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Brief communication

Cryogenic 3D printing for tissue engineering

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ABSTRACT

We describe a new cryogenic 3D printing technology for freezing hydrogels, with a potential impact to tissue engineering. We show that complex frozen hydrogel structures can be generated when the 3D object is printed immersed in a liquid coolant (liquid nitrogen), whose upper surface is maintained at the same level as the highest deposited layer of the object. This novel approach ensures that the process of freezing is controlled precisely, and that already printed frozen layers remain at a constant temperature. We describe the device and present results which illustrate the potential of the new technology.

Tissue engineering is a specialized field of biomedical engineering established to address failing human organs and tissues, by replacing them [2]. Recently, additional applications of tissue engineering have emerged, as replacement for animal use in research, in the form of micro-organs or organs on a chip [3]. A key element in tissue engineering is the manufacture of a tissue scaffold, an extracellular matrix [7]. Various methods are used for generating a tissue scaffold [1]. Freezing of a hydrogel, is the tissue scaffold engineering method relevant to this work [5,10]. Here we describe a new freezing technology for generating tissue scaffolds using 3D additive methods. 3D additive methods are of increasing interest in tissue engineering in general, and scaffold design in particular [4,6,8].

3D printing produces a three dimensional object through the addition of layers. There are a variety of manufacturing methods that can be classified as 3D printing. The additive method of manufacturing, layer over layer, is a central element to all of them. One conventional 3D printing technology employs a printer head that delivers the material to be printed, in a molten form, at a controlled rate and temperature. The head has the ability to move in the XY plane and the printing table can move on the z-axis, under computer control, allowing the manufacturing of complex shapes. The molten material is deposited on the printing table where it solidifies; the process continues until a layer is completed, then the printing table moves downwards and another layer is deposited on the previous. In conventional 3-D printing the material is a plastic and the printing process usually occurs in open air and at room temperature. The phase transition temperature of the molten plastic is higher than the room temperature.

This study describes a new addition to 3D printing that, while simple to implement, facilitates 3D printing with freezing for tissue engineering. We found that the outcome of a freezing process during 3D printing can be controlled by immersing the 3D printed object in a liquid with a lower temperature than the phase transition temperature of the printed material. As consecutive layers are printed, the level of the immersion liquid should be continuously raised to be flush with the highest printed layer i.e. the dispensing printing head.

Fig. 1, is a schematic showing the key elements of this 3-D printing method. A central element is the printing head that can dispense the printing fluid at a controlled rate, through a syringe. The printing head is attached to a carrier that can move in the x–y plane. Both the x–y motion and the motion of the syringe plunger are computer controlled. Another element is the printing surface. The printing surface has a computer controlled z-direction motion. A container for the immersion fluid and the printed object rests on the printing surface. The immersion fluid temperature should be controlled and lower than the freezing temperature. The immersion fluid level changes during the printing process to continuously match the top of the last printed layer. A valve is used to meter the addition of the immersion liquid, maintaining the desired level.

To demonstrate the technology, we have modified a conventional 3D printer (FlashForge, Creator Model, Los Angeles, CA USA). The original printer head that melted and dispersed a plastic was replaced with a new printer head assembly. A photograph of the

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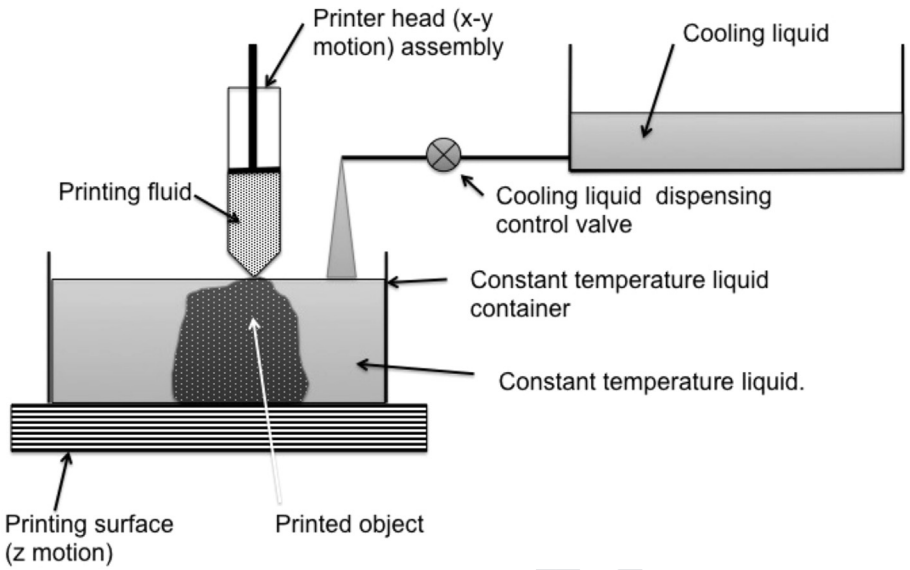

