Direct-Write Deposition of Thermogels

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Sara Maria Giannitelli[1], Valeria Chiono[2], Pamela Mozetic[1,3]*

[1] Università Campus Bio-Medico di Roma, via Álvaro del Portillo 21, 00128 Rome, Italy

[2] Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Corso Duca degli Abruzzi 24, Turin, Italy

[3] Center for Translational Medicine, International Clinical Research Center, St. Anne's University Hospital (FNUSA-ICRC), Brno, Czechia

* Corresponding author. E-mail address: p.mozetic@unicampus.it

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Abstract

The use of biocompatible hydrogels has widely extended the potential of additive manufacturing (AM) in the biomedical field leading to the production of 3D tissue and organ analogues for in vitro and in vivo studies.

In this work, the direct-write deposition of thermosensitive hydrogels is described as a facile route to obtain 3D cell-laden constructs with controlled 3D structure and stable behavior in physiologic conditions.

Keywords: Thermogels, Direct write deposition, 3D constructs, Tissue Engineering (TE), Additive Manufacturing (AM).

Introduction

Additive manufacturing (AM) of hydrogel-based materials has attracted growing interest as it enables the production of complex functional living tissues incorporating cells and/or bioactive

molecules into three-dimensional structures. This emerging tool appears to be promising for advancing tissue engineering (TE) toward the fabrication of functional tissue and organ analogues for transplantation [1], as well as for drug screening and cancer or disease *in vitro* modeling [2,3]. Thus, several research groups have adapted different AM techniques to generate cell-laden constructs [4,5]. Such constructs can be obtained starting from a bio-ink, which is a suspension of cells in an aqueous solution based on hydrogels precursors, both of natural and synthetic origin, made insoluble in water through crosslinking processes (chemical or physical crosslinking). However, to achieve an accurate reproduction of the designed architecture, hydrogels have to meet specific requirements in terms of viscosity and gelation rate, which limits the number of formulations that can be processed by AM [6]. Moreover, the crosslinking process must be non-cytotoxic for embedded cells and should guarantee adequate structural integrity and mechanical properties for *in vitro* culture and *in vivo* implantation. Indeed, the low viscosity of the deposited fluid and the time necessary for its gelation often lead to low reproducibility, control and resolution of the filament structure.

The authors have disclosed [7] the use of heat-sensitive hydrogels characterized by a sol-gel transition in a temperature range between 20 °C and 30 °C to prepare cell-laden constructs which are particularly stable under physiologic conditions.

In the present work, the Pluronic/alginate gel system has been proposed as prototypal thermosensitive gel to describe the procedure for the fabrication of cell-laden constructs with controlled 3D structure. This combination of polymers with distinct phase-transition mechanisms has been chosen with the aim of integrating the advantages of Pluronic gel in terms of printability [8] with the stability of the temperature-insensitive alginate component. The present system has been tested in combination with several cell lines (BALB/3T3, C2C12 and Human Dermal Fibroblast) [9,10] and successfully adopted for the engineering of muscle cell alignment

through the use of C2C12 murine myoblast cell line [9].

Materials

To minimize the risk of contamination, AM equipment should be placed in a biological safety cabinet, and all the pieces of equipment in contact with the bioink should be properly sterilized before processing.

Preparation of the bioink

1. Pluronic F127 (BASF)
2. Sodium alginate (PROTANAL® XP 3499, FMC BioPolymers)
3. Dulbecco's Modified Eagle Medium (DMEM)
4. Deionized water
5. Autoclavable glass vial with lid and magnet
6. Cell source (e.g. human dermal fibroblasts from adult skin, HDF) with appropriate medium and supplements
7. Autoclave
8. Ice bath
9. Magnetic stirrer

Additive manufacturing

1. A 3-axes motion control system (see **Note 1**).
2. Pressure-driven syringe barrel and piston system (Optimum system, Nordson EFD) terminated with a 250 μm ID blunt needle.

