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High-resolution 3D printing of xanthan gum/nanocellulose bio-inks

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ABSTRACT

The current study provides a comprehensive rheology study and a survey on direct ink writing of xanthan gum/nanocellulose nanocrystal (XG/CNC) bio-inks for developing 3D geometries that mimic soft tissue engineering scaffolds’ physical and mechanical properties. The presence of CNC was found to be a critical prerequisite for the printability of XG bio-inks; accordingly, the hybrid XG/CNC bio-inks revealed the excellent viscoelastic properties that enabled precise control of hydrogel shaping and printing of lattice structures composed of up to eleven layers with high fidelity and fair resolution without any deformation after printing. The lyophilized 3D scaffolds presented a porous structure with open and interconnected pores and a porosity higher than 70%, vital features for tissue engineering scaffolds. Moreover, they showed a relatively high swelling of approximately 11 g/g, facilitating oxygen and nutrient exchange. Furthermore, the elastic and compressive moduli of the scaffolds that enhanced significantly upon increasing CNC content were in the range of a few kPa, similar to soft tissues. Finally, no significant cell cytotoxicity was observed against human liver cancer cells (HepG2), highlighting the potential of these developed 3D printed scaffolds for soft tissue engineering applications.

1. Introduction

Tissue engineering, which combines material and life science with engineering, has provided a viable therapeutic solution to repair tissue functions by utilizing scaffolds, cells, and bioactive molecules. An ideal tissue engineering scaffold should possess some requirements, including high porosity with suitable pore size, mechanical stability like the target tissue, controllable geometry, and adjustable biodegradability during tissue regeneration. Furthermore, it should facilitate cell attachment, migration, proliferation, and in some cases, cell differentiation [1,2].

Although various techniques such as solvent casting, solute leaching, phase inversion, freeze-casting, and electrospinning have been used to develop 3D scaffolds, they face limitations in architectural design and development time. Furthermore, they are restricted to incorporating fine internal architectural details and controlled porosity [3,4]. Printing is a revolutionary method that holds promise for the fabrication of 3D scaffolds with highly complex geometries and precisely adjusted pore sizes uniformly distributed throughout the objects [5–7]. So far, different printing techniques, including Inkjet printing, laser-based printing, extrusion-based printing, and direct ink writing (DIW)-based printing, have been used to print tissue engineering 3D scaffolds [8]. The latter, direct ink writing (DIW), has attracted significant attention as a versatile method for 3D printing hierarchical geometries with different thicknesses by way of layer-by-layer deposition of an ink through a fine nozzle [9]. It has also demonstrated considerable interest in biomedical applications since it enables 3D printing of cells/biomaterials at ambient temperature. Accordingly, different tissue engineering 3D scaffolds have been developed using viscoelastic materials such as hydrogels, colloidal suspensions, gels, or pastes as an ink [10–13].

Hydrogels, natural 3D structures with high water retention capacity, are considered attractive candidates in biomedical applications due to their similarity to the extracellular matrix of native tissues and tailored mechanical properties mimicking a wide range of soft tissues. In some cases, high interaction with cells enables hydrogels to support cell attachment, proliferation, and differentiation [1,2,5]. Furthermore, the tunable rheological properties of hydrogels make them an attractive candidate for DIW. However, developing a bio-ink with good printability to create complex geometries is challenging [8,14]. Ink rheology

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is one of the essential features for the successful printing of objects. The ink must behave as a shear-thinning fluid and exhibit a viscoelastic response to the applied pressure to extrude from a nozzle and fabricate a 3D object after gel deposition. In other words, the shear-thinning behavior of the ink grants that the needle does not clog during deposition, while viscoelastic performance enables the printed object to maintain its structural integrity after extrusion from the nozzle [15–18]. So far, different natural and synthetic hydrogels such as gelatin [8], gelatin methacryloyl [19], sodium alginate [20], silk [21], nanocellulose [22], polycaprolactone [23], and polyvinyl alcohol [10] have been used to develop tissue engineering 3D scaffolds by DIW technique. Nonetheless, natural ones tend to have more outstanding inherent biocompatibility with cells and host tissues. Furthermore, they can promote cell adhesion and growth, while synthetic hydrogels lack bioactivity and may produce acidic by-products during degradation that can cause tissue necrosis [24–26].

Xanthan gum (XG) is an extracellular polymer produced from Xanthomonas campestris bacteria through submerged aerobic fermentation. This most important commercial microbial polysaccharide consists of the α-glucopyranose unit as a backbone, like cellulose, with a side chain of D-mannose and D-glucuronic acid. It has been widely used in various fields such as food (as a stabilizer and thickening agent), food packaging, waste-water treatment, agriculture, petroleum (e.g., oil drilling operation), pharmaceutical, cosmetic, advanced drug delivery, protein delivery, and tissue engineering. This broad and diverse range of applications is due to the extraordinary properties of XG, including outstanding physicochemical properties, stability under a wide range of temperatures and pH levels, high viscosity at low concentration, high degree of pseudoplastic behavior, biodegradability, and non-toxicity. Moreover, XG is an FDA-approved material regarding its biocompatible, bio-adhesive, and wound healing properties, allowing its biomedical and pharmaceutical application [27–30].

