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Bani Asadi, Hossein; Teixeira Polez, Roberta; Kimiaei, Erfan; Madani, Zahraalsadat; Rojas Gaona, Orlando; Österberg, Monika; Seppälä, Jukka **3D printing and properties of cellulose nanofibrils-reinforced guince seed mucilage bio-inks**

Published in: International Journal of Biological Macromolecules

DOI: 10.1016/j.ijbiomac.2021.10.078

Published: 01/12/2021

Document Version Publisher's PDF, also known as Version of record

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Please cite the original version:

Bani Asadi, H., Teixeira Polez, R., Kimiaei, E., Madani, Z., Rojas Gaona, O., Österberg, M., & Seppälä, J. (2021). 3D printing and properties of cellulose nanofibrils-reinforced quince seed mucilage bio-inks. *International Journal of Biological Macromolecules*, *192*, 1098-1107. https://doi.org/10.1016/j.ijbiomac.2021.10.078

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Contents lists available at ScienceDirect



International Journal of Biological Macromolecules

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



3D printing and properties of cellulose nanofibrils-reinforced quince seed mucilage bio-inks



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ARTICLE INFO

Keywords: Hydrogel 3D printing Quince seed mucilage Cellulose nanofibrils

ABSTRACT

Plant-based hydrogels have attracted great attention in biomedical fields since they are biocompatible and based on natural, sustainable, cost-effective, and widely accessible sources. Here, we introduced new viscoelastic bio-inks composed of quince seed mucilage and cellulose nanofibrils (QSM/CNF) easily extruded into 3D lattice structures through direct ink writing in ambient conditions. The QSM/CNF inks enabled precise control on printing fidelity where CNF endowed objects with shape stability after freeze-drying and with suitable porosity, water uptake capacity, and mechanical strength. The compressive and elastic moduli of samples produced at the highest CNF content were both increased by ~100% (from 5.1 ± 0.2 kPa and 32 ± 1 kPa to 10.7 ± 0.5 and 64 ± 2 kPa, respectively). These values ideally matched those reported for soft tissue; accordingly, the cell compatibility of the printed samples was evaluated against HepG2 cells (human liver cancer). The results confirmed the 3D hydrogels as being non-cytotoxic and suitable to support attachment, survival, and proliferation of the cells. All in all, the newly developed inks allowed sustainable 3D bio-hydrogels fitting the requirements as scaffolds for soft tissue engineering.

1. Introduction

Hydrogels are three-dimensional (3D) networks obtained from crosslinked polymer chains that can absorb and hold a significant volume of water or aqueous solutions. They are intrinsically soft and semi-solid materials with excellent oxygen permeability and high porosity, enabling them as carrier and delivery systems based on hydrophilic therapeutic agents designed to treat diseases. Furthermore, they are biodegradable and biocompatible in some cases, displaying tailorable mechanical properties that can mimic the natural extracellular matrix (ECM) and soft tissues. As such, hydrogels have found many applications in different biomedical fields, including tissue engineering, regenerative medicine, drug delivery, and wound dressing [1-4]. Hydrogels can be obtained from a wide range of natural or synthetic hydrophilic polymers; nevertheless, those derived from natural sources exhibit unique properties, such as biocompatibility, sustainability, and in some cases, biodegradability, that are not easy to be reproduced in synthetic materials [5-7].

As the most abundant biopolymers in nature, polysaccharides are an example of natural polymers with hydrogel-forming ability [8,9]. Plant seeds, like quince seeds, are rich sources of polysaccharides. Among these, quince (Rosaceae family), a yellow fruit similar to apple, original from West Asia and Middle East regions, is traditionally used as a medicine to treat diseases such as sore throat, intestinal colic, constipation, bronchitis, skin burns, and wounds [10]. When soaked in water, quince seeds swell and form an extraordinary hydrocolloid/ hydrogel, known as quince seed mucilage (QSM), due to the abundant hydrophilic functional groups, including hydroxyl (-OH), carboxylic acid (-COOH), and amide (-CONH2) groups. QSM is mostly composed of cellulose, unsaturated fatty acids, amino acids, and polysaccharides such as glucuronoxylan. Furthermore, compared to other commercial hydrocolloids like xanthan gum, gellan gum, locust bean gum, and guar gum, QSM is richer in polysaccharides with relatively higher molecular weight. Moreover, its anti-inflammatory, anti-hemolytic, antioxidant, antimicrobial, anti-infection, anti-ulcerative, and regenerative features are well known [11,12]. Moreover, according to the ISO safety

https://doi.org/10.1016/j.ijbiomac.2021.10.078

Received 25 August 2021; Received in revised form 5 October 2021; Accepted 11 October 2021 Available online 16 October 2021 0141 9120 (© 2021 The Authors, Publiched by Elequier B V. This is an open access article under the CC BV

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standards, QSM is considered safe for biomedical use [13]. All in all, this renewable, sustainable, green, and inexpensive biomaterial, obtained from a residual biosource, quince fruit seeds, can be used as a promising material for biomedical applications [10,11,13]. Nevertheless, like most biopolymers, on its own, QSM exhibits poor mechanical performance, limiting its application in certain biomedical fields [14,15]. Hence, reinforcing fillers are considered here to modify the mechanical properties of the hydrogels. Particularly, cellulose nanofibrils (CNF) are proposed to reinforce the hydrogels given CNF's high aspect ratio, high stiffness and modulus, tunable surface chemistry, and biocompatibility [16–19].

