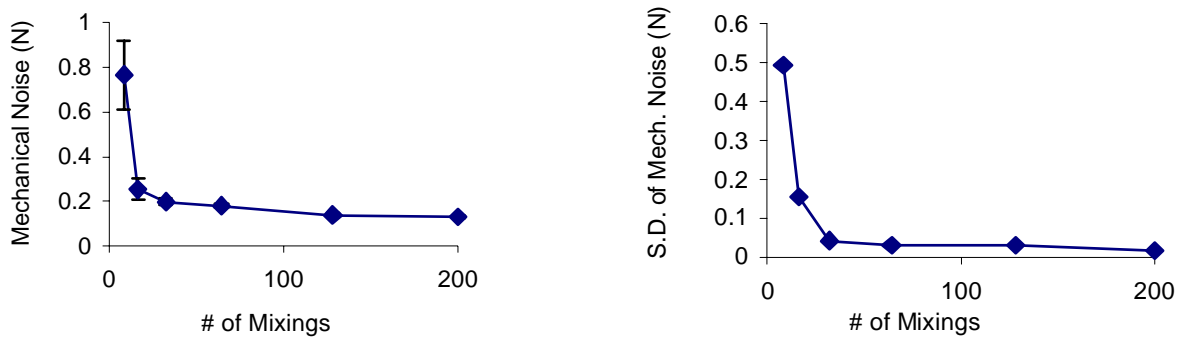

Fig. 6 Effect of mixing intensity on gel homogeneity. (left) "Mechanical noise," i.e. standard deviation of temporal variation in load necessary to print; (right) Sample-to-sample variation in mechanical properties of gel, i.e. standard deviation of mechanical noise.

Gels mixed to a lesser extent (i.e. 8 times) exhibited substantial mechanical noise, whereas those mixed to a greater extent (i.e. 200 times) exhibited significantly less mechanical noise. Moreover, sample-to-sample variation decreased significantly as the mixing duration increased. This was judged from the standard deviation of the mechanical noise experienced for each treatment.

Cell Viability

Increased magnitudes of mixing did not appear to exert a significant impact on cell viability in the seeded gels. Indeed, across magnitudes of 8, 64, and 200 times of mixings, cell viability appeared to remain relatively constant, at a magnitude of 74% ($\pm$ 10-15% S.E.M.) when viability was normalized to the initial cell viability (Fig. 8). Moreover, decreasing the extrusion tip diameter from 0.200 inch to 0.010 inch also appeared to have negligible impact on cell viability. Finally, increasing the gel curing time across magnitudes of 15, 30, and 45 minutes appeared to have insignificant impact on cell viability.
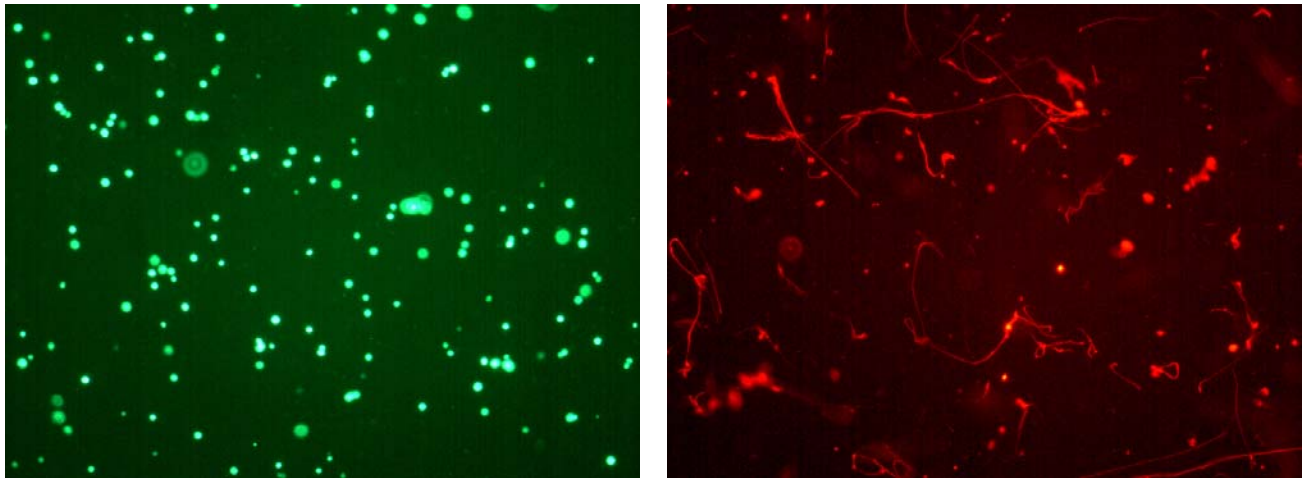
Fig. 7 Representative images of fluorescent microscopy using LIVE/DEAD viability assay. (left) Live; (right) Dead.