Thanks to its excellent versatility, rheology, and structure, xanthan gum has been used widely for food 3D printing [31–33]. Furthermore, recently, it has been considered a promising material in printing biomedical devices. In one recent work, Yang et al. [34] developed a 3D printed porous antibacterial dressing composed of XG, gelatin methacrylate, and titanium dioxide, illustrating an ideal swelling and excellent water uptake efficiency. Similarly, Moura et al. [35] 3D printed wound patches composed of XG/sodium alginate bio-inks illustrated significant cell compatibility against human Dermal Fibroblast cells over 14 days. Moreover, 3D printing of methacrylated xanthan gum and its compatibility against L929 mouse fibroblasts has been reported by Patricio et al. [36]. Shapira et al. [37] outlined high-resolution 3D printing of complex constructs and ECM structures composed of xanthan gum/calcium-alginate nanoparticles with excellent biocompatibility, stability at a wide range of temperatures, and high transparency, making them promising support mediums for 3D printing tissues and organs. Despite such favorable properties, similar to most bio-based hydrogels, to its own, XG has poor mechanical strength, possessing some limitations in specific applications, such as tissue engineering scaffolds [27]; therefore, it has been mostly blended with other polymers/particles. Accordingly, in the current study, we developed a bio-ink composed of XG and cellulose nanocrystals (CNC). Our results revealed that the printability of the XG improved significantly with the addition of CNC, and the swelling behavior and physical and mechanical properties influenced positively, whereby fitting the requirements as scaffolds for soft tissue engineering. Furthermore, the scaffolds supported the attachment and proliferation of human liver cancer cells (HepG2), a positive feature for the claimed application.

2. Materials and methods

2.1. Materials

Xanthan gum from Xanthomonas campestris and phosphate buffer solution (PBS, pH = 7.4) was purchased from Sigma-Aldrich. Cellulose nanocrystalline freeze-dried form was purchased from the University of Maine. WST-1, a cell proliferation reagent, was purchased from Roche Diagnostics. Ethanol Etax B was obtained from ALTIA Industrial (Finland). The ultrapure (Type 1) water was used as a solvent.

2.2. 3D printing

Xanthan gum (XG) was dissolved in water at room temperature under mild mechanical stirring overnight to prepare an 8 wt% gel. Cellulose nanocrystal (CNC) was dispersed in water by mechanical mixing and sonication to prepare an 8 wt% suspension. To obtain the uniform inks, different weight ratios of XG and CNC were mixed and mechanically stirred for 1 h. The mixture was homogenized more using an Ultra Turrax (IKA T25) digital homogenizer at 18000 rpm in an ice bath to enhance CNC dispersion within the XG inks and avoid clogging the nozzle while printing. Prior to 3D printing, the final ink was centrifuged at 2000 rpm at room temperature to remove the air bubbles. The weight ratios of XG and CNC were selected as 70/30, 60/40, and 50/50, and the samples were designated as XG70, XG60, and XG50, respectively.

Printing was done by a BIOX (CELLINK, Sweden) printer at room temperature using a 3 mL clear pneumatic syringe equipped with a 250 μm (G25) plastic nozzle. The pneumatic pressure was varied between 70 and 90 kPa, while the printing speed was fixed at 20 mm·s⁻¹ for all the inks. To investigate the multilayered geometries, rectangular 3D lattice structures (with a cross-section of 30 mm × 30 mm) and disk-shaped 3D geometry (with a diameter of 25 mm) were printed in 9 layers. The printing was performed with 15% and 20% infill density. Moreover, a hollow cylinder with a diameter of 10 mm composed of 30 layers was printed to illustrate the ink stability. Disk-shaped geometries with an infill density of 50% were printed for the characterizations. The printed scaffolds were dried under two different conditions, freeze-drying and air-drying. After that, they were crosslinked thermally by heating at 165 °C for 7 min [38].

2.3. Characterization

2.3.1. Rheology

The apparent viscosity of the inks was determined using a parallel-plate rheometer (Anton Paar MCR 301, Austria) under an oscillatory shear rate at 23 °C. The shear rate was first increased logarithmically from 0.01 to 1000 s⁻¹ using PP25 geometry; then, it was decreased from 1000 to 0.01 s⁻¹ with the same conditions. This loop was selected to simulate the shear history of the ink undergoing extrusion, whereby the increasing shear rate causes the ink to overcome the yield stress and begin to flow, while the decreasing one allows the ink to relax until it stops flowing [13,15]. A Power-law model (Eq. 1) was adjusted to the experimental data of the increasing ramp, in which η is the viscosity of the ink (Pa·s), K is the consistency coefficient, γ is the shear rate (s⁻¹), and n is the power-law or flow index [39,40].