On the other hand, recent advances in 3D printing technologies have opened up new and rapidly growing opportunities to develop 3D hydrogel structures with precise control on the architecture, as needed for biomedical applications [20,21]. Accordingly, 3D scaffolds for different tissues, from soft [22,23] to hard ones [24-26], have been developed successfully through 3D printing. Among the different techniques developed for the printing of the materials, direct ink writing (DIW) or robocasting, consisting of the layer-by-layer deposition of the ink through a computer-controlled fine needle at ambient conditions, has gained much attention for biomedical applications since most hydrogel biomaterials may contain temperature-sensitive components [27-30]. In other words, DIW is unique in its ability to extrude continuous filaments at room temperature. Furthermore, this printing technique has the advantages of low cost, simple construction, and the ability to work with various inks or pastes [31,32]. Nevertheless, the inks suitable for DIW are limited since the ink must easily flow through a fine nozzle while resisting deformation immediately after deposition. It is known that a hydrogel ink with shear-thinning behavior, exhibiting a viscoelastic response to applied pressure, can be extruded from a nozzle and be deposited to fabricate 3D objects [33,34]. Several studies have confirmed the shear-thinning behavior of nanocellulose-based inks and their excellent viscoelastic characteristics that enable successful 3D printing via DIW [35-38]. However, there is no report on the 3D printing of quince seed mucilage. Hence, we introduced a new, fully biobased ink comprising quince seed mucilage and CNF with excellent viscoelastic properties for 3D printing with high fidelity, multilayered lattice structures. CNF was employed as a reinforcing phase to modify the mechanical and viscoelastic properties of QSM hydrogels. Our results confirmed the printability of the developed inks and their suitable porosity, water uptake capacity, and mechanical properties when printed into 3D structures, matching the characteristics of soft tissues. Additionally, they were shown for their biocompatibility with HepG2 cells, opening the opportunity for these fully bio-based 3D structures in soft tissue engineering.

2. Materials and methods

2.1. Materials

Fresh quince fruits were provided, and their seeds were removed. Calcium chloride was supplied from Sigma-Aldrich. Ethanol (92.4%, ETAX B) was purchased from ALTIA Industrial, Finland. Cell proliferation reagent WST-1 was purchased from Roche Diagnostics.

2.2. Ink preparation and printing

It has been reported that TEMPO-mediated oxidation introduces carboxylate groups that reduce the adhesion between the cellulose fibrils and provides interfibrillar electrostatic repulsion, facilitating their colloidal dispersion [39]; therefore, TEMPO-CNF was used in this study. It was prepared (2 wt%) via the method reported in our previous work [40]. For preparing QSM, 10 g quince seeds were washed gently with ethanol and then soaked in distilled water (120 ml) for 24 h. The obtained mucilage was filtered to eliminate seeds and solid impurities. It was then centrifuged at 4 °C for 30 min at 12000 rpm to further extract

solid impurities. The final brownish QSM (Fig. S1) was kept in a refrigerator at 4 $^{\circ}$ C. A certain amount of the gel was freeze-dried to calculate the solid content of the gel. It was found to be 2 wt%. The different weight ratios of the QSM and TEMPO-CNF, including 1:0.25, 1:0.5, 1:0.75, and 1:1, were mixed physically, followed by further mixing with an IKA Ultra Turrax T25 digital homogenizer to have entirely homogenous hydrogels. The prepared inks were coded as Q1T0.25, Q1T0.5, Q1T0.75, and Q1T1, respectively. Pure QSM and TEMPO-CNF were coded as Q1T0 and Q0T1, respectively, and used as control samples.

3D printing of the developed inks was done using a CELLINK Bioprinter (BIO X, Sweden) with a pneumatic syringe equipped with a 20-gauge blunt needle (diameter and length of 840 μ m and 25 mm, respectively). The pneumatic presser varied between 14 kPa to 18 kPa. A lattice 3D model (30 mm \times 30 mm) composed of five layers was printed with an infill density of 25%. Furthermore, a disc-shape sample with a diameter of 25 mm and infill density of 50% was printed for characterizations, including porosity and microstructure studies, swelling and degradation rate tests, mechanical strength measurement, and cell compatibility evaluation. All printed samples were frozen at -20 °C for 48 h prior to lyophilization. The freeze-dried samples were then cross-linked using 1 M calcium chloride solution.

