**TITLE**

3D Printed Porous Cellulose Nanocomposite Hydrogel Scaffolds

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**SUMMARY**

The three critical steps of this protocol are i) developing the right composition and consistency of the cellulose hydrogel ink, ii) 3D printing of scaffolds into various pore structures with good shape fidelity and dimensions and iii) demonstration of the mechanical properties in simulated body conditions for cartilage regeneration.

**ABSTRACT**

This work demonstrates the use of three-dimensional (3D) printing to produce porous cubic scaffolds using cellulose nanocomposite hydrogel ink, with controlled pore structure and mechanical properties. Cellulose nanocrystals (CNCs, 69.62 wt%) based hydrogel ink with matrix (sodium alginate and gelatin) was developed and 3D printed into scaffolds with uniform and gradient pore structure (110-1100 µm). The scaffolds showed compression modulus in the range of 0.20-0.45 MPa when tested in simulated in vivo conditions (in distilled water at 37 °C). The pore sizes and the compression modulus of the 3D scaffolds matched with the requirements needed for cartilage regeneration applications. This work demonstrates that the consistency of the ink can be controlled by the concentration of the precursors and porosity can be controlled by the 3D printing process and both of these factors in return defines the mechanical properties of the 3D printed porous hydrogel scaffold. This process method can therefore be used to fabricate structurally and compositionally customized scaffolds according to the specific needs of patients.

**INTRODUCTION**

Cellulose is a polysaccharide consisting of linear chains of β (1-4) linked D-glucose units. It is the most abundant natural polymer on Earth and is extracted from a variety of sources, including marine animals (e.g., tunicates), plants (e.g., wood, cotton, wheat straw), and bacterial sources, such as algae (e.g., Valonia), fungi, and even amoeba (protozoa)1,2. Cellulose nanofibers (CNF) and cellulose nanocrystals (CNC) with at least one dimension on nanoscale are obtained through mechanical treatments and acid hydrolysis from cellulose. They not only possess the properties of cellulose, such as potential for chemical modification, low toxicity, biocompatibility, biodegradable and renewable, but it also has nanoscale characteristics like high specific surface area, high mechanical properties, rheological and optical properties. These attractive properties have made CNFs and CNCs suitable for biomedical applications, mainly in the form of 3-dimensional (3D) hydrogel scaffolds3. These scaffolds require customized dimensions with controlled pore structure and interconnected porosity. Our group and others have reported 3D porous cellulose nanocomposites prepared through casting, electrospinning and freeze-drying4-8. However, control on the pore structure and fabrication of complex geometry is not achieved through these traditional techniques.

3D printing is an additive manufacturing technique, in which 3D objects are created layer by layer through the computer-controlled deposition of the ink9. The advantages of 3D printing over traditional techniques includes design freedom, controlled macro and micro dimensions, fabrication of complex architectures, customization and reproducibility. In addition, 3D printing of CNFs and CNCs also offers shear-induced alignments of nanoparticles, preferred directionality, gradient porosity and can easily be extended to 3D bioprinting10-15. Recently, the dynamics of CNCs alignment during 3D printing has been reported16,17. Advances in the field of bioprinting have enable 3D printed tissues and organs despite the involved challenge such as choice and concentration of living cells and growth factors, composition of the carrier ink, printing pressures and nozzle diameters18-20.

The porosity and compressive strength of cartilage regenerative scaffolds are important properties that dictates its efficiency and performance. Pore size plays an important role for the adhesion, differentiation, and proliferation of cells as well as for the exchange of nutrients and metabolic waste21. However, there is no definite pore size that can be considered as an ideal value, some studies showed higher bioactivity with smaller pores while others showed better cartilage regeneration with larger pores. Macropores (<500 µm) facilitate tissue mineralization, nutrient supply and waste removal while micropores (150-250 µm) facilitate cell attachment and better mechanical properties22,23. The implanted scaffold must have sufficient mechanical integrity from the time of handling, implantation and until the completion of its desired purpose. The aggregate compressive modulus for natural articular cartilage is reported to be in the range of 0.1-2 MPa depending on age, sex and tested location4,24-29.