\[
\eta = K\gamma^{n-1}
\]

(1)

The zero-shear rate viscosity, the viscosity of the ink after extrusion, was extracted from the descending ramp, where the shear rate was close to zero. On the other hand, the ink viscosity at printing conditions was obtained from the ascending ramp due to the method reported by smith et al. [15]. First, the applied shear rate while printing was calculated using Eq. 2, where n is the power-law index, V is the print speed (20 mm·s⁻¹), and D is the nozzle diameter (250 μm, G 25). It was then used to calculate the ink viscosity at printing conditions.

\[
\gamma = \frac{(3n + 1) \times 2V}{4nD}
\]

(2)

The static (τₛ) and dynamic (τ_d) yield stresses were extracted from the
flow curves drawn from the shear rate sweep test data. \( \tau_{y,s} \) was extracted from the change in the slope of the stress during the initial increase in shear rate, while \( \tau_{y,s} \) was measured from the y-intercept \((\approx 0 \text{ s}^{-1})\) of the same plot under decreasing rate \([15]\). \( \tau_{y,s} \) was also obtained from the stress-sweep test curves, where the loss modulus \((G')\) intersected the storage modulus \((G')\) \([41,42]\). The yield stress was then compared with the maximum applied shear stress \((\tau_{\text{max}})\) during printing to investigate the flowability of the ink. \( \tau_{\text{max}} \) was calculated using Eq. 3, where \( \Delta P \) is pneumatic pressure during printing \((70-90 \text{ kPa})\) and \( r \) and \( L \) are the needle radius \((0.265 \text{ mm})\) and length \((31.75 \text{ mm})\), respectively.

\[
\tau_{\text{max}} = \frac{\Delta P \cdot r}{2L}
\]  

A frequency sweep test was used to investigate the ink stability between 0.1 and 100 rad s\(^{-1}\) at a linear viscoelastic region, identified by a strain sweep test between the deformation of 0.01 to 100% at a fixed frequency of 10 rad s\(^{-1}\). The tests were performed using a PP25 geometry at 23 °C. The printed scaffolds were examined under the strain and frequency sweep tests under the same conditions, except that the scaffolds were first immersed in a PBS solution for 24 h and then subjected to tests at 37 °C. The \( G' \) values were then used to calculate the elastic modulus of the scaffolds \((E)\) by Eq. 4, where \( \nu \), the Poisson ratio, was considered as 0.5, similar to that of rubber-like materials \([43]\).

\[
E = (1 + 2\nu)G'
\]  

2.3.2. Fourier transform microscopy

The mechanism of crosslinking and the chemical structure of the scaffolds were studied with Fourier transform microscopy (FTIR) using PerkinElmer FTIR with an ATR instrument. The spectra were recorded between 4000 and 500 cm\(^{-1}\) with a scan rate of 16 and a resolution of 4 cm\(^{-1}\).

2.3.3. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was conducted to confirm the presence of the components in the hydride samples by investigating the thermal degradation behavior of the pure and composite samples. It was done by a TA Instruments model Q500 with an increasing temperature ramp from 30 to 700 °C under a nitrogen atmosphere.

2.3.4. Scanning electron microscopy

The microporosity of the printed scaffolds was observed using scanning electron microscopy (SEM). The samples were first sputtered with a thin layer \((\sim 4 \text{ nm})\) of gold-palladium and then subjected to imaging with a Zeiss Sigma VP microscope. The imaging was performed from the surface and cryo-fracture surface area of the samples. The average pore size and pore size distribution were reported by measuring the size of at least 90 pores using ImageJ software.

2.3.5. Porosity

The porosity of the printed scaffolds \((\Phi)\) was calculated by measuring the weight of adsorbed ethanol. A dried sample weighing \( m_0 \) and volume of \( V \) was immersed in ethanol. The porosity was calculated by Eq. 5, where \( m_1 \) is the sample's saturated weight, and \( \rho \) is the density of ethanol \((0.789 \text{ g mL}^{-1})\).

\[
\Phi(\%) = \frac{m_1 - m_0}{\rho \times V}
\]  

2.3.6. Swelling and gel release

The water uptake capacity of the printed scaffolds was studied by monitoring the weight gain of a dried sample \((m_d)\) in a PBS solution \((\text{pH} = 7.4)\) within 7 days. The swelling \((S)\) was calculated with Eq. 6, where \( m_w \) is the weight of the wet scaffold at time \( t \). \( m_w \) was measured after 30 min and 1 h, and then every day for 7 days. It was performed on freeze-dried and air-dried scaffolds.

\[
S(g/g) = \frac{m_w - m_d}{m_d}
\]  

The gel release (GR) was calculated 7 days after immersion in a PBS solution using Eq. 7, where \( m_d \) is the weight of the dried scaffold and \( m_f \) is the weight of the dried scaffold after 7 days of immersing in the PBS solution.

\[
\text{GR(\%)} = \frac{m_d - m_f}{m_d}
\]  

2.3.7. Shrinkage

The printed scaffolds' shrinkage was measured by comparing the apparent volume of the sample before and after drying, i.e., freeze- and air-drying.