2.3. Characterizations

2.3.1. Rheology

The viscosity of the ink and oscillatory dynamic rheological properties were studied using a rotation Anton Paar MCR 301 rheometer with parallel geometries (PP25 and CP25). The ink measurements were done at 23 °C, while the printed samples were measured at 37 °C. The apparent viscosity of the ink was measured as a function of shear rate between 0.01 and 100 s⁻¹ with a logarithmic interval. Afterward, the stress (τ) sweep test was performed at a constant frequency of 10 rad·s⁻¹ and logarithmically increasing stress from 0.1 to 10³ Pa to evaluate the printability of the ink. The yield stress (τ_{y}) was extracted from the G', G"- τ curve, where the loss modulus intersected the storage modulus. It was then compared with the maximum stress (τ_{max}) applied on the ink, originating from the pneumatic pressure during printing. τ_{max} was calculated using Eq. (1) [40], in which ΔP is the applied pneumatic pressure (14–18 kPa), r is the radius of the needle (420 µm), and L is the length of the needle (3.175 cm).

$$\tau_{\rm max} = \frac{\Delta P.r}{2L} \tag{1}$$

The linear viscoelastic region of the printed samples, which was first immersed in the distilled water for 24 h, was obtained through a strain sweep test between 0.01 and 100% with a logarithmic interval at a fixed angular frequency of 10 rad·s⁻¹. Afterward, their viscoelastic performances, namely the storage modulus (G') and loss modulus(G''), were recorded as a function of frequency between 0.1 and 100 rad·s⁻¹ with a logarithmic interval at a fixed strain rate of 0.1% (within the linear viscoelastic region). The elastic modulus (E) of the printed samples was determined using the storage modulus values by Eq. (2), in which ν is the Poisson ratio. It was considered as 0.5 since the samples behaved similarly to rubber-like material [41].

$$E = 2G'(1 + 2\nu)$$
(2)

2.3.2. Fourier transform infrared (FTIR) spectroscopy

The chemical structure of the pure QSM and TEMPO-CNF and the composite inks before and after crosslinking were studied using a PerkinElmer FTIR with ATR with a scan number and resolution of 32 and 4 cm⁻¹, respectively, between wavenumber of 4000 to 500 cm⁻¹.

2.3.3. Scanning electron microscope (SEM)

The microstructure of the freeze-dried printed samples was

evaluated using SEM images taken from the surface and cross-section surface area. The images were taken with a Zeiss Sigma instrument. The samples were coated with a gold-palladium layer (LECIA EM ACE600 sputter coater) prior to imaging.

2.3.4. Porosity

The porosity was calculated by monitoring the amount of ethanol absorbed by the lyophilized printed sample soaked in ethanol solution using Eq. (3). Ethanol was used since it penetrates easily into the pores without significantly inducing hydrogel shrinkage or swelling [11].

$$\Phi(\%) = \frac{m_f - m_0}{\rho \times V} \times 100 \tag{3}$$

where m_0 and *V* are the weight (g) and the apparent volume (cm³) of the dried sample, m_f is the weight of the sample 24 h after immersion in ethanol, and ρ is the density of ethanol (0.789 g·ml⁻¹).

2.3.5. Swelling study

The swelling ratio (SR) was calculated by monitoring the gained weight of the dried sample (m_0) immersed in PBS solution (pH = 7.4) at 37 °C for 24 h using Eq. (4). The sample was taken out at certain times, its surface solution was removed using blotting paper, and its weight (m_i) was recorded.

$$SR(\%) = \frac{m_i - m_0}{m_0} \times 100$$
(4)

2.3.6. Degradation test

The weight loss of the sample immersed in PBS solution (pH = 7.4) at 37 °C for seven days was reported as its degradation rate (DR). The dried sample was weighted (m_0) and soaked in PBS solution. It was then removed from the solution every 24 h, dried in a vacuum oven at 60 °C, and weighted again (m_d) . The degradation rate was calculated using Eq. (5).

$$DR(\%) = \frac{m_0 - m_d}{m_0} \times 100$$
(5)

2.3.7. Shrinkage

The dimensional changes of the printed sample after freeze-drying were presented as its shrinkage. The apparent volume of the sample was measured before (V_b) and after (V_a) lyophilization, and the shrinkage percentage was calculated by Eq. (6).

$$Shrinkage(\%) = \frac{V_b - V_a}{V_b} \times 100$$
(6)

2.3.8. Compression test

The compression modulus and strength of the printed sample were measured using a TA Instruments DMA Q800 at a controlled humidity and temperature condition. The cross-linked disc-shape printed sample was first immersed in the distilled water for 24 h to reach its equilibrium swelling. It was then subjected to a controlled force with a fixed compression rate of 0.001 N·s⁻¹ at 37 °C and 50% RH. The stress-strain curve was drawn, and the compression modulus (tangent modulus at the linear region) and compression strength were extracted.