3. Heated holder for the pressure-driven syringe (operating at 37 °C) (see **Note 2**).
4. Programmable pressure controller (OB1-MK3, Elveflow, 0-8000 mbar range).
5. Petri dishes, glass slides, or other printing substrates.

Methods

Although the procedures are described for a Pluronic/alginate solution of selected concentration, the proposed methods can be extended to each heat-sensitive hydrogel having a transition from the sol to the gel phase upon increasing the temperature, at values included between 20 °C and 30 °C. Information on the sol-gel transition kinetic, gelation time at physiological conditions should be determined for each novel printing ink. A detailed description of all these characterization steps is out of the scope of the present chapter. However, a representative example of the set of analyses required for the optimization of a novel thermogel can be found in Gioffredi et al.[8].

Preparation of Pluronic/Alginate solution

The Pluronic/alginate solution can be prepared according to the so-called "cold method" proposed by Schmolka [11].

1. In a glass vial add 100 mg of sodium alginate to 3.9 mL of an ice-cold 0.2x solution of DMEM under mild stirring.
2. While keeping the solution in an ice bath, slowly disperse 1 g of Pluronic F127 in the alginate solution under mild stirring (see **Note 3**).
3. Sterilize the solution by autoclaving, and store sterilized solution at 4 °C.

Printing process

Main steps of scaffold manufacturing by direct write deposition are summarized in **Figure 1**. Subconfluent HDFs can be routinely processed by trypsinization and centrifugation to obtain a cell pellet (see **Note 4**).

1. Working in a biosafety cabinet, homogeneously suspend HDFs in the sterile Pluronic/alginate solution kept at 4 °C, at a final concentration of 1×10^6 cells/mL.
2. Transfer the bioink into the pressure-driven syringe.
3. Mount the syringe on the heated holder, connect it to the pressure controller, and bring it at 37 °C (see Note 5).
4. Extrude the bioink at a pressure of 1.2 bar (see Note 6) with a needle/substrate relative speed of 10 mm/s. Several layers can be superimposed to build a 3D construct of the desired geometry.
5. Gently dispense few mL of the crosslinking solution on the construct surface to induce alginate crosslinking.
6. After 5 minutes, remove the crosslinking solution, and gently wash the crosslinked scaffold in cell culture medium to terminate the gelation process.
7. Check the constructs under an inverted microscope. Two representative cell-laden constructs with different printing patterns are reported in **Figure 2**.
8. Incubate constructs at 37°C in a 5% CO_2 atmosphere with frequent medium changes (see **Note 7**). At selected time-points, constructs can be retrieved and used for biological characterization.

[**Fig 1** here]

[**Fig 2** here]

Notes

1. Several commercial or custom motion control systems can be adapted to direct write deposition process. Please refer to [12] for an example of a custom-built apparatus.
2. This piece of equipment shall be customized upon the dimensions of the chosen syringe system to provide firm handling and homogeneous heating. Depending on the hydrogel composition, the use of an additional heated plate (set at 36-38 °C) as a holder for the printing substrate could be helpful to stabilize the gel structure after printing.
3. It is advisable to perform stepwise addition (5 or more aliquots) of Pluronic F127. Complete dissolution can take several hours.
4. The process can be adapted to different cell types, and the reported use of HDFs should be intended as a representative example. Given the mild conditions used in the processing method for preparing cell-laden constructs, the bioink can be supplemented with biomolecules of interests (e.g. growth factors).The time necessary to induce bio-ink gelation is generally between 10 and 15 minutes.
5. Depending on the rheological properties of the printing solution, the extrusion pressure can be varied between 1 and 3 bar.
6. Pluronic F127 is rapidly eluted from the constructs during the first hours in culture and can be removed by frequent medium changes.