2.3.8. Compression test

The compressive stress-strain curves were drawn with the data obtained from a compression test performed under a controlled force condition. The test was conducted with a TA Instruments model DMA Q800 with a preload of 0.001 N and a compression force rate of 0.2 N min\(^{-1}\) at 37 °C. All the scaffolds were first immersed in a PBS solution for 24 h and then compressed up to 18 N. The slope of the stress-strain curve in the elastic region was reported as sample compressive modulus. Furthermore, the compressive strength at 30% strain was extracted and compared for different scaffolds.

2.3.9. Cell compatibility

Cell compatibility of the printed scaffolds was assessed by liver hepatocellular carcinoma cells \((\text{HepG2 cell line})\). HepG2 cells were cultured according to ATCC protocol \((\text{HB-8065 American Type Culture Collection})\). They were cultured in a mixture of Dulbecco's modified Eagle's medium \((\text{DMEM, Gibco, 41966029})\) and 10% fetal bovine serum. The water uptake capacity of the printed scaffolds was studied by monitoring the weight gain of a dried sample \((m_d)\) in a PBS solution \((\text{pH} = 7.4)\) within 7 days. The swelling \((S)\) was calculated with Eq. 6, where \( m_w \) is the weight of the wet scaffold at time \( t \). \( m_w \) was measured after 30 min and 1 h, and then every day for 7 days. It was performed on freeze-dried and air-dried scaffolds.

\[
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\]  

The gel release (GR) was calculated 7 days after immersion in a PBS solution using Eq. 7, where \( m_d \) is the weight of the dried scaffold and \( m_f \) is the weight of the dried scaffold after 7 days of immersing in the PBS solution.

\[
\text{GR(\%)} = \frac{m_d - m_f}{m_d}
\]  

In vitro cytotoxicity and proliferation tests were performed following standard specifications \((10993-5 \text{ Biological Evaluation of Medical Devices - Part 1, 2009})\) \([44]\) by colorimetric assay using WST-1 reagent kit \((\text{Roche, Mannheim, Germany, 11644807001})\). One milliliter of HepG2 cells with a density of 50000 cells-\text{mL}^{-1} was seeded on scaffolds and cultured for 1, 3, and 7 days. 100 \( \mu \text{L} \) of WST-1 reagent were added to each scaffold with 1 mL of cell medium. After 30 min of incubation, the solution was transferred into a new 96-well plate, and the optical density was read at 450 nm using a Synergy H1 multimode microplate reader \((\text{BioTek, Bad Friedrichshall, Germany})\). Active cells react with the WTS-1 reagent and form a formazan dye whose color intensity determines the cell viability of the scaffolds. All experiments were carried out in triplicate.

3. Results and discussion

3.1. Direct ink writing 3D printing

The ink's rheological properties are the most critical parameters determining successful processing in DIW. Although a shear-thinning viscosity profile is the main requirement for DIW, a rapid elastic recovery, well-defined yield stress, and high enough elastic modulus are other substantial parameters directing successful extrusion from a nozzle with no distortion of single printed filaments, which finally leads to having a 3D object with high shape fidelity \([12,15,41]\). Therefore, different rheological properties of the developed inks, including viscosity, viscosity recovery, flowability, and strength, were investigated via oscillatory tests. Fig. 1a to Fig. 1c present the inks' viscosity and their recovery as a function of shear rate at 23 °C. All inks revealed a
systematic decrease over shear rate increasing, where the viscosity dropped approximately 4 orders of magnitude at 1000 s⁻¹ shear rate, indicating the ink destruction under shear [13]. Nevertheless, the inks recovered their initial high viscosity after removing the applied shear rate, i.e., after extrusion, allowing the material to maintain a high shape fidelity [45]. The experimental flow viscosity values in increasing ramp were fitted to a power-law model using Eq. 1. The consistency coefficient (K) and power-law index (n), as well as the coefficient of determination (R²) values, are summarized in Table 1. The relevant values for plain XG are provided in Fig. S1. All the inks had relatively high R² values, revealing the strong correlation between the experimental data and the power-law model [46,47]. Furthermore, the power-law index values were varied between 0.201 and 0.175, indicating non-Newtonian behavior with a remarkable shear-

![Fig. 1. Rheological properties of the inks at 23 °C.](image)

### Table 1

| Ink   | Viscosity (Pa·s) | n   | K        | R²  | j² | Viscosity'' (Pa·s) | τy,s (Pa) | τy,d (Pa) | τy,d'' (Pa) | τmax (°) |
|-------|------------------|-----|----------|-----|----|-------------------|-----------|-----------|-------------|----------|
| XG70  | 2345             | 0.193 | 1.07 × 10⁵ | 0.928 | 151 | 1.52               | 240       | 150       | 188         | 290      |
| XG60  | 3232             | 0.186 | 1.48 × 10⁵ | 0.981 | 158 | 2.29               | 280       | 200       | 214         | 375      |
| XG50  | 3650             | 0.175 | 1.21 × 10⁵ | 0.947 | 164 | 2.49               | 290       | 210       | 282         | 375      |

¹ Zero-shear rate viscosity was obtained from rate decreasing curves.
² At printing conditions.
³ Obtained from rate increasing curve.
⁴ Determined from flow curves.
⁵ Determined from stress-sweep curves.
⁶ Determined by Eq. 3.
thinning behavior. The reduction in the power-law index upon increasing CNC content suggests the positive effect of CNC addition on the shear-thinning behavior in hybrid inks [39].