2.3.9. Cell culture and cytocompatibility test

The cytocompatibility of the samples was evaluated by culturing HepG2 cells according to the protocol recommended by the ATCC (HB-8065 American Type Culture Collection). Briefly, cells were grown in a 75 cm² culture flask with 10 ml of DMEM culture medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). The cells were maintained at 37 °C and 5% CO₂ In 90% of confluence, the cells were detached with TrypLE Express Enzyme (Triple X, Gibco) at a ratio of 1:5. Cell viability was assessed by WST-1 cell proliferation assay (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H

tetrazolium salt). Samples were washed with Dulbecco's phosphatebuffered saline (DPBS+, Gibco) at 4 °C and sterilized under UV light for 25 min in a laminar flow cabinet (KOJAIR Biowizard Silver SL-130 Blue Series) before seeding. One milliliter of cells with a density of 50,000 cells/ml was seeded and cultured for 1, 3, and 7 days. After this period, samples were washed with DPBS+, 1 ml of cell medium, and 50 µl of WST-1 reagent were added to each well. Afterward, the samples were incubated for 1 h, and the content was transferred to a 96-well plate, 100 µl per well. The optical density (OD) was read at 420 nm using a Synergy H1 multimode microplate reader (Biotek, Bad Friedrichshall, Germany). The background was measured with the same amount of cell medium and WST-1 reagent. All experiments were carried out in duplicate.

3. Results and discussion

3.1. Ink rheology and 3D printing

The apparent viscosity of the composite inks was measured as a function of shear rate. The pure QSM (2 wt%) and TEMPO-CNF (2 wt%) were subjected to tests for comparison behavior. As Fig. 1a illustrates, the viscosity revealed a progressive reduction in all samples, known as shear-thinning viscosity. Upon increasing the shear rate from 0.01 to 100 s^{-1} , the apparent viscosity fell approximately three orders of magnitude. This non-Newtonian behavior, which was further observed in the shear stress-shear rate curves (Fig. S2), has been reported to be due to dis-entanglement and alignment of the microstructures and breaking of the three-dimensional structure in the flow direction [42]. Shear-thinning behavior is often favored for extrusion-based 3D printing since it guarantees the ink viscosity undergoes a substantial reduction under shear stress, facilitates extrusion of inks from fine or micronozzles under pressure, ensures the smooth flow of ink, and provides a high printing fidelity [43,44]. On the other side, the viscosity of the hybrid inks was between the values for pure QSM (Q1T0) and TEMPO-CNF (Q0T1). In other words, the addition of CNF increased the viscosity of the sample, suggesting the formation of a more robust structure, owing to the inter- and intra-molecular hydrogen bonds within nanocellulose



Fig. 1. (a) apparent viscosity versus shear rate, (b) stress-sweep test at a fixed angular frequency of 10 rad·s⁻¹; the photographs of (c) and (g) Q1T0.25, (d) and (h) Q1T0.5, (e) and (i) Q1T0.75, and (f) and (j) Q1T1 hydrogels before and after freeze-drying, respectively. Q1T0.25, Q1T0.5, Q1T0.75, and Q1T1 represent samples compose of different weight ratios of the QSM and TEMPO-CNF, including 1:0.25, 1:0.5, 1:0.75, and 1:1, respectively. The solid and blank symbols represent the storage modulus and loss modulus, respectively.

[45] and between the micro- and nanofibrils and QSM. This improvement was further confirmed by the storage and loss moduli curves (Fig. S3), where both G' and G" were increased significantly upon increasing the CNF content. It is worth noting that the ink viscosity should not be too high since it then cannot extrude out from a syringe [46]; accordingly, the apparent viscosity of the hybrid inks was compared with that reported in the literature, indicating a suitable range of apparent viscosity for direct ink writing 3D printing [33,44,46,47].

The yield stress (τ_y) is another critical feature that describes ink flow during the printing process. When the applied shear stress (τ_{max}) exceeds τ_y , the ink initiates to flow from a static state due to the breaking down of the ink polymer superstructure [28,46]. Fig. 1b presents results from stress sweep tests for the pure QSM hydrogels and the hybrid inks. All samples revealed a gel-like or elastic behavior at low shear rates (G' >G'') followed by a liquid-like or viscous behavior at higher shear (G' <G''). Accordingly, τ_y was extracted at the crossover point between G' and G'', 35 to 80 kPa. According to Eq. (1) and concerning the applied pressure and needle diameter and length, the τ_{max} varied between 92 Pa and 120 Pa, which was significantly higher than the yield stress values, suggesting the flowability of the inks under the printing conditions. It is noteworthy that the yield stress should be high enough for selfsupporting and shape retention after deposition; otherwise, the printed ink would deform easily [48,49]. Coffigniez et al. [47] suggested a yield stress threshold of 50Pa, under which DIW is not possible. Accordingly, the neat QSM had poor printability (yield stress of ca. 35 Pa), and the structure collapsed few seconds after printing.

