3D printing of shape-morphing and antibacterial anisotropic nanocellulose hydrogels

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ABSTRACT

We report on a procedure for the preparation, printing and curing of antibacterial poly(N-isopropylacrylamide) nanocellulose-reinforced hydrogels. These composites present a highly anisotropic microstructure which allows to control and modulate the resulting mechanical properties. The incorporation of such nanoparticles enables us to modify both the strength and the humidity-dependent swelling direction of printed parts, offering a four-dimensional property to the resulting composite. Antibacterial properties of the hydrogels were obtained by incorporating the functionalized peptide ε-polylysine, modified with the addition of a methacrylate group to ensure UV-immobilization. We highlight the relevance of well-adapted viscoelastic properties of our material for 3D printing by direct ink writing of self-supporting complex structures reaching inclination angles of 45°. The addition of cellulose nanoparticles, the overall ink composition and the printing parameters strongly determine the resulting degree of orientation. The achieved control over the anisotropic swelling properties paves the way to complex three-dimensional structures with programmable actuation.

1. Introduction

Hydrogels are materials with a hydrophilic character capable of holding large amounts of water within their three dimensional network of crosslinked polymers (Billiet, Vandenhaute, Schellnhub, Van Vlierberghe, & Dubruel, 2012; Hoffman, 2012). In fact, hydrogels can swell up to 1000-fold their initial volume when immersed in water whilst retaining their form and some strength, thus enabling the design of mechanical actuators (Cheng, Jia, & Li, 2020; Liu et al., 2016). Due to some physico-chemical similarities with biological soft tissues, and the ease of functional chemistry incorporation within their composition and structure, hydrogels have attracted the attention of the medical field as wound dressings (Gupta et al., 2020) and smart drug delivery systems (Caballero-Aguilar, Silva, & Moulton, 2020).

A number of publications have shown that hydrogels and hydrogel composites can be formulated as inks suitable for 3D printing by several methods such as stereolithography or direct ink writing (DIW), facilitating their use in a wide variety of applications (Billiet et al., 2012; Jang et al., 2018; Koffler et al., 2019; Lee, Bristol, Preul, & Chae, 2020), while continuous research develops on controlling their physicochemical properties such as viscosity, dispersion of additives, size and shape (Duan, Hockaday, Kang, & Butcher, 2013; Wüst, Godla, Müller, & Hofmann, 2014). The ease with which the physical state of hydrogels can be modified (as smart materials) by external factors such as pH, humidity, temperature, light, or biochemical signals (Gaharwar, Peppas, & Khademhosseini, 2014; Xu et al., 2008) further supports their biomedical uses as e.g. in artificial muscles (Park & Kim), but has also opened doors in the field of soft robotics (Han et al., 2018).

However, commonly used hydrogels have rather poor mechanical properties when hydrated and this has led to intense research efforts to develop tougher hydrogels. Among the different strategies explored, a general trend tends to blend reinforcements materials (such as clays (Gao, Du, Sun, & Fu, 2015) or oxides (Erh, Sander, Grisch, & Studart, 2013; Li et al., 2013)) with the hydrogels to improve their mechanical strength, stiffness and toughness. Alternatively, the incorporation of bio-based materials, such as cellulose nanocrystals and cellulose nanofibers revealed not only to increase the strength and stiffness of the resulting hydrogels (both pre- and post-cure) but also to enable a better control of the viscoelastic properties of the inks (Dai et al., 2019; Liu et al., 2019). Additionally, because of the anisotropic nature of the

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reinforcement, it enables the introduction of properties varying with orientations and at different length scales (Hausmann et al., 2020; Markstedt et al., 2015; Müller, Oztürk, Arlov, Gatenholm, & Zenobi-Wong, 2017; Sydney Gladman, Matsumoto, Nuzzo, Mahadevan, & Lewis, 2016).

Usually, the response of smart hydrogels to external stimuli is an isotropic change in volume, but the incorporation of an anisotropic mechanical response to environmental stimuli would enable the design of more complex ‘smarter’ actuators, and allow a better mimicry of biological structures (Sano, Ishida, & Aida, 2018; Sydney Gladman et al., 2016). It has been shown that one way to introduce mechanical anisotropy into hydrogels is to incorporate stiff elements with a high aspect ratio within the hydrogel structure. These high aspect ratio elements then adopt a preferred orientation when experiencing the high shear and extensional forces associated with passing through a nozzle for 3D printing (Hausmann et al., 2018; Siqueira et al., 2017). Incorporating programmable shape changes into synthetic hydrogels has to date most commonly been achieved with inorganic materials (Erz et al., 2013) or cellulose nanofibers combined with nano-clay (Sydney Gladman et al., 2016). The use of pure oriented cellulose nanoparticles without any other anisotropic building blocks (e.g. laponite, carbon fibers or alumina platelets) to give the directional reinforcement, used to recreate the self-morphing strategy of natural materials, has to the best of our knowledge not been previously reported.