The applied shear rate at the printing conditions, calculated by Eq. 2, is provided in Table 1. These values were then employed to extract the inks' viscosity. As Table 1 shows, all inks revealed relatively low viscosity allowing them to flow easily through the single nozzle tip [9]. On the other side, all inks had relatively high zero-shear rate viscosity (Table 1), proposing a fast recovery of the initial high viscosity. This behavior hinders droplet formation caused by the surface-tension-driven droplet formation and the collapse of the printed structure. Furthermore, it can guarantee that the printed layer partially solidifies and supports the subsequent printed layers, causing higher shape fidelity [9,12].

Another parameter determining ink suitability for DIW is yield stress. Two different yield stresses should be considered; the static yield stress ($\tau_{y,s}$), the stress required to make a flow from rest, and the dynamic yield stress ($\tau_{y,d}$), the minimum stress required for a fluid in motion to keep its flow. On the one hand, the ink needs to overcome the static yield stress to initiate extrusion from a nozzle; on the other hand, it needs to present high enough dynamic yield stress to prevail over the gravity caused by the whole printed structure and the capillary forces. The latter promises the continuous extrusion of filament with minimum deformation and enables the ink to preserve its shape after printing and support the following layers [13,15]. Accordingly, $\tau_{y,s}$ and $\tau_{y,d}$ were determined from shear stress-shear rate curves (Fig. 1d to Fig. 1f), $\tau_{y,s}$ was also extracted from stress sweep curves (Fig. 1g). The results are provided in Table 1. All samples presented yield stress at a low shear rate, followed by ink flow at higher shear values. Although there were differences between the yield stress values calculated from flow curves and stress sweep curves, in all inks $\tau_{y,s}$ was considerably lower than $\tau_{\text{max}}$, maximum shear stress applied during printing, indicating that the ink flows through the deposition nozzle like a liquid [11,41,48]. On the other hand, $\tau_{y,d}$ was varied between 150 and 210 kPa, significantly higher than 50 Pa reported as a threshold under which DIW is not possible [13]. The previously observed high dynamic shear rate and high viscosity at low shear rates suggest that the developed inks can provide shape fidelity and structural stability for additive manufacturing [49].

It should be noted that the $\tau_{y,d}$ of plain XG ink was around 80 Pa.

![Fig. 2. Photograph of 3D printed scaffolds: (a) and (d) XG70, (b) and (e) XG60, (c), and (f) XG 50 before and after freeze-drying (with an infill density of 15%). (g) G50 with an infill density of 20%, (h) and (i) XG50 after freeze-drying and air-drying, respectively. (j) extruded filament, (k) side view of XG50, and (l) a hollow cylinder printed by XG50 bio-ink.](image-url)
and G′ was always higher than G″, revealing that the inks behaved like a solid or gel material under low shear strain [49]. Furthermore, both G′ and G″ increased upon increasing CNC content, indicating the ink strength enhancement established by hydrogen bonds forming between the hydroxyl groups of CNC and hydroxyl and carboxyl groups of XG.

The tendency for nozzle clogging, shape fidelity, and integrity are serious challenges during hydrogel printing, which can significantly influence the overall performance of 3D objects [52,53]. Fig. 2 illustrates the photograph of printed scaffolds with 15 and 20% infill density before and after drying (freeze-drying and air-drying). Although the pure XG scaffold collapsed a few seconds after printing (Fig. S2b), all hybrid inks presented the same printability, shape fidelity, and structural stability. No clogging of dispensing nozzle and no ink droplets, as well as no inappropriate structures with filaments merging or collapsing, caused by gravity during or after printing, were observed [49,52]. Moreover, no evidence of filaments broadening (Fig. 2j and Fig. S3), caused by gravity and the weight of the higher layers, was observed, suggesting a dense polymer network and entanglements of polymer chains [6,45]. Furthermore, good shape accuracy was observed from the top (Fig. 2a–2c) and side (Fig. 2k) views of the printed scaffolds. A high multilayer structure was printed to demonstrate shape stability and fidelity in the z-direction (Fig. 2l). The formulated ink (XG50) led to the formation of a hollow cylinder with up to 30 layers of height and a multilayer structure was printed to demonstrate shape stability and fidelity in the z-direction. The interconnected and porous structure is a fundamental issue in scaffold design, enabling enough space for cell compatibility and growth [54].