A 3D grid structure was printed at ambient temperature using five layers to investigate the printability of the developed inks. Fig. 1 displays the printed structures before and after lyophilization. All inks were extruded out easily through an 840-µm needle and presented a stable jetting characteristic, with no signs of ink spreading, arche formation, and nozzle clogging [35,50,51]. Consequently, a precise fabrication of a multilayered structure with proper adhesion between the printed layers was obtained. In all samples, the extruded filament retained its shape after deposition, and no evidence of delamination or filaments flattening was observed, suggesting a quick shift from a shear-thinning fluid into a solid-like material, which is an essential viscoelastic feature of inks suitable for DIW 3D printing [32,47].



Fig. 2. SEM images from the surface and cross-section area of the lyophilized samples. (a) and (b) Q1T0.25, (c) and (d) Q1T0.5, (e) and (f) Q1T0.75, and (g) and (h) Q1T1 (magnification $50 \times$).

3.2. Chemical, microstructural features, and porosity

The chemical features of the neat QSM, TEMPO-CNF, and the printed hybrid hydrogels were studied through FTIR. Fig. S4 illustrates the FTIR spectra of the samples before and after crosslinking. The pure QSM and TEMPO-CNF revealed characteristic peaks similar to those reported in the literature [9,11]. Furthermore, these characteristics peaks appeared in the hybrid hydrogels, confirming the presence of each component, while no new peaks were detected after crosslinking, suggesting that calcium ions established simple hydrogen bonding between polymer chains [52].

A porous structure with uniform pore size and interconnecting porosity is favorable for tissue engineering. For instance, it should facilitate cell proliferation and migration, secretion of ECM materials, and transfer of nutrients, oxygen, and water vapor. It should also promote vascularization and be suitable for drug delivery applications. Moreover, an ideal porous structure should accelerate wound healing by absorbing the wound exudate, keeping the wound moist [53-57]. Accordingly, the surface and cross-section SEM images were used to examine the surface morphology and network distribution of the freezedried 3D hydrogels (Fig. 2). All samples displayed a lamellar porous interconnected structure, resulting from the ice templating process in which the frozen trapped water molecules later sublimated during the freeze-drying. This honeycomb-like, regular porous structure is also enabled by crosslinking with the calcium ions, forming regular pores and an orchestrated network in the hydrogels [52,58]. It is worth indicating that a compact film-like morphology has been reported for the surface of the lyophilized hydrogels due to a higher crosslinking density at the surface, restricting polymer chain mobility and, consequently, limiting the space available for ice crystal formation during the ice templating process [59]. However, in the current study, the surface of the lyophilized hydrogels revealed both film-like and porous morphologies, particularly at low CNF content, suggesting that only mild crosslinking, and no formation of covalent bonds, happened throughout the samples. On the other hand, the pores were evenly distributed in the structure, with a pore size of approximately 50 to 350 µm (Table 1), in agreement with that reported for QSM-based hydrogels [8,9] and in a range that is suitable for tissue engineering and wound dressing applications [54,60,61]. Nevertheless, the architecture and pore size depended on the hydrogel composition. Increasing CNF content developed a denser and more organized network, with smaller pore size and higher surface area. These observations are also in good agreement with observations from the literature, which indicated a reduction of pore size with the increased nanocellulose concentration [62]. This reduction is attributed to the lower mobility of cellulose fibrils at higher concentrations during ice templating [59]. Finally, the SEM images revealed good dispersion of CNF, without any visible aggregation (up to $1000 \times$ magnification) and confirmed excellent integration of cellulose fibrils with QSM due to the absence of any noticeable phase separation; all results suggest excellent compatibility between the two components [45.63].

Porosity is another geometrical feature that affects the diffusion of soluble nutrients, growth factors, and cells; the larger porosity initiates

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Sample	Pore size (µm)	Porosity (%)	Shrinkage (%)	Swelling ratio ^a (%)	Degradation ^b (%)
Q1T0.25	$\begin{array}{c} 250 \pm \\ 100 \end{array}$	76 ± 3	13 ± 1	1120 ± 60	10.6 ± 0.6
Q1T0.5	188 ± 75	78 ± 3	10 ± 0.7	1290 ± 80	$\textbf{9.9} \pm \textbf{0.5}$
Q1T0.75	175 ± 60	81 ± 5	8 ± 0.5	1470 ± 90	$\textbf{7.1} \pm \textbf{0.3}$
Q1T1	88 ± 40	82 ± 4	7 ± 0.4	1690 ± 120	$\textbf{6.4} \pm \textbf{0.3}$