Fig. 1. Cryogenic 3D printer. Schematic of the immersion cooling fluid concept.

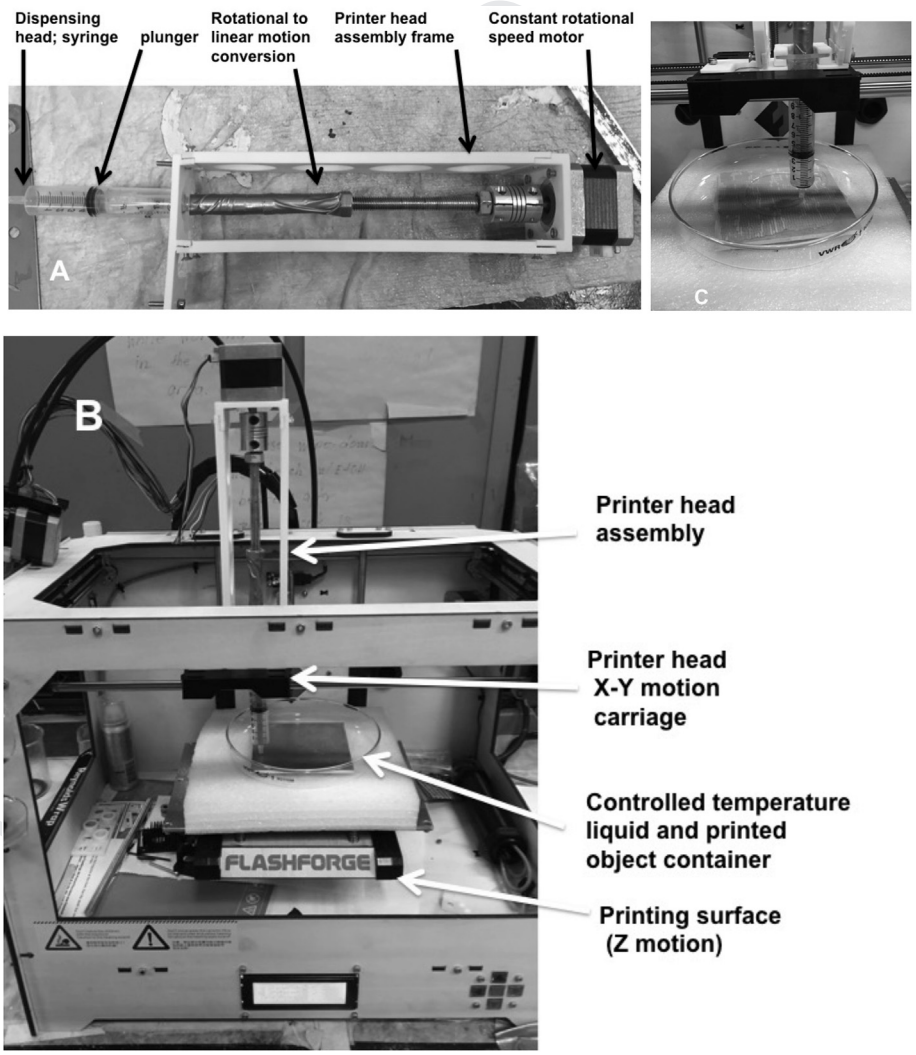

Fig. 2. Photographs of the 3-D cryogenic printer. A) A photograph of a new printer head assembly in which the printing liquid is dispensed through a syringe. B) A photograph of the assembled cryogenic printing device showing the original printer, the new printer head and the container in which the object is printed. C) A higher magnification of the printing head in the container.