The porosity and water uptake capacity of the scaffolds, which are critical for cell survival, are considerably influenced by the shrinkage of the hydrogel; a larger volumetric changes a lower porosity and water uptake capacity [16,55]. Hydrogels are usually subjected to relatively high dimensional changes, i.e., shrinkage, after drying, owing to a large amount of preserved water in their network. Fortunately, freeze-drying as a straightforward method for drying the hydrogels can significantly eliminate the dimensional changes by the sublimation of ice crystals during lyophilization [10]. Accordingly, no structural distortion with a minimal dimensional shrinkage of less than 3% was observed after freeze-drying 3D printed scaffolds (Fig. 2d to 2f). In contrast, the air-dried ones collapsed in the z-direction (Fig. 2i) with a high shrinkage of more than 90% (Table 2). It is worth noticing that the relatively high-volume changes did not cause any wrinkles on the surface of the scaffold, contrary to what was previously reported in the literature [6], suggesting a robust polymer network formation due to the entanglement of CNC chains and intermolecular hydrogen bonding between XG molecules and CNC chains. The scaffolds’ shrinkage slightly decreased upon the increasing of CNC portion in both air- and freeze-dried samples, which could be explained by the improved mechanical strength of scaffolds. A similar result has been reported by Fourmann et al. [56], where nanocelluloses constrained the shrinkage of the poly(N-isopropylacrylamide) structures in the direction of reinforcement.

### 3.2. Chemical structure and composition

FTIR spectra were recorded to compare the chemical structure of the printed scaffolds with the plain XG and CNC and to investigate the crosslinking mechanism during the heating of the sample (Fig. 3 and S4). The characteristic peaks of XG powder were in line with those reported in the literature [28]; -OH group peak at 3300 cm⁻¹, C–H group peak (present in CH₂, CH₃, and CHO) at 2900 cm⁻¹, C=O group peaks (present in ester, aldehyde, ketone, and acid) at 1730 cm⁻¹ and 1630 cm⁻¹, C–H group peak at 1410 cm⁻¹, and C–O group peak at 1040 cm⁻¹. Similarly, CNC presented cellulose characteristic peaks as follows: -OH and -CH groups peaks at 3350 cm⁻¹ and 2890 cm⁻¹, -OH group peak of the adsorbed water at 1645 cm⁻¹, H–C–H, O–C–H, and C–H groups peaks at 1430 cm⁻¹ and 1375 cm⁻¹, and the half-ester sulfate group peak, generated during acid hydrolysis reaction, at 1160 cm⁻¹ [57]. The previous peaks were repeated in the scaffolds, revealing the presence of XG and CNC in them. Fig. 3a represents the FTIR spectra of the scaffolds after crosslinking. For comparison, the XG70 scaffold before crosslinking is also included in this figure. All the characteristic peaks were repeated in the printed scaffolds after crosslinking. Nevertheless, the acid/ester areas ratio decreased (dashed line rectangle), confirming that the acid converted to the ester overheating at 165 °C [38].

Since each XG and CNC had its own thermal degradation behavior, the TGA thermograms further investigated each component’s presence in the printed scaffold. Fig. 3b illustrates the thermograms of plain XG and CNC as well as the printed scaffolds. Three degradation stages were differentiated in the XG thermogram; an initial weight loss of approximately 13% below 200 °C, attributed to the release of absorbed moisture bonded to the saccharide structure and volatile matter, followed by a two-step weight loss between 200 and 700 °C due to main chain degradation [58,59]. On the other side, CNC revealed a typical polysaccharide thermal degradation; a minor weight loss of approximately 3% below 200 °C, followed by a major one between 200 and 700 °C, corresponding to the depolymerization of cellulose chains and break of glycosidic bonds [60]. All scaffolds presented a combination of the heat degradation behavior of the plain XG and CNC; neither showed the relatively pronounced weight loss of XG before 200 °C nor just the two-step degradation of CNC, suggesting the presence of each component in the printed scaffolds.

### 3.3. Microstructure and porosity

The interconnected and porous structure is a fundamental issue in scaffold design, enabling enough space for cell compatibility and growth.
and oxygen and nutrient diffusion. Moreover, it facilitates the spreading of signal transduction and regulation [61,62]. Hence, the surface morphology and porous structure of freeze-dried printed scaffolds were examined using SEM imaging (Fig. 4). Although the surface of all the samples showed a network structure, the cross-section images revealed a microporous structure, with smooth surfaces and highly interconnected open pores with an average pore size ranging from 60 to 90 μm. Accordingly, they revealed a relatively high porosity of more than 75% (Table 2). The XG70 and XG60 scaffolds partially collapsed during freeze-drying, which was eliminated in the XG50 scaffold. An increase in

Fig. 3. a) FTIR spectra of the printed scaffolds after crosslinking, b) TGA thermograms of the plain XG, CNC, and printed scaffolds after crosslinking.