^a After 24 h.

the diffusion of cells to the interior. Furthermore, it is advantageous for exchanging oxygen and nutrients [9,64,65]. All samples revealed relatively high porosity (Table 1), which is in good agreement with that reported for neat QSM [9,11] and was in the typical range reported for tissue engineering and wound dressing applications [64,66]. The porosity increased slightly upon the addition of CNF, which is expected to result in more favorable interactions with the surrounding tissues [66]. This increase could be attributed to the supporting effect of mico-and nanofibrils in the spatial network skeleton that promotes the retention of the gel shape during freeze-drying and the formation of smaller pore sizes, as previously observed in SEM images. A similar observation has been reported for graphene oxide-reinforced silk fibroin hydrogels [67].

3.3. Shrinkage, swelling, and degradation

The hydrogel shrinkage directly affects the porosity and water content, which are critical features in their application, especially for tissue engineering, drug delivery systems, and wound patches. Accordingly, a large volumetric shrinkage induces the deformation of the whole structure and considerably reduces the porosity and water uptake capacity [68,69]. Owing to the high amount of trapped water in the respective polymeric networks, hydrogels are usually subjected to dimensional changes upon drying. Nevertheless, crosslinking and freeze-drying are two well-known methods that minimize shrinkage in hydrogels [21]. The shrinkage of the samples is presented in Table 1, where all hydrogels revealed relatively low volume shrinkage, < 13%. In other words, the dimensional stability was well preserved due to intermolecular hydrogen bonding between the polysaccharide chains established with the incorporation of CNF. The TEMPO-CNF reinforced the hydrogel structure, diminished the hydrogel volume collapse, and rendered the structures more resistant to shrinkage, resulting in better shape fidelity after freeze-drying [21,35].

The swelling of hydrogels plays a vital role in tissue engineering applications because it affects the proliferation and differentiation of cells by facilitating the diffusion of nutrients and growth factors. It is also an essential feature in exudating wounds, e.g., in wound dressing applications [70-72]. The trend of the swelling ratio of freeze-dried hydrogels versus time is depicted in Fig. 3a. The equilibrium swelling ratio (after 24 h) is provided in Table 1. All samples revealed very fast water uptake during the first two hours, followed by minor water absorption up to 24 h. Moreover, they presented a relatively high swelling ratio in line with the values reported for QSM-based hydrogels used in biomedical applications [9,70]. The relatively high water absorption capacity could be due to the intrinsic hydrophilicity of the two components, QSM and TEMPO-CNF, which arose from the abundant hydrophilic functional groups such as hydroxy, carboxyl, and amine groups [64]. It could also be attributed to the sample's high porous structure, favoring water diffusion within the homogeneously porous internal structure [66]. Meanwhile, the presence of the CNF significantly augmented the swelling ratio, with the equilibrium swelling ratio increasing approximately 50% in the Q1T1 sample compared to the Q1T0.25 one. This was most likely a result of the higher porosity of the samples. All in all, the developed porous hydrogels can be suggested as 3D structures for tissue engineering and wound dressing applications since they ease the diffusion of nutrients and growth factors as well as the absorption exudates due to their relatively high-water uptake capacity.

Degradation is another important feature for a hydrogel for tissue engineering applications. On the one hand, an ideal structure for tissue engineering needs to be biodegradable, e.g., to provide sufficient space for cell growth and tissue formation. On the other hand, the degradation rate should be synchronized with the regeneration rate of the tissue [66,73–75]. Accordingly, the degradation rate of the lyophilized cross-linked hydrogels was characterized by monitoring the mass loss over 7 days, Fig. 3b. All the hydrogels revealed the highest network

^b After 7 days.



Fig. 3. Swelling ratio and degradation rate in PBS solution (pH 7.4) at 37 °C.

degradability at the end of the first day, which gradually reduced with time, up to 7 days. None of the hydrogels showed noticeable shape changes during the whole degradation experiment, with a maximum degradation rate of less than 11%. Cellulose is not degraded in the organism/body; therefore, the observed loss weight could be due to the degradation of QSM and/or release of the components, which were not effectively crosslinked by Ca⁺² ions [70]. The degradation rate decreased upon increasing the CNF content, attributing to the reinforcing effect of the cellulose particles, their intrinsically lower degradation rate than QSM, and increasing the cross-linking density established by the calcium ions [66,76]. Some tissues, such as skin, require a longer time for re-epithelialization [11]; therefore, the developed hydrogels with relatively low degradation rate and more physical stability could be effective in this field.

