Materials Science and Engineering C 80 (2017) 313–325

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Printability study of hydrogel solution extrusion in nanoclay yield-stress bath during printing-then-gelation biofabrication

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ARTICLE INFO

Article history:
Received 26 March 2017
Received in revised form 25 May 2017
Accepted 31 May 2017
Available online 1 June 2017

Keywords:
Filament formation
Printability
Nanoclay support bath
Material extrusion
Printing-then-gelation

ABSTRACT

Yield-stress support bath-enabled extrusion printing is emerging as a promising filament-based direct-write strategy for different applications in tissue engineering and regenerative medicine. Central to the printing quality of complex three-dimensional structures fabricated by the support bath-enabled fabrication approach is the formation of a continuous filament with well-defined geometry. The objective of this research is to study the printability of hydrogel precursor solutions in a Laponite nanoclay yield-stress bath during extrusion printing where the printed hydrogel precursor solutions remain liquid. The printability herein is mainly evaluated based on the morphology and dimensions of printed liquid filaments. Seven filament types are observed during extrusion in the nanoclay bath: three types of well-defined filaments (swelling, equivalent diameter, and stretched) and four types of irregular filaments (rough surface, over-deposited, compressed, and discontinuous). When the alginate concentration increases, the diameter of filaments made of alginate-gelatin blends decreases. The nanoclay concentration significantly affects the morphology of deposited filaments: low concentration Laponite bath (such as 0.5% (w/v)) may lead to the formation of irregular filaments such as rough surface and over-deposited filaments while high concentration bath (such as 8.0% (w/v)) may result in the formation of compressed filaments. Operating conditions affect the filament diameter and morphology similar to those as observed during conventional extrusion printing. The printability knowledge enables the successful fabrication of cellular vascular constructs in the Laponite nanoclay bath.

1. Introduction and background

The disparity between increasing demand for transplantable human organs and significant shortage thereof motivates tissue engineering and regenerative medicine. Fortunately, layer-by-layer additive manufacturing-based organ printing [1] provides a promising solution to achieve the on-demand fabrication of three-dimensional (3D) human organ constructs to address this issue [2–6]. Of three commonly used bioprinting approaches [4–10], material extrusion, a continuous filament-based fabrication approach, is widely utilized due to its easy implementation, high efficiency in terms of greater deposition and printing speed, and wide range of extrudable materials [11]. During conventional extrusion bioprinting, each deposited layer is rapidly solidified *in situ* prior to the deposition of the next layer, which is a gelation-while-printing approach. While conventional extrusion bioprinting works for various applications [10,12–13], it is limited by possible nozzle clogging due to the short standoff distance [11,14] and weak interfacial strength between two sequentially deposited layers due to the phase change in the gelation process [14]. To address these challenges, a printing-then-gelation approach [14] has been proposed: the printed structure remains liquid during fabrication, and the structure is solidified only after the whole structure is completely printed.

The printing-then-gelation approach is mainly enabled by utilizing some complex fluids with a yield-stress property as the support bath material. Various support bath materials have been used for extrusion printing, such as hydrophobic fluids [15], various hydrogel matrices [16,17] and hydrogel particles [18], and thixotropic yield-stress materials [14,19–20] based on different supporting mechanisms from buoyancy-based [15], to entrapment-based [16–18], to yield-stress fluid-based [14,19–20]. Thus far, only the yield-stress material has been explored for the printing-then-gelation concept [14] for complex 3D structure printing.

Since the extruded filament during extrusion printing is the basic building block to form complex 3D structures, the better understanding of filament formation process during extrusion is of great significance. During conventional extrusion, the filament formation process is mainly influenced by the surface tension and gravity in nature, and the effects of build material properties and operating conditions on the filament diameter have been well investigated [10,21–24]. However, during the yield stress material-enabled printing-then-gelation process [14], the

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http://dx.doi.org/10.1016/j.msec.2017.05.144
0928-4931/

effects of surface tension and gravity on filament formation are negligible. A filament being deposited during yield stress-enabled extrusion printing is adequately supported by the yield-stress bath and since the liquid filament and the yield-stress bath are both aqueous in most cases, their interfacial tension is negligible. Instead, the yield stress and elastic behavior of the yield-stress bath material may play a more important role during the formation of filaments in addition to build material properties and operating conditions.

For filament formation in a support bath in general, Kolesky et al. [25] reported that by only varying the printing pressure, filament diameter could be adjusted in a wide range using only one nozzle with a given diameter; microchannel arrays with diameters increasing from 45 μm to 500 μm were fabricated using the same nozzle. Highley et al. [17] changed operating conditions including the nozzle diameter, flow rate, and nozzle translation rate in an extrusion process and found that increasing the nozzle diameter and flow rate caused an increase in filament diameter while increasing the translation rate led to a decrease in filament diameter. As expected, these general conclusions reached are similar to those observed during conventional extrusion. Furthermore, the filament printing performance in a Carbopol yield-stress bath was reported during PDMS filament printing [20]. Depending on different Carbopol products used, printed filaments were smooth and cylindrical or had a rough surface. Unfortunately, there is still no systematic investigation dedicated to study the filament formation in yield stress bath-enabled extrusion.