Fig. 4. SEM images (magnification = 60×) and pore size distribution of the printed scaffolds.
the CNC content increased the physical crosslinking density, i.e., the hydrogen bonding force between the polymer chains and the rigid nanofiller, restricted the chain backbone’s motion, and thereby formed a strong and dense complex composite structure that did not collapse during freeze-drying. It furthermore changed the surface of the scaffold to a denser one and decreased the porosity and average pore size, similar to that reported for the CNC-reinforced hydrogels [63–65]. Overall, the observed porous structure, porosity, and average pore size resembled the majority of scaffolds for tissue engineering [34,66,67], proposing the high potential of the developed 3D objects as tissue-engineered scaffolds. It is worth notifying that no apparent agglomeration of CNC and any phase separation were observed, suggesting a homogeneous distribution of the CNC in the XG matrix and good compatibility between components.

3.4. Swelling behavior and gel release

Water uptake capacity is one of the essential characteristics of the scaffolds for tissue engineering applications, enabling the structures to have a high uptake of culture media and thereby supply nutrients to the cells, enhancing cells’ viability and proliferation [28]. The swelling (S) of the freeze-dried and air-dried scaffolds is illustrated in Fig. 5. Furthermore, the S of the printed scaffolds after 7 days is summarized in Table 2. All freeze-dried samples had a very high swelling ratio above 8 g/g in the first 30 min of the test, which gradually increased over time. The observed S was in good agreement with that previously reported for XG-based hydrogels [68,69]. The slightly reduced upon increasing the CNC content, which could be explained by the increase in crosslinking points obtained from hydrogen bonding between XG chains and CNC, limiting the mobility of the hydrogel network chains and restricting the penetration of water molecules. The reduced pore size and forming a denser network structure with more compact porosity upon the addition of CNC, as previously observed in the SEM images, could be another piece of evidence for this behavior [28,58,64]. A similar profile was observed for the air-dried scaffolds; however, the SR was considerably higher than the freeze-dried ones (more than 30 g/g). Accordingly, significant dimensional changes were observed for air-dried scaffolds (Fig. 5c). Although both air-dried and freeze-dried scaffolds were crosslinked thermally at 165 °C, the latter revealed more stability. It has been reported that ice crystals are formed inside the sample upon lyophilization, while the polymer chains are concentrated in the unfrozen liquid domains and interact with each other and consequently form a robust physical network structure [10], thereby leading to lower swelling. The benefit of crosslinking was more investigated by measuring the scaffolds gel release (GR) 7 days after immersion in the PBS at 37 °C. The results are presented in Table 2. Although both XG and CNC are hydrophilic polymers that easily dissolve/disperse in water, the freeze-dried and air-dried scaffolds revealed relatively low gel release of less than 10% after, proving the successful thermally crosslinking of the components, as well as the efficient hydrogen bonding between hydroxyl and carboxyl functionalities.

3.5. Mechanical properties

The compressive stress-strain curves and the compressive modulus and compressive strength at 30% strain are illustrated in Fig. 6. The compressive stress gradually increased at the beginning of the deformation in all scaffolds, while they presented a significantly higher strength against deformation at a higher compressive rate. The former was due to the release of the massive amount of water trapped in the scaffolds’ porous structures, while the latter was attributed to the XG and CNC structural resistance against deformation [11]. Upon increasing the CNC content, compressive modulus and compressive strength at 30% strain improved significantly, approximately 200% and 500%, respectively, in the XG50 scaffold, for instance, which is quite comparable to the typical polysaccharides-reinforcing strategy [70]. This improvement, which suggests the formation of more robust network structures, could be attributed to the proper interaction between CNC and XG polymer chains through hydrogen bonding between hydroxy functionalities of CNC with the hydroxyl and carboxyl functionalities of the XG

![Fig. 5. a and b) Swelling behavior of the freeze-dried and air-dried scaffolds. c) Photograph of freeze-dried and air-dried scaffolds seven days after immersion in PBS solution at 37 °C.](image-url)
[71] A similar trend has been reported for the polyethylene glycol-grafted chitin (PEG-g-ChNCs) reinforced polyvinyl alcohol (PVA) hydrogel, where the interfacial interactions between PVA chains and PEG-g-ChNCs via forming hydrogen bonds played a key role in enhancing the mechanical properties of the developed hydrogels [72]. The higher modulus of CNC rather than the XG could be another reason for increasing the compressive modulus of the scaffolds upon increasing the CNC content [73,74]. The observed mechanical properties of the developed scaffolds, in line with those reported for XG-based hydrogels [75], were similar to soft tissues [6], making them suitable scaffolding materials for soft tissue tissues. It is worth notifying that all scaffolds deformed significantly in the lateral direction to preserve the volume (Poisson’s ratio \( \approx 0.5 \)); however, they still maintained the regular structure without any visible damage and rupture, indicating the good structural integrity and mechanical stability of the FIM scaffold [76,77].