3.4. Compression strength and viscoelastic performances

The ability of a hydrogel to withstand mechanical load mainly determines the field of application [77]. For instance, for tissue engineering applications, the hydrogel's mechanical properties should be matched with the mechanical properties of the native tissue [78]. Accordingly, compression tests were conducted under controlled loading to evaluate the mechanical performances of the developed 3D hydrogels. Fig. 4a presents the obtained compressive stress-strain curves. The compressive modulus and ultimate compressive strength are provided in Fig. 4b. None of the samples failed under the test conditions and revealed a typical hydrogel compressive stress-strain curve; a linear deformation behavior at low strain, followed by a continuous load increase, leading to a densification region where the stress increased sharply. The linear region is assigned to a large amount of water trapped in the hydrogel matrix. The continuous loading increase is attributed to the porosity reduction and pores walls upon increasing the applied load [40,79]. Moreover, a sharp increase at the end of the curve corresponds to the maximum deformation, making the subsequent deformation more complex [80]. The incorporation of CNF increased the compressive modulus and ultimate strength by approximately 50%and 70%, respectively, indicating a stiffer hydrogel and a reinforcing function of CNF, as previously reported for other hydrogels [66,81]. Of note, the developed hydrogels had a comparable mechanical performance with skin and soft tissues [78].

The rheology studies were carried out to further investigate the mechanical properties of the developed hydrogels. The mechanical strength of the lyophilized cross-linked hydrogels was examined by



Fig. 4. Compressive stress-strain curves at 27 °C and RH of 50%, (b) comparison of compression and tensile moduli, (c) strain sweep test at a fixed frequency of 10 rad s⁻¹, and (d) frequency sweep test at a fixed shear strain of 0.1% at 37 °C. The solid and blank symbols represent the storage modulus and loss modulus, respectively.

monitoring the storage (G') and loss (G'') loss moduli versus strain and frequency in an oscillatory mode at 37 °C. The linear viscoelastic region first was determined through a strain sweep. Fig. 4c depicts the trend for G' and G'' versus strain rate at a fixed frequency of 10 rad·s⁻¹. The moduli were constant up to a threshold strain value, approximately 1%, where G' was significantly higher than G'', demonstrating the linear viscoelastic region and suggesting the formation of network structures. With further increase in strain, both G' and G'' began to present strain-dependent behavior, indicating the onset of the non-linear viscoelastic behavior. Finally, at a critical shear strain value, the moduli profiles intersected, and G'' exceeded G', presenting a deformation point due to system structure breaking down [42,82]. Although the linear viscoelastic region did not change significantly, both moduli increased noticeably upon increasing the CNF content, suggesting the reinforcing effect of nanocellulose [18].

In the next step, the extent of viscoelasticity of the hydrogels was determined through a frequency sweep test within the linear viscoelastic region (a fixed strain rate of 0.1%). Fig. 4d shows the behavior of the G' and G'' versus angular frequency at a fixed strain of 0.1%. The storage modulus was nearly independent of frequency and varied between 10.5 and 21.5 kPa, in line with that reported for nanocellulose-reinforced hydrogels [18]. Furthermore, G' was always superior to G'', with roughly an order-of-magnitude, indicating a solid-like or gel-like behavior. In other words, the curves demonstrated that all hydrogels had viscoelastic characteristics, with the elastic component being dominant over the viscous one due to the mechanical entanglements, electrostatic forces, and the formation of hydrogen bonds between the two components and between each individual component [42,83]. They also confirmed excellent compatibility between the components since, unlike some literature [18], G' and G" never tend to dissociate at higher oscillating frequencies. On the other hand, increasing CNF enhanced both storage and loss moduli significantly, confirming a more elastic system and the reinforcing effect due to the hydrogen bonds formed within the hydrogel structure established by the CNF [82,84]. The storage modulus was used to calculate the elastic modulus of the hydrogels through Eq. (2). The results are presented in Fig. 4b, where the elastic modulus varied between 30 kPa to 65 kPa. Although the elasticity of soft tissues has been reported from 0.3 to 220 kPa, many types of soft tissues, like brain tissue, liver, adventitial layer, and connective tissue, have an elasticity of approximately 10 kPa [85-88]. Accordingly, the newly developed 3D bio-hydrogels possessed the mechanical properties required by soft tissue engineering applications.

3.5. Cell compatibility

Cell growth and survival are essential parameters in tissue engineering [70]. The cell compatibility of the quince seed mucilage against different cell lines has been reported in the literature [9-11]. TEMPO-CNF based biomaterials have also been studied and shown to present good biocompatibility, with no toxicological effects when cultured with myoblasts, fibroblasts, and liver cancer cells [38,89]. Accordingly, a cell viability test was carried out to investigate the biocompatibility of the hybrid 3D hydrogels. Hepatocellular carcinoma cell line (HepG2) was used as a model cell line in this work due to its high sensitivity to toxins, proving to be a good tool for biocompatibility assessment [90,91]. Furthermore, it has been shown that CNF is suitable for 3D cultures of various cell lines, allowing them to form spheroids, small tissue-like cell aggregates. Namely, it can facilitate the formation of hepatic (HepaRG and HepG2) and stem cell spheroids in three-dimensional (3D) cell cultures [92-94]. Cells were seeded onto hybrid hydrogels, and cell proliferation was assessed by monitoring the maintenance of