In our previous work11, 3D printing was used to fabricate porous bioscaffolds of a double crosslinked interpenetrating polymer network (IPN) from a hydrogel ink containing reinforced CNCs in a matrix of sodium alginate and gelatin. The 3D printing pathway was optimized to achieve 3D scaffolds with uniform and gradient pore structures (80-2125 µm) where nanocrystals orient preferably in the printing direction (degree of orientation between 61-76%). Here, we present the continuation of this work and demonstrates the effect of porosity on the mechanical properties of 3D printed hydrogel scaffolds in simulated body conditions. CNCs used here, were earlier reported by us to be cytocompatible and non-toxic (i.e., cell growth after 15 days of incubation was confirmed30). Moreover, scaffolds prepared via freeze-drying using the same CNCs, sodium alginate and gelatin showed high porosity, high uptake of phosphate buffer saline and cytocompatibility toward mesenchymal stem cells5. The goal of this work is to demonstrate the hydrogel ink processing, 3D printing of porous scaffolds and the compression testing. Schematics of the processing route is shown in **Figure 1**.

**PROTOCOL**

**1. Preparation of precursors**

* 1. Preparation of cellulose nanocrystals suspension

NOTE: Isolation of cellulose nanocrystals is done according to the procedure reported by Mathew, et al30.

1.1.1 Dilute 17 wt% suspension of cellulose nanocrystals to 2 wt% by adding distilled water to make a total volume of 2 L. Mix thoroughly using ultra sonication and use smaller batches (250-300 mL) for efficient mixing.

1.1.2 Pass the sonified suspension through the homogenizer 10 times at a pressure of 500-600 bar. At this point, a thick transparent gel of 2 wt% cellulose nanocrystals is obtained.

1.1.3 Concentrate 2 wt% cellulose nanocrystals gel to 11 wt% through centrifugations at 24,500 x *g* for 1.5 h. Decant water out in between every 30 min.

NOTE: Experiment can be paused here.

1.2 Preparation of matrix phases

1.2.1 Prepare homogeneous solution of 6 wt% sodium alginate (SA) in distilled water at 60 °C under continuous stirring.

1.2.2 Prepare homogeneous solution of 12 wt% gelatin (Gel) in distilled water at 60 °C under continuous stirring.

NOTE: Prepare a volume of 20 mL for matrix solutions and store in refrigerator.

1.3 Preparation of crosslinkers

1.3.1 Prepare the solution of 3 wt% calcium chloride in distilled water at room temperature under continuous stirring.

1.3.2 Prepare the solution of 3 wt% glutaraldehyde in distilled water at room temperature under continuous stirring.

NOTE: Prepare a volume of 50 mL for crosslinking solutions and store in room temperature. Refer to the **Table of Materials** for vendor information. Experiment can be paused here.

**2. Preparation of hydrogel ink**

2.1 Prepare 40 mL of hydrogel ink in a polystyrene container by mixing 11 wt% CNC, 6 wt% SA and 12 wt% Gel to obtain a wet (wt%) composition of CNC/SA/Gel/Water: 6.87/1.50/1.50/90.12.

2.2 Heat the mixture to 40 °C and mix with a spatula until a smooth paste is obtained.

2.3 Transfer the mixture into a 60 mL syringe. Pass the mixture through a series of nozzles with different diameters into another 60 mL syringe, with the help of mechanical clamp. Repeat the process until smoothly extruded filaments of hydrogel ink are obtained. Start with nozzle with biggest diameter of 800 µm, followed by 600 µm and 400 µm.

2.4 Gently centrifuge (4000xg) the syringe filled with hydrogel ink to remove trapped air.

NOTE: Experiment can be paused here.

**3.** **Measurement of rheological properties of hydrogel**

NTE: Perform the rheological properties by using a smooth cone-on-plate geometry, CP25-2-SN7617, diameter 25 mm, 2° nominal angle and gap height 0.05 mm at 25 °C.

3.1 Turn on the rheometer, air compressor and temperature control box. Initialize the software.

3.2 Mount the measuring tool in the rheometer and set zero-gap.

3.3 Extrude approximately 1 mL of the hydrogel ink onto the rheometer platform.

3.4 Measure the viscosity as a function of shear rate. Select the shear rate range from 0.001 to 1000.

3.5 After the measurement is done, clean the rheometer platform and measuring tool. Extrude 1 mL of fresh hydrogel ink again on the rheometer platform.