Acknowledgement

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Figure Legend

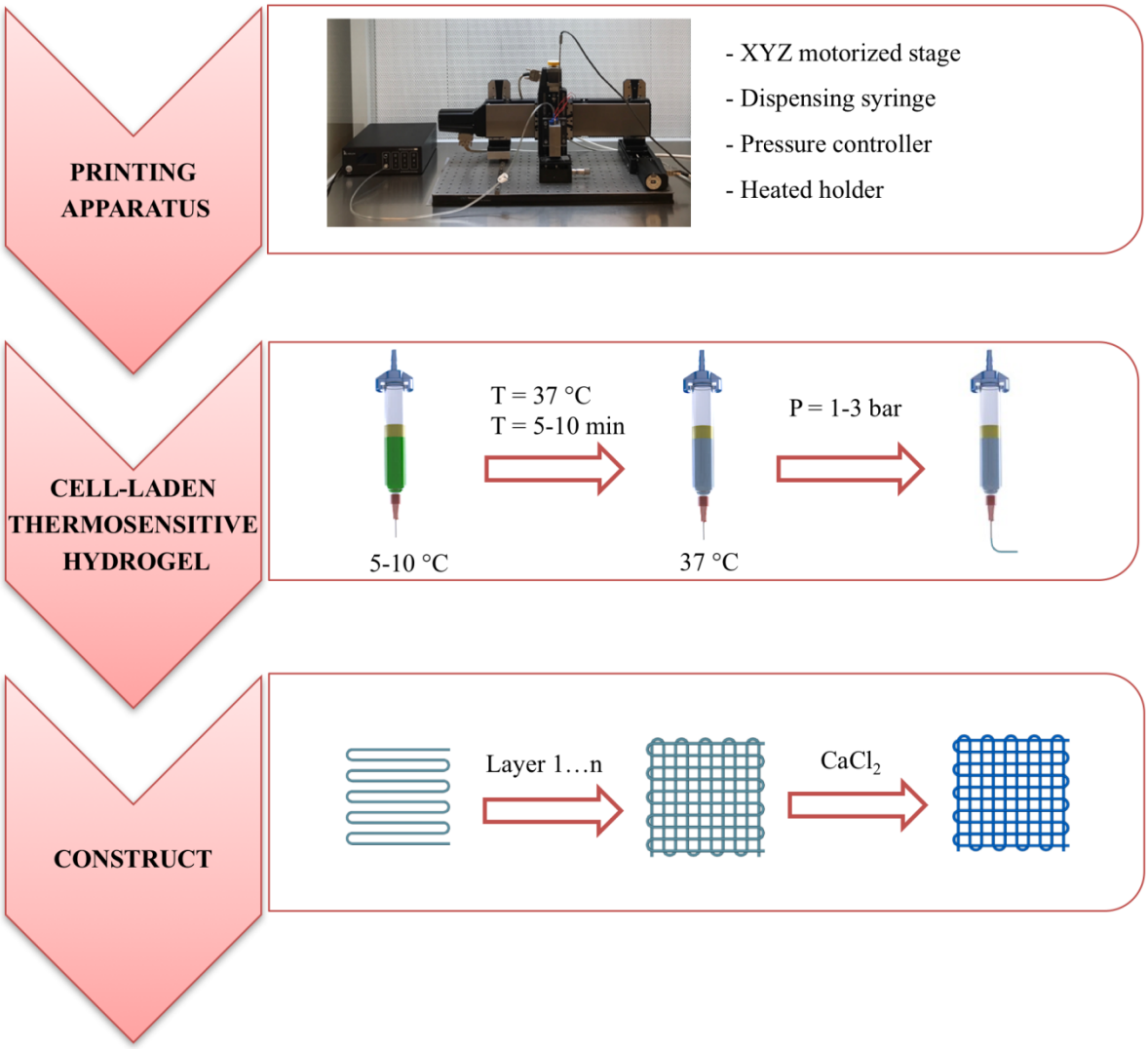

Figure 1. Workflow for the fabrication of 3D constructs via direct-write deposition of thermosensitive hydrogel: (i) 3D printing apparatus, (ii) syringe loading and sol-gel transition, (iii) scaffold manufacturing and crosslinking.