The objective of this study is to study the printability of hydrogel precursor solutions in a nanoclay yield-stress bath during extrusion-based printing-then-gelation where the printed hydrogel precursor solutions remain liquid until a whole structure is fabricated. The printability herein is mainly evaluated based on the morphology and dimensions of printed liquid filaments. In particular, Laponite nanoclay, a member of the smectite mineral family, was selected for the preparation of yield-stress baths for its unique properties such as the ionic insensitivity, thermal stability and ultraviolet transparency. These properties enable the Laponite nanoclay as a versatile yield-stress support bath material for the fabrication of complex 3D human tissue constructs from various hydrogels. Alginate-gelatin blends were selected herein as the viscoelastic bioink/build material to be printed in the Laponite nanoclay bath. Alginate, a natural polysaccharide isolated from brown algae, consists of a family of unbranched binary copolymers of 1,4 linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units). Gelatin is derived from collagen, a stiff helical protein with the repeating amino acid sequence glycine-X_1-X_2 in which X_1 and X_2 are often proline and hydroxyproline. These two hydrogels are biocompatible and widely used in bioprinting, and alginate-gelatin blends have been applied as bioinks for cellular artificial tissue fabrication [26] and utilized to make sponges for tissue matrices [27], drug delivery carriers [28,29], wound dressing fibers [30], and enzyme immobilization beads [31]. By varying the concentration of alginate solution and nanoclay suspension, respectively, the effects of their rheological properties on the morphology of extruded filaments are investigated in this study. In addition, the effects of operating conditions including the dispensing pressure, nozzle size, and nozzle path speed on the filament diameter and morphology are studied and discussed systematically. Furthermore, a complex 3D fibroblast-based vascular structure is printed and then gelled in the bath, and the cell viability and metabolic activity are measured to show the biocompatibility of the proposed printing-then-gelation biofabrication approach.

2. Materials and experimental setup

2.1. Nanoclay yield-stress bath preparation

Laponite, a synthetic nanoclay, is widely used in personal care products, coatings, and industrial applications. Laponite nanoclay ($Na_{0.7}Si_8Mg_{5.5}Li_{0.3}O_{20}(OH)_4$) is usually in the form of nanoscale platelets, approximately 1 nm thick and 25 nm in diameter, with very low polydispersity. When dispersed in aqueous solutions, sodium ions dissociate from individual platelets, leaving the faces of each disc negatively charged; hydroxide ion dissociation at platelet edges results in a slight positive charge. This charge distribution drives Laponite nanoclay platelets to adopt a stable "house-of-cards" arrangement as aqueous nanoclay suspension equilibrates to form a colloidal suspension with a yield stress. At rest or when a stress applied on the nanoclay suspension is lower than the yield stress, it behaves as a solid, otherwise it behaves as a liquid.

Laponite EP nanoclay (BYK Additives Inc., Gonzales, Texas) was used herein to function as the yield-stress support bath for filament formation investigation and cellular structure fabrication due to its neutral pH value. When a nozzle translates in a Laponite EP nanoclay bath, nanosilicates around the nozzle experience shear stress higher than its yield stress, resulting in a transition from a gel state to a sol state. Thus the liquefied Laponite nanoclay suspension can easily fill the crevasse behind the nozzle and entrap the deposited filaments in place. After the nozzle travels away from a given location, the local shear stress recovers below the yield stress, which leads to a rapid reverse transition from the sol state to the gel state to hold deposited filaments *in situ*.

Laponite EP nanoclay suspensions (pH ≈ 7.0) at different concentrations were used as the support yield-stress bath materials for this filament printability study. Nanoclay suspensions were prepared by dispersing the appropriate amount of dry Laponite EP nanoclay powder in deionized (DI) water with continuous mixing for a minimum of 60 min to ensure thorough hydration of the nanoclay solids, and were stored in the dark in sealed containers to prevent degradation and evaporation and aged for one day before use. Specifically, 0.5%, 1.0%, 2.0%, 4.0%, and 8.0% (w/v) Laponite EP nanoclay suspensions were prepared to investigate the effects of support-bath material properties on filament formation; and 2.0% (w/v) Laponite EP nanoclay suspensions were used for 3D cellular construct fabrication.

2.2. Alginate-gelatin bioink preparation

The bioink blends were prepared by dispersing the appropriate amount of dry sodium alginate (NaAlg) (low molecular weight (20–40 kDa), Acros Organics, Waltham, MA) powder and gelatin (Type A, 300 bloom, from porcine skin, MP Biomedicals, Solon, OH) powder in hot DI water (~50 °C) with continuous stirring until completely dissolved. Then the blend precursor solutions were cooled down to 37 °C for use. Specifically, bioink blends consisting of 0.5%, 1.0%, 2.0% (w/v) NaAlg and 10.0% (w/v) gelatin were prepared and printed respectively to investigate the effects of bioink properties on filament formation.

2.3. Cellular bioink preparation

For cellular construct fabrication, bioink made of the alginate and gelatin precursors and mouse fibroblasts was prepared for extrusion printing. The mixed stock solution (4.0% (w/v) alginate and 20.0% (w/v) gelatin) was prepared by dispensing the required amount of each powder in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) at 37 °C with continuous stirring until completely dissolved, then combined with suspended NIH-3T3 mouse fibroblasts (1×10^7 cells/mL, ATCC, Rockville, MD) in DMEM at 1:1 (v:v) to make the final bioink with cells (2.0% (w/v) NaAlg, 10.0% (w/v) gelatin, and 5×10^6 cells/mL 3T3 fibroblasts). Specifically, the fibroblasts were cultured in DMEM supplemented with 10.0% Fetal Bovine Serum (FBS; HyClone, Logan, UT) in a humidified 5.0% CO_2 incubator at 37 °C. The culture medium was replaced every 3 days as required. Then the freshly confluent flasks of 3T3 fibroblasts were washed twice with Dulbecco's phosphate buffered saline (PBS; Cellgro, Manassas, VA), and incubated with 0.25% Trypsin/EDTA (Sigma-Aldrich) for 5 min at 37 °C to detach the cells from the culture flasks. After that, the cell suspension was centrifuged at 1000 rpm for