3.6 Measure storage moduli (G′) and loss moduli (G″) as a function of shear stress at a frequency of 1 Hz. Select the shear stress range from 103 to 107.

3.7 Once the tests are completed, copy the data into text file and plot rheological curves in logarithmic scale.

**4. File preparation for 3D Printing**

NOTE: Cura 2.4.0 software is used for designing 3D scaffolds (20 mm3) having three types of pores. 1- Uniform pores of 0.6 mm, 2- uniform pores of 1.0 mm and 3- gradient pores of range 0.5-1 mm.

4.1 Download stereolithography (stl) file of a solid cube from thingsinverse.com and open the file in Cura.

4.2 Click the loaded model and move it to X/Y/Z : 0/0/0 mm. Click **Scale**, uncheck box for **Uniform scaling** and set the dimensions to X/Y/Z : 20/20/20 mm. Click **Rotate** and rotate the cube by 45° in XY plane.

4.3 In the side panel, in **Nozzle & Material**, select 0.4 mm and paste the profile. Select **Discov3ry complete** as the printer.

4.4 In the side panel, select **Custom** for **Print Setup**. Under **Quality** section, enter 0.2 mm for all sub sections. Under **Shell** section, enter 0 mm for all sub sections. Under **Material** section, enter 26 °C for temperature, 1 mm Diameter and 100 % Flow. Under **Speed** section, enter 30 mm/s as **Print Speed** and 120 mm/s as **Travel Speed**. Under **Support** section, uncheck the box for **Enable Support**. Under **Build Plate Adhesion** section, select **Skirt**, enter 3 mm as **Skirt Distance** and 150 mm as **Skirt/Brim Minimum length**.

4.5 For scaffolds with uniform pore size, enter 0.6 or 1 mm **Infill Line Distance** and select **Grid Infill Pattern**.

4.6 For gradient porosity scaffolds, Merging and Grouping tool is used. Right click the loaded model, select **Multiple Models**, enter 2 and press **OK**. Scale each model as X/Y/Z : 20/20/7 mm. Place the models on top of each other. Enter **Infill Line Distance** as 0.3, 0.5 and 0.7 mm for bottom, middle and top model, respectively. Select all three models (**Ctrl + A**), right click and click **Group Models**.

4.7 Save the models on the Sure Digital (SD) card. Cura automatically save the file as gcode that is read by the printer.

**5. 3D printing porous scaffolds**

5.1 Insert the transfer tube into the nozzle holder and connect 400 µm nozzle to it. Level the build plate to get the correct distance between the build plate and nozzle.

5.2 Load the centrifuged syringe into the cartridge and connect it to the other side of the transfer tube.

5.3 Insert the SD card into the printer, select **Purge fast** and start purging the hydrogel ink until it starts to extrude from the nozzle. Continue purging for 2-3 min to obtain a homogeneous flow.

5.4 From the SD card, select the saved files for uniform and gradient porosity scaffolds and start printing. Keep an eye on the extrusion rate. If needed, adjust the speed and flow rate accordingly. For smaller pore size, use faster speed combined with low flow rate (50 mm/s and 70%).

NOTE: Do not touch the 3D printed scaffolds.

**6. Crosslinking of 3D printed scaffolds**

6.1 After the 3D printing is complete, gently add drops of 3 wt% CaCl2 to the scaffold until it becomes completely wet. Wait for 5 min.

6.2 Very carefully transfer the scaffold from the printer to a 50 mL container filled with 3 wt% CaCl2. Leave it overnight.

6.3 Wash thoroughly with distilled water and transfer the scaffold to a 50 mL container filled with 3 wt% glutaraldehyde. Leave it overnight.

6.4 Wash thoroughly and store the 3D printed scaffold in distilled water.

**7. Compression testing**

NOTE: Perform compression tests with 100 N load cell in water at 37 °C.

7.1 Fill the container equipped with submersible compression base plate with 2 L of water and start the heating system to reach 37 °C.