While the number of mixings, the printing tip diameter, and the curing time all did not negatively impact cell viability, it appeared that increasing mixing may actually improve cell viability in special circumstances. Gels mixed 200 times exhibited higher levels of cell viability than those mixed 8 times, when the gels were allowed to cure for a period of 45 minutes. One explanation for this phenomenon is that higher levels of mixing facilitate a better distribution of the cross-linking agent, calcium ions, throughout the alginate gel and allow for more homogeneous solidification during the 45-minute curing period. Thus, better-mixed gels experience lower peak-shear during extrusion and have a lesser tendency to kill cells throughout the printing process.
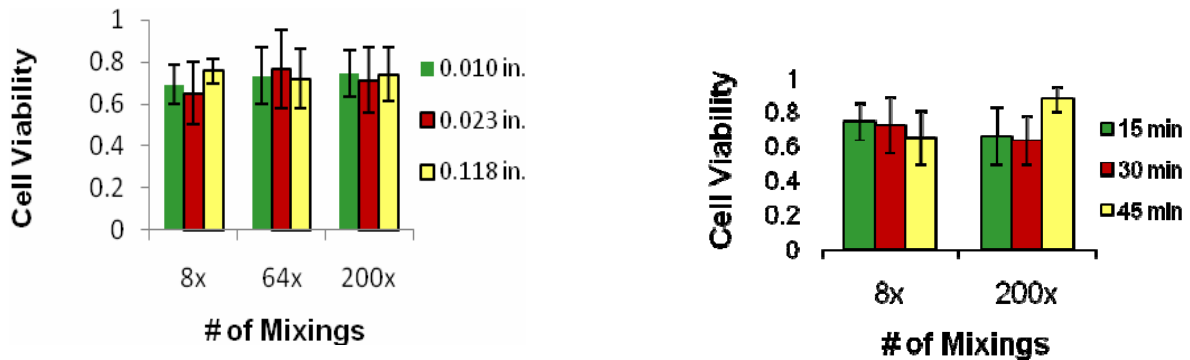

Fig. 8 Effect of (left) tip diameter and (right) curing time on cell viability as measured 24 hours after printing.

Geometric Fidelity

Gels mixed for greater duration had a tendency to deposit at a constant rate, rather than agglomerating at the extrusion tip before falling uncontrolled onto the print surface; this continuous flow resulted in complete parts without material voids (Fig. 9, top-right).
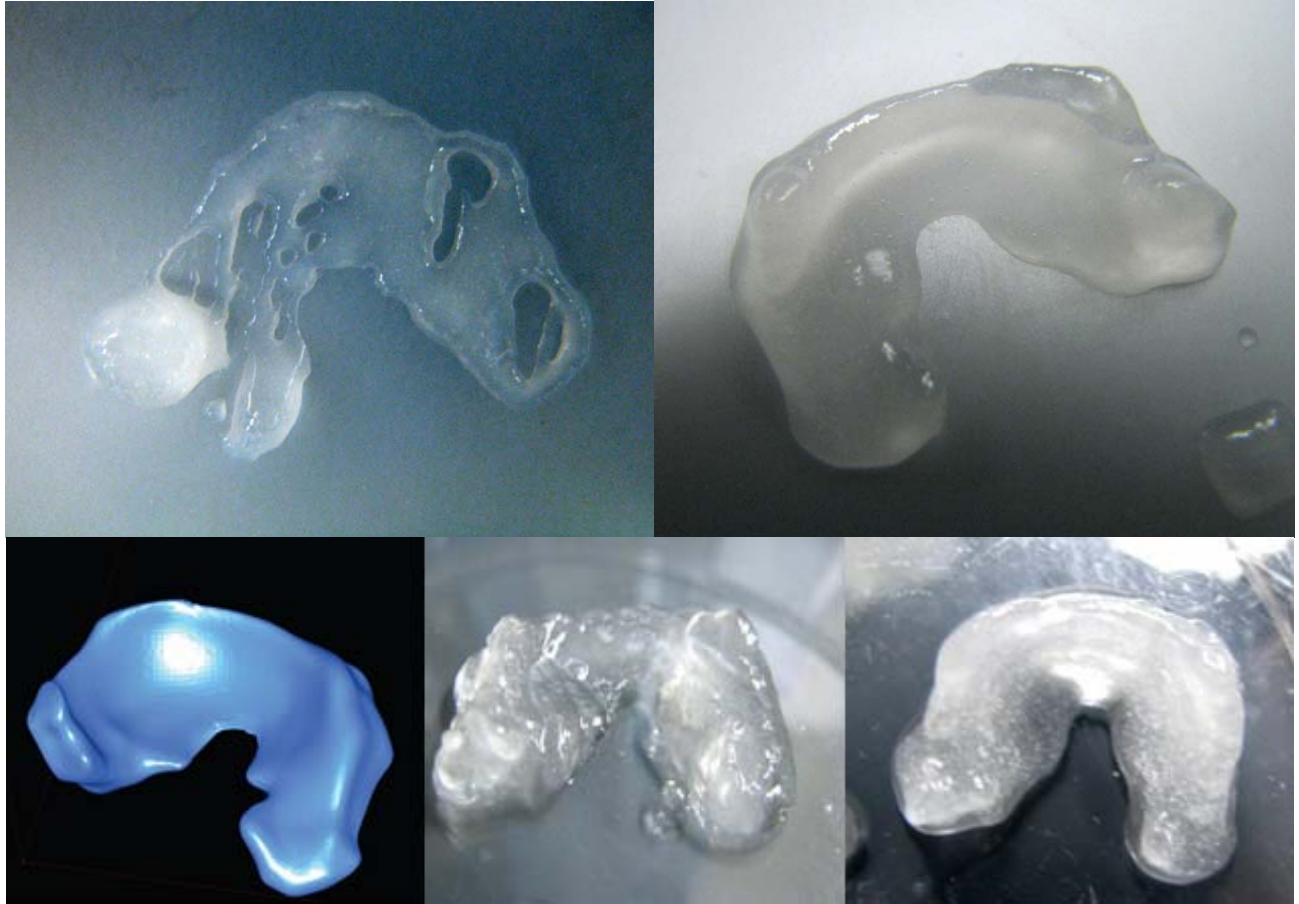
Fig. 9 (top-left) A sample print of a gel mixed 32 times exhibiting discontinuous material deposition streams and a resultant incomplete geometry with material voids (top-right) Complete sample print of a gel mixed 200 times. (bottom-left) CAD model of ovine meniscus, (bottom-center) printed ovine meniscus with 32X mixed gel, and (bottom-right) printed ovine meniscus with 200X mixed gel.