Fig. 5. Cell viability of HepG2 cells in the presence of hybrid hydrogels after 1, 3, and 7 days of incubation assessed in 420 nm. * indicates p < 0.05.

mitochondrial dehydrogenases. Fig. 5 displays the cytotoxicity from WST-1 results after 1, 3, and 7 days of culture measured by colorimetric analysis. From a cell viability point of view, cytotoxicity can be categorized into four levels; below 30% reveals severe cytotoxicity; between 30% and 60% illustrates moderate cytotoxicity; between 60% and 90% represents mild cytotoxicity, and above 90% shows no-cytotoxicity [95]. All samples presented cell viability higher than 90%, suggesting no signs of toxicity. The HepG2 cells metabolic activity increased during the cell culture periods, confirming that all hydrogels were non-cytotoxic and considerably supported cell attachment, survival, and proliferation [9,70]. Furthermore, there were significant differences in cellular metabolic activity on day 1 and day 7 (p < 0.05) for all hydrogels. Moreover, significant differences were observed between the hydrogels and the tissue culture plate (TCP) as a control (p < 0.05), attributing to the porosity of the hydrogels that provided a higher surface area for cell growth and improved seeding by capillarity effect. This improvement could also be due to the samples' swelling capability, which favored the high nutrient and cell waste exchange in the environment [11,64,96]. Altogether, the hydrogels presented better physicochemical properties (porosity, pore size, chemical surface, and surface topography) than TCP and consequently improved cell adhesion, viability, and proliferation. It is worth notifying that the cell viability increased upon increasing CNF loading, which can be explained by the higher porosity, swelling ratio, and physical integrity of the structures with higher fibril content, improving the surface area available for cell growth [9].

Mucilage from plant-derived polysaccharides has become trending in tissue engineering applications, attributing to their accessibility, biocompatibility, and gelation ability. Nevertheless, finding an ideal plant-based hydrogel that can mimic human tissue properties in terms of structure, function, and performance is still challenging. On the other hand, there has been exponential growth in the 3D printing of tissue engineering scaffolds recently since complex structures can be obtained quickly and simply through this technique. However, the ink used in 3D printers is still one of the most discussed areas. Here we successfully developed natural, sustainable, and biocompatible bio-hydrogels composed of quince seed mucilage and TEMPO-cellulose nanofibrils, which could be printed precisely in lattice geometries through direct ink writing 3D printing. These developed hydrogels possessed the physical and mechanical performances required for soft tissue engineering applications and furthermore presented biocompatibility against HepG2 cells. Our findings can open insight on 3D printing of plant-based mucilage with desirable geometries for potential biomedical applications, namely engineered scaffolds for soft tissues.

4. Conclusion

This study developed a series of bio-hydrogels composed of quince seed mucilage and TEMO-cellulose nanofibrils to investigate their printability and evaluate their characteristics for biomedical applications. Although the pure QSM presented desirable shear-thinning viscosity for direct ink writing 3D printing, the presence of CNF was found to be essential for filament self-supporting and shape retention after deposition. Accordingly, using hybrid hydrogels, 3D lattice geometries with honeycomb-like regulated porous structures were printed successfully with a suitable resolution. The addition of CNF had significant impacts on hydrogels' water uptake capacity, shrinkage, degradation, and porosity. Moreover, the mechanical properties and viscoelastic performances of the hydrogels improved significantly upon increasing the ratio of cellulose particles, while no evidence of particle aggregation of phase separation was observed. The physical and mechanical performances of the hydrogels were in line with that reported for soft tissues like skin. Furthermore, they revealed excellent biocompatibility against HepG2 cells, making them interesting candidates for biomedical applications.

CRediT authorship contribution statement

Hossein Baniasadi: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, and Visualization.

Roberta Teixeira Polez: Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, and Visualization.

Erfan Kimiaei: Formal analysis, Investigation.

Zahraalsadat Madani: Formal analysis, Investigation.

Orlando J. Rojas: Supervision, Funding acquisition, Review & Editing.

Monika Österberg: Supervision, Funding acquisition, Review & Editing.

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Acknowledgment

The authors would like to acknowledge the Academy of Finland funding; No. 327248 (ValueBiomat) and 327865 (Bioeconomy). This work was a part of the Academy of Finland's Flagship Programme under Projects No. 318890 and 318891 (Competence Center for Materials Bioeconomy, FinnCERES). The authors would also like to thank Ms. Marja Kärkkäinen for providing TEMPO-CNF and the Biohybrid Materials Research Group (Aalto University) for providing the HepG2 cells.

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