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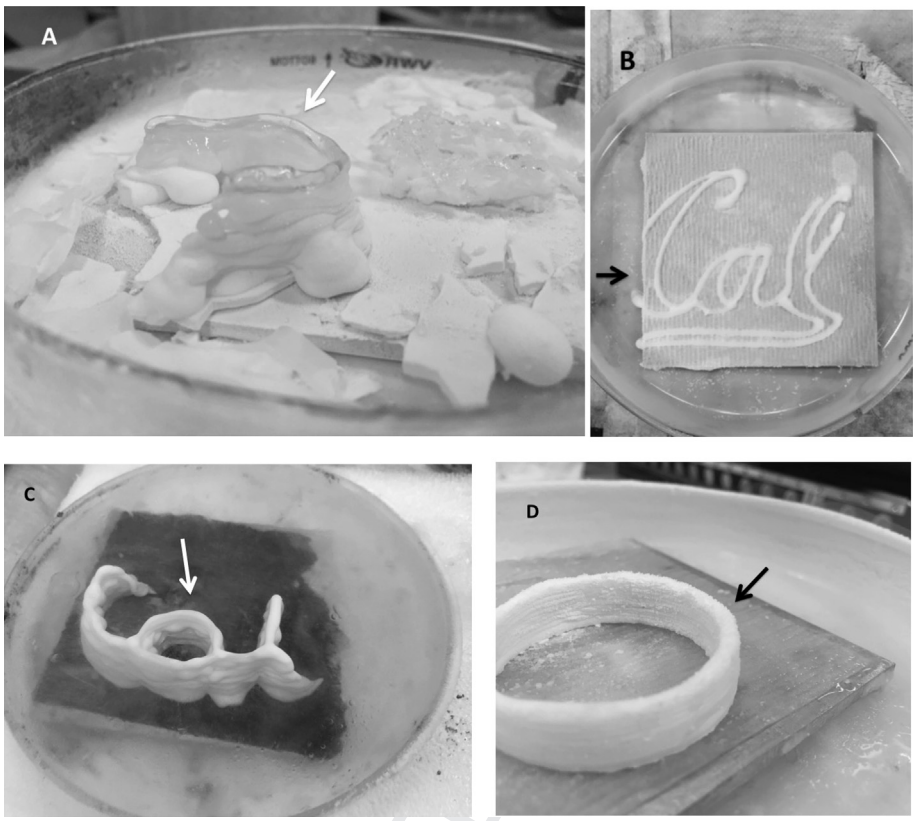

Fig. 3. Cryogenic 3D printed objects. A) A collapsed ring like structure (arrow) whose cryogenic 3D printing has failed because the object was not immersed during printing in liquid nitrogen. Only the base was cooled by liquid nitrogen. B) A cryogenic 3D printed object. Left hand side of the letter C, (arrow) was not printed because it lacked an ice nucleating layer. C) Manual control cryogenic 3D printing of a multilayer, complex object. D) A ring like objected made of several layers (arrow) with computer controlled cryogenic 3D printing.

new printer head is shown in Fig. 2A. The new printer head is centered around a syringe, loaded with a liquid hydrogel as the printing material, whose plunger is controlled by a NEMA17 stepper motor. The stepper motor was taken from the original printer where it dispersed the molten plastic under computer control. In this design the rate of hydrogel delivery onto the printing surface is controlled by the original printer software. The printing head components are attached to a 3-D printed assembly frame. The assembly frame was designed to attach to the original X–Y printer head carriage, at the same location and in the same way as the original printing head. This also allows the use of the original software to control the X–Y motion of the new printer head, without any modification. Optionally the temperature of the printing hydrogel inside the syringe can be controlled through resistive heating, to ensure the desired temperature and viscosity.

Fig. 2B shows a photograph of the modified printer. In addition to the new printing head, a glass dish was placed on top an insulation layer on the printing table. It served as the container for the immersion fluid and the printed object. Fig. 2C shows a higher magnification of the printing head in the container. The immersion fluid addition was done manually in this study.

The following software was used: Blender (http://www.blender.org/) was used to create 3D models in STL format. Slic3r (http://slic3r.org/) was used to convert them into GCODE instructions which were translated by GPX (https://github.com/whpthomas/GPX) into X3G files for use on the 3D printer. The FlashForge printer was running the preinstalled Sailfish Firmware.