Not only was deposition constant, but the texture of the printed material was more uniform for those gels mixed to greater extent. Finally, gels printed after increased mixing generated shapes closer to the desired geometry, i.e. had greater geometric fidelity (Fig. 10).

Fig. 10 Sample, high-fidelity print of a space shuttle (1" long).

Discussion

The goal of this research was to identify a platform for quantitatively evaluating the heterogeneity of printing inks and to facilitate the translation of alginate scaffold construction from the domain of injection molding to that of SFF. The latter goal was facilitated by studying the impact of mixing on gel homogeneity, as well as on the retained viability of cells seeded throughout the scaffold.

Analyzing the load cell response during extrusion of alginate gels appeared to generate a good measure of the material's heterogeneity. The EnduraTEC was highly sensitive to the mechanical noise of the material, as well as interference from the syringes used during the test and the machine itself. These irrelevant components were removed using band-pass filtering of the raw data, while still retaining information about the material response. When processing the data, the option of subtracting baseline data—obtained from trials run using the same syringes filled with atmospheric air or water—was also considered. However, attempts to do so resulted in processed data which still exhibited the downward trend incurred during the test as a result of the syringe's construction. One possible reason for why subtracting baseline data may have been an inadequate means of dealing with the trend of increasing force magnitudes is the fact that the starting position of the syringe varied from test to test. Additionally, the resistance experienced by each syringe varied depending upon the number of mixings the barrel had previously experienced. Thus, it was decided that using a band-pass filter to eliminate the high-frequency noise (resultant from the machine's interference) and low-frequency noise (resultant from the syringe draft) was necessary.

Each syringe contained sufficient gel (6 mL) to run two separate tests. These two tests were run immediately after one another. There was no apparent pattern in behavior separating the gel's performance between the first and second tests. Consequently, to analyze the behavior from each syringe, we averaged the mechanical noise values from both tests and took the average to be the representative value from that particular sample.

Increasing the duration of mixing appeared to better distribute the cross-linking agent throughout the gel, thus accounting for the improved uniformity of the material texture and viscosity. Qualitatively, the gel appeared to be smoother and more uniform in color and texture. Additionally, the material printed more easily on the Fab@Home than gels mixed for a low level of mixings. The sputtering and unpredictable deposition patterns previously observed were mitigated by increasing the duration of mixing. Finally, it appeared that gels which were mixed more experienced less phase-separation upon, and after, deposition.

Results from mechanical testing indicate that less mechanical noise is experienced during the printing process for those gels mixed for greater duration. These gels also tend to require less force for extrusion. This suggests that those gels mixed for shorter duration and with less uniform distribution of cross-linking agent likely experience clogging at the extrusion tip. When clumps of gel solidify within the barrel of the depositing syringe, the solid clumps tend to be trapped in the syringe tip during the extrusion process. This reduces the overall volume of alginate gel leaving the syringe for a given force, thus requiring the machine to exert greater force to extrude the gel at the same volumetric flow rate.

Mixing did not appear to have a significant detrimental impact on cell viability, despite concerns from experts in the field that more mixing would cause the seeded cells to induce apoptosis.

Conclusions

This study demonstrated a novel framework for quantifying material property homogeneity of printing inks. Furthermore, increased mixing was found to drastically increase ink uniformity, and consequently, improve the geometric fidelity of printed parts while not affecting cell viability. By mixing the gel an order of magnitude more than previously accepted and published limits, the print quality was substantially improved. These resulting improvements clear the way for extended application of this tissue engineering technology with greater precision and reliability, while also introducing a novel method for optimizing other types of printing inks that present challenges related to material property inconsistency.

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