The mechanical stability of the printed scaffolds was further studied by the rheological measurements. Fig. 6c presents the results of the shear strain sweep test. \( G' \) and \( G'' \) revealed an independent trend in all scaffolds at small deformation, known as linear viscoelastic behavior, followed by a non-linear viscoelastic behavior, where the magnitude of the deformation influenced the moduli due to the internal structure disruption [51]. It is amply clear that the linear viscoelastic regime increased with increased CNC concentration, which is highly demanded in diverse applications [11]. Overall, a shear strain of 1% was considered a safe value, which guaranteed that the angular frequency sweep measurements were in the linear viscoelastic region, Fig. 6d. All the scaffolds exhibited similar viscoelastic behavior within the entire frequency range of 0.1–100 rad s\(^{-1}\). Namely, both the moduli were parallel and independent of the frequency in the whole frequency range. Moreover, \( G' \) was always significantly higher than \( G'' \), indicating that the elastic properties of the scaffolds prevailed over their viscous properties, and they behaved as robust gel-like or solid-like materials under the test conditions [75,78,79]. Furthermore, the \( G' \) and, accordingly, elastic modulus (Eq. 4) enhanced upon increasing the CNC portion, from 110 kPa in XG70 to 190 kPa in XG50 (Fig. 6b), suggesting the enhancement of mechanical strength and elastic properties resulting from the synergistic reinforcement effect of CNC and dynamic cross-linking bonds formed in the hydrogel network [80]. On the other hand, both \( G' \) and \( G'' \) were considerably higher than those previously observed for the inks (Fig. 1h and i), proposing the successful crosslinking of the inks by heating as well as improving the structural properties after freeze-drying.

3.6. Cell compatibility

The cell compatibility of xanthan gum hydrogel and cellulose nanocrystals against different cell types has been previously investigated and proved [28,63,81–83]. However, a preliminary test was performed to evaluate the in vitro cytocompatibility of the hybrid XG/CNC scaffolds against the hepatocellular carcinoma cell line (HepG2). HepG2 cells were selected due to their high toxicity sensitivity, proving to be a good tool for biocompatibility assessment [16]. The cytotoxicity of the freeze-dried printed scaffolds assessed from WST-1 after 1 day is shown in Fig. 7a. After 24 h, all printed scaffolds displayed similar cell viability levels, indicating similar metabolic activity levels [28]. Furthermore, the cell viability level was higher than 90% in all samples, slightly higher than that observed for the control group, indicating no detectable cytotoxicity and, consequently, good cell biocompatibility [82,84]. The higher cell viability level in the printed scaffolds than the control group could be due to the porous interconnected network structures promoting cellular activity and allowing better cell proliferation [84]. Fig. 7b displays the optical density of the scaffolds at
440 nm after 1, 3, and 7 days of incubation by colorimetric analysis. High absorbance values indicate increased production of the formazan dye product resulting from the reaction of WST-1 with the mitochondrial succinate-tetrazolium reductase. Therefore, elevated optical density values can be directly correlated to cell viability. All samples exhibited significantly enhanced optical density between day 1 and day 7 (p-value < 0.05), demonstrating increased metabolic activity attributed to the cell multiplication and greater cell growth rate with higher living cell numbers. In other words, the printed scaffolds provided a convenient microenvironment for cell attachment, viability, and proliferation and could be considered safe for biomedical applications [85,86]. It is worth noting that upon increasing CNC content, the scaffold provided a less favorable environment for cell proliferation which could be explained by the reduction of swelling and porosity that are essential for cell migration and cell spreading. It could also be attributed to the higher bioactivity of XG against HepG2 cells rather than CNC [87,88].

4. Conclusions

The current paper developed a series of hydrogels composed of xanthan gum and cellulose nanocrystals with excellent potential as bioink for printing 3D objects via the direct ink writing technique. Lattice geometries composed of eleven layers were printed with high resolution, shape fidelity, and structural stability without facing any common challenges in DIW of high-viscosity inks such as nozzle clogging and filament merging or collapsing. The ink stability was further proved by printing a hollow cylinder with a diameter of 10 mm composed of 30 layers. Different properties of the developed scaffolds were evaluated, and it was found that their porosity, swelling ratio, pore size, and mechanical properties were in line with those required for soft tissue engineering applications. Furthermore, it was shown that all the developed scaffolds supported the attachment and proliferation of human liver cancer cells (HepG2), making them more exciting options as scaffolding materials. Overall, the XG50 scaffold had the highest mechanical properties; however, it revealed the lowest swelling ratio, porosity, and cell viability, proposing complementary tests might be needed due to the target application to select the best formulation.

CRediT authorship contribution statement

Hossein Baniasadi designed the research, conducted the experiments, analyzed the results, and wrote the manuscript; Erfan Kimiaei and Roberta Teixeira Polez conducted the experiments; Rubina Ajdary edited the manuscript; Orlando J. Rojas, Monika Osterberg, and Jukka Seppälä supervised and edited the manuscript.

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Appendix A. Supplementary data

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