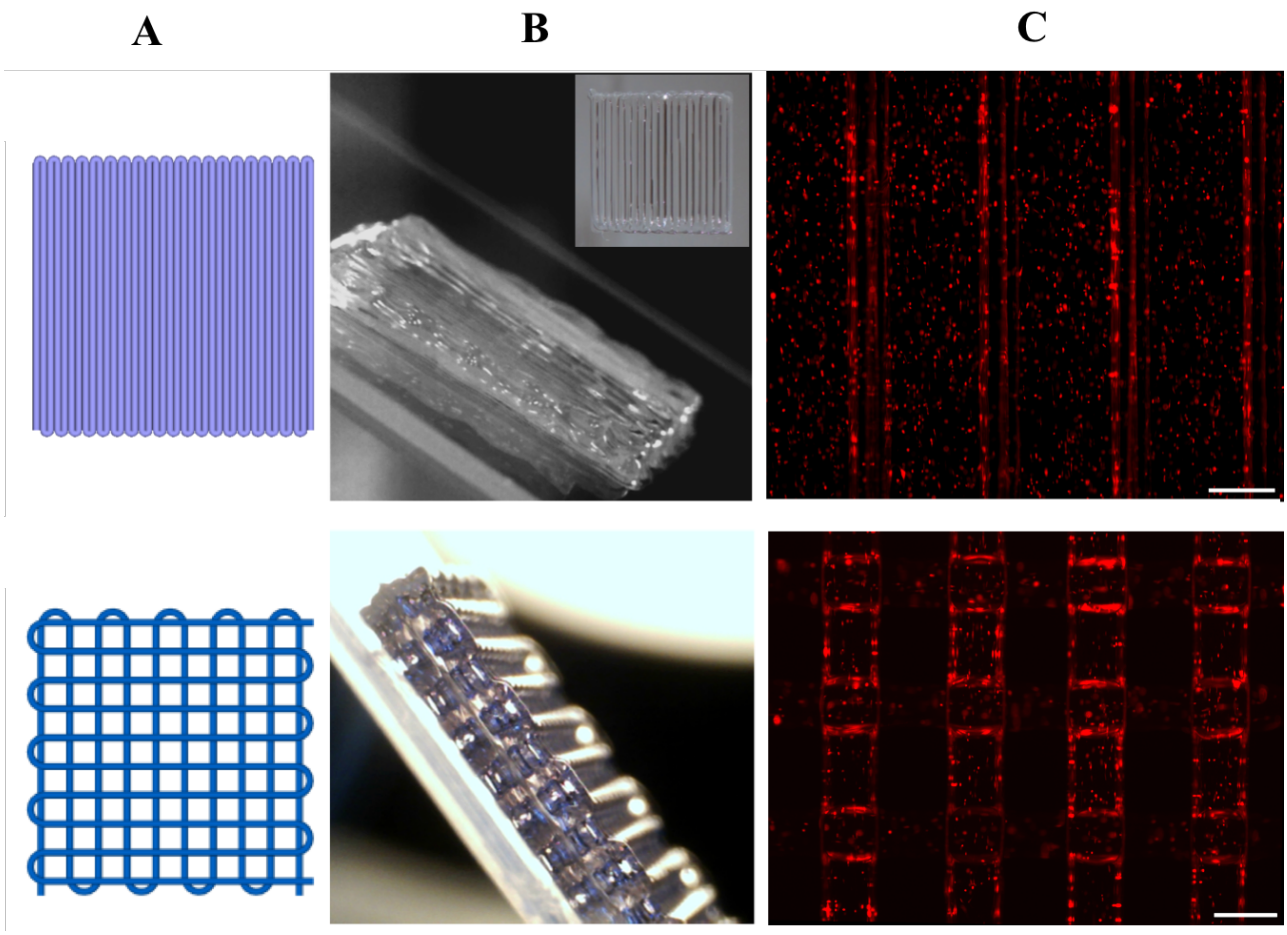

Figure 2. Schematization of construct architectures (A), macrographs (B) and fluorescence micrographs (C) of the cell-laden constructs for two different geometries. HDF cells were labelled with PKH26 red-fluorescent dye. Scale bar: 500 μm.