7.2 Initialize Bluehill Universal software and set up the testing method. Select rectangular specimen geometry and choose the option to enter dimensions before testing each sample. Set the strain rate to 2 mm/min and end of result as 80% compressive strain together with 90 N force.

7.3 In the **Measurement** section, select force, displacement, compressive stress and compressive strain. Choose the option to export data as text files for future plotting.

7.4 Set the zero extension point by using the jog controls to lower crosshead plate as close as possible to base plate.

7.5 Measure and note the dimensions of the samples to be tested.

7.6 When the water temperature reaches to 37 °C, place the sample on the base plate. Secure the sample by moving the crosshead plate so that it starts to touch the sample.

7.7 Move the water bath up, so that the plates with the sample in-between them are immersed in water.

7.6 Enter sample name and dimensions. Start the test.

7.7 After the test is complete, first move the water bath down and then raise the crosshead plate.

7.8 Remove the sample and its pieces, if any, clean both the plates and load a new sample.

7.9 After all the samples are tested, export the raw data. Plot compressive stress vs. compressive strain curves and determine the compressive tangent modulus at strain values of 1-5 % and 25-30 %.

NOTE: Place the gradient cube in such a way that the larger holes face the stationery base plate.

First secure the scaffold in between the grips and then start/stop the measurement.

**REPRESENTATIVE RESULTS**

CNCs based nanocomposite hydrogel ink shows a strong non-Newtonian shear thinning behavior (**Figure 2a**). The apparent viscosity of 1.55 × 105 Pa.s at a low shear rate (0.001 s-1) drops by five orders of magnitude to a value of 22.60 Pa.s at a shear rate of 50 s-1 (≈50 s-1 being a typical shear rate experienced during 3D printing)31. The hydrogel ink exhibits a viscoelastic solid behavior, as the storage modulus G’ (4.42×107 Pa) is an order of magnitude greater than the loss modulus G’’ (8.26×106 Pa) at low shear stress, with a well-defined dynamic yield stress value (G’=G’’) of 5.59×104 Pa (**Figure 2b**). The 3D printed porous nanocomposite hydrogel scaffolds are in shown in **Figure 3**. For all the printed scaffolds, the shape and dimensions are very well retained after printing as well as after double crosslinking. The pore sizes of the scaffolds, 110-1100 µm, are in the range of 100-400 µm that is considered a benchmark for cartilage regeneration32.

The 3D printed scaffolds were tested in compression mode. This is the preferred mode of mechanical testing for cartilage materials because the role of natural cartilage is to bear loads in compression. To mimic the in vivo conditions, scaffolds were tested in water at 37 °C. **Table 1** and **Figure 4a** represents the compressive data obtained for different porous nanocomposite hydrogel scaffolds at a strain rate of 2 mm/min. At low strain rates (1-5%), the compressive modulus (~ 0.17 MPa) is more or less similar for all types of porous scaffolds. This shows that the elastic nature of the hydrogel ink is preserved even in the presences of the macropores. However, at high strain rates (25-30%), the highest modulus of 0.45 MPa is obtained for reference scaffold with no porosity. However, as soon as the pore size increases, the modulus decreases, due to the decrease in density indicating the expected relationship between porosity of the scaffolds and the corresponding mechanical properties. In case of the gradient porous scaffolds, the modulus is higher (0.34 MPa) as compared to uniform porous scaffolds (0.20 and 0.26 MPa) because of the presence of smaller pore sizes and more solid walls. Furthermore, the compressive modulus of the 3D hydrogel scaffolds increases as the compression rate increases (**Figure 4b**), exhibiting and mimicking the viscoelasticity of natural cartilage tissues that is considered favorable for load bearing scaffolds33. The compressive modulus of 0.20 MPa at strain rate of 2 mm/min increases to 0.35 MPa at 5 mm/min and further increases to 0.47 MPa at 120 mm/min and is in the range reported for natural cartilage (i.e., compressive modulus of 0.1-2 MPa).

**FIGURE AND TABLE LEGENDS**

**Figure 1. Schematics of the processing route.** (**a**) Preparation of the nanocomposite hydrogel ink. (**b**) 3D printing porous scaffolds. (**c**) Double crosslinking of 3D printed scaffolds. (**d**) Compression testing of 3D porous scaffolds in water at 37 °C.