Results are shown in Fig. 3. For this study we have used agar gel in distilled water, 40 g/l, as the printing fluid, and liquid nitrogen as the immersion constant temperature fluid. We will begin by showing different failures of 3D printing by freezing; to illustrate the value of our simple but yet central concept of printing in an immersion cooling liquid. Panel A, shows the results of printing through gaseous nitrogen cooled air onto a liquid nitrogen cooled printing surface. This example does not use immersion in a cooling liquid after printing. The arrow points to a top layer of the printed object. It shows that with this mode of cooling, higher layers of the 3-D printed object cannot solidify, and, therefore, the ability to print large organs is limited. Panel B shows another important aspect of frozen structures printing related to ice nucleation. Nucleation is an important aspect of solidification, in particular with small droplets. We have found that an ice nucleating layer (seed) is required to initiate the frozen object printing process. We have used tissue paper saturated with water and frozen on aluminum in liquid nitrogen as the nucleation surface. In addition to serving as a nucleation site, this configuration secures the printed object to the printing surface, preventing it from floating away in the immersion fluid. The arrow in Panel B points to a missing part of the letter C. It is seen that this part of the letter did not print, because the printing was not done on an ice nucleating surface. Panel C, illustrates the value of using the precisely computer controlled motion of the 3-D printer. The object in Panel C was printed under a layer of liquid nitrogen that was continuously flush with the highest layer of the printed object. However, here the motion of the X–Y carriage was done by manual control. The results show that complex structures can be produced. However, printing with manual control is a lengthy process and the printing is not precise. Finally Panel D shows the outcome of a computer controlled 3-D printing of a freezing object with computer control over the X-Y-Z motion, with the use of a ice nucleating layer and

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immersion of the printed object in liquid nitrogen. The layers are printed precisely one on top of the other and the top layer is as solid as the bottom layer. In the future, the object in Panel D could become a large blood vessel, with precisely computer controlled composition and structure.

This Brief Communication, is a first order feasibility study of a new method for 3-D printing of frozen objects, with potential applications in tissue engineering. We have shown that the simple immersion of the printed object in a cooling liquid, with a variable height fitted to the last printed layer, can produce high quality and rapid printing of frozen objects. To the best of our knowledge, this simple but enabling addition to the 3-D printing technology was not reported before. We will discuss some advantages of this technology.

The cryogenic 3D printing technology described in this study allows precise control over the thermal conditions during freezing of the deposition layer. The cooling liquid, controls the temperature of the solid layer on which the melt is deposited. The rate of deposition of the melt is also known and computer controlled. A key advantage of this technology is that complex and well defined structures of frozen materials can be manufactured through these controls. The immersion of the printed object into a constant temperature cooling liquid has several additional advantages. One is the elimination of thermal stresses. Another, relates to the issue of long term storage of engineered tissues. Currently, it is envisioned that the manufacturing strategy in tissue engineering is comprised of two steps. First the engineered tissue is produced. Than, means are sought for long term preservation of the product, tentatively by freezing. With our method the engineered tissue is produced in a frozen state, ready for long term storage, thereby eliminating a complex step in the manufacturing process of engineered scaffolds. In this proof of feasibility we have used agar as the printing materials and liquid nitrogen as the immersion fluid. Liquid nitrogen is convenient because it maintains a constant temperature in atmospheric pressure, without the need for controls. However, obviously, other aqueous printing materials and immersion fluids can be used. In fact, the immersion cooling liquid can be a preserving solution or a cryoprotectant solution and the printing material can also be a (stem) cell.

In the future, this technology may find application in other aspects of tissue engineering. Currently, attempts are made to incorporate cells, such as stem cells, into the tissue engineered scaffold [7]. The technology described here may be suitable for 3D printing of tissues with cells. Cryopreservation of cells by rapid freezing, in small volume droplets, is known [9]. The cryogenic 3D printing method described in this study could be used to produce cooling rates typical of splash freezing, and may result in vitrification of cells. It would be interesting to develop cryopreservation methods for cells deposited onto an engineered tissue scaffold produced by cryogenic 3D printing.

Conflict of interest

None declared.

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