**Figure 2. Log–log plots of nanocomposite hydrogel ink.** (**a**) Viscosity vs. shear rate and (**b**) G’ and G’’ vs. shear stress.

**Figure 3. 3D printed porous scaffolds.** Scale: 500 µm. (**a**) Reference with no holes. (**b**) 1 mm pore size. (**c**) 0.60 mm pore size. (**d**) Gradient porosity 110-800 µm.

**Figure 4. Representative stress-strain curves for 3D printed porous nanocomposite hydrogel scaffolds.** (**a**) At constant strain rate of 2 mm/s. (**b**) At different strain rates for 1 mm pore size scaffold.

**Table 1. Compression data for 3D printed nanocomposite hydrogel scaffolds.**

**DISCUSSION**

3D printing requires suitable rheological properties of the hydrogel ink. The high viscosity ink will require extreme pressures for its extrusion while low viscosity ink will not maintain its shape after extrusion. The viscosity of the hydrogel ink can be controlled through the concentration of the ingredients. As compared to our previous work11, the solid content of the hydrogel ink is increased from 5.4 to 9.9 wt% resulting in concentrated hydrogel ink which helps to improve the resolution of the printed scaffold. It may be noted that, unlike long flexible CNFs, rigid rod like CNCs can produce inks with higher solid contents at a given viscosity due to the absences of physical entanglements14. Another important aspect that affect printability is the homogeneity of the ink. It was noted that heating the hydrogel ink at a temperature of 40 °C promotes the homogeneous mixing of CNCs with the matrix phase. To further ensure the smoothness of the hydrogel ink, it was passed through a series of nozzles, starting with the biggest diameter of 800 µm, then 600 µm and finally 400 µm. During these passes, the nozzle can be clogged which indicates the presences of big lumps but after these passes the hydrogel ink extruded effortlessly in the form of a continuous filament. The nozzle movement to obtain 3D printed constructs is also of great importance as indicated by our previous work11. The nozzle pathway should avoid repetitive movements and excess depositions of the hydrogel ink so that the resolution of the 3D print is preserved.

The porosity obtained in the 3D printed hydrogel scaffolds is in the acceptable range as compared to the targeted porosity (**Table 1**). An exact match cannot be expected because of the swelling nature of the hydrogel ink. The consistency of the hydrogel ink is an important factor especially when *ex-situ* crosslinking has to be done, i.e. crosslinking after the printing of the 3D construct. It was noted that the hydrogel ink was concentrated enough (solid content of 9.9 wt%) to maintain its shape, structure and dimensions during and after the printing process.

The pore size of the scaffold plays an essential role in cell interactions, oxygen diffusion and waste removal together with its mechanical properties to perform and support the desired functionality. Scaffolds with gradient porosity have the ability to better represent the actual in vivo conditions where cells are exposed to layers of different tissues with varying structural properties22,23,34. The porosity and mechanical properties are inversely related but the composition of the hydrogel scaffold can play an important role. CNCs has been selected as the main ingredient of the hydrogel ink because of its well-known mechanical properties2,35,36. The hydrogel ink fabricated here, possess its elasticity even in the presences of the pores, has an optimal pore size (110-1100 µm) and a suitable compressive modulus (0.20-0.45 MPa) required for cartilage regeneration applications.

Compression testing was done in water and at body temperature to mimic the in vivo conditions as much as possible. There was no drying step involved between 3D printing and mechanical testing. In natural tissues, a porosity gradient is observed rather than one uniform pore size. The same is true for compression values for load bearing natural tissues, as the compressive modulus depends on the age, sex and on the tested location.

The advantage with the study presented here is that the final porosity and compressive modulus values of 3D porous scaffold can be controlled and customized through hydrogel ink composition and 3D printing process. This protocol is flexible and can be modified according to the specific requirements. The 3D printing is a powerful technique and can be explored in future to develop scaffolds with complex structural and compositional features. Multi material dispensing can introduce revolution by controlling the composition of the scaffolds, concentration of cells or growth factors, structural features such as directionality or porosity, mechanical properties and degradation rate in different parts of the 3D constructs.

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**DISCLOSURES**

The authors have nothing to disclose.

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