As alluded to above, the polymer network of hydrogels can quite easily be functionalized to have desired chemical/biological properties such as antimicrobial activity (Mauri, Rossi, & Sacchetti, 2016; Yigit, Sanyal, & Sanyal, 2011) which is required in many biomedical applications, but especially for tissue scaffolds and wound dressings. A number of ways of achieving antimicrobial action including the use of antibiotics and antimicrobial particles such as silver and zinc-oxide nanoparticles has been reported (Gupta et al., 2020; Li et al., 2018; Stojkovska et al., 2014). However, the use of naturally occurring molecules such as antimicrobial peptides and proteins (AMPs) has attracted particular interest (Lei et al., 2019; Neves, Pereira, Araújo, & Barrias, 2018; Zhang & Gallo, 2016) because of their broad spectrum efficacy even at low concentration, the ease with which they can be incorporated into hydrogels and because they are often more durable against micro-organism adaptation than synthetic agents (Zhou et al., 2011). A promising example of such AMPs is ε-polylysine (EPL). EPL is usually derived from Streptomyces albalbus and has found widespread use in food additives as it is non-toxic, biodegradable and can be produced at low cost (Shih, Shen, & Van, 2006). Being water-soluble, EPL is a good candidate for covalent chemical modification of hydrogels, conferring upon them good antimicrobial properties against fungi, gram-positive and gram-negative microorganisms. The immobilization of EPL in hydrogels or coatings is not expected to affect its antimicrobial efficacy (Hylgaard et al., 2014; Zhou et al., 2011).

In this report, we focus on the synthesis of functionalized polymer-hydrogel inks reinforced with cellulose nanocrystals and nanofibers appropriate for direct ink writing. Cellulose nanocrystals are the main reinforcing elements (up to 35 wt%), while cellulose nanofibers, employed at a much lower concentration (1 wt%) are included to significantly enhance the shape retention and tune the rheological properties of the inks. N-isopropyl acrylamide (NIPAM), a photopolymerizable monomer, was chosen to be chemically and physically crosslinked with the nanocellulose particles to produce biocompatible hydrogels. We chose to create inks suitable for DIW 3D printing because of the lack of constraints on material composition (polymer and reinforcing content) and because it is easier to control the local orientation of stiff reinforcing elements by this approach than with other 3D printing methods.

2. Experimental section

2.1. Materials

N-isopropylacrylamide (NIPAM) 97%, photo initiator Irgacure 2959 (98%), crosslinker ethylene glycol dimethacrylate (EGDMA) 98%, glucose (99.5%), sodium bromide (NaBr ≥ 99%) and sodium hydroxide (NaOH ≥ 99%) were purchased from Sigma-Aldrich (Buchs, Switzerland). Glucose oxidase (high purity), 2,2,6,6-Tetramethyl-1-piperidinylxoyl (TEPDO), sodium hydrochlorite (NaClO) solutions (12–14 % chloride) and dimethylformamide DMF (≥ 99.8%) were purchased from VWR International. ε-poly-lysine (99.4%) was bought from Handary S.A.. Methacrylic acid MA (≥ 99%) and N,N-Dicyclohexylcarbodiimide – DCC (99%) were purchased from Alfa Aesar. N-Hydroxy-succinimide NHS (≥ 99%) was acquired from Merck. Cellulose nanocrystals from sulfuric acid hydrolysis of eucalyptus pulp produced at the USDA Forest Service – Forest Products Laboratory (Madison, WI) were purchased from University of Maine as freeze-dried powder (z-potential −47.3 mV – Supplementary Information). Never-dried elemental chlorine free (ECF) cellulose fibers (81.3 % cellulose, 12.6 % hemi cellulose, lignin 0% and ash 0.3%) from bleached softwood pulp (Picea abies and Pinus spp.) were obtained from Stendal GmbH (Berlin, Germany) and used for the production of cellulose nanofibers (CNFs).

2.2. Methods

2.2.1. CNF preparation

Never dried cellulose fibers were oxidized following previously established protocols from Saito and Isogai (2004) with slight modification. The cellulose fibers were suspended in water in order to form a suspension with a concentration of 2 wt%. TEMPO and sodium bromide (NaBr) were dissolved in water to concentrations of 0.1 and 1.0 mmol per gram of cellulose pulp, respectively, and mixed with the fiber suspension. The pH of the suspension was adjusted to 10 with NaOH solution (1 mol L⁻¹). A concentration of 10 mmol NaClO was chosen per gram of cellulose pulp. The TEMPO-oxidized cellulose fibers were thoroughly washed until the conductivity was similar to that of distilled water. The oxidized and purified cellulose fibers were dispersed in water to a concentration of 2 % (w/v) and ground using a Supermass Colloidier (MKZA10-20 J CE Masuko Sangyo, Japan) to obtain cellulose nanofiber suspension. The energy applied to the grinding process was 9 kW h/kg of cellulose. The oxidized fibers presented COOH content, determined by conductometric titration with NaOH, of 1.1 mmol/g, and z-potential of −53.2 ± 2.7 mV (Supplementary Information).

2.2.2. Preparation of inks

2.2.2.1. CNC-based inks. To prepare an ink containing 20 wt% of CNC, 4 g of cellulose nanocrystals CNCs were mixed with 14.1 g of deionized water (bubbled with N2 for one hour to remove oxygen). A dispersion of the CNCs in water with dissolution of NIPAM has been achieved by mixing the ingredients with the speedmixer (SpeedMixer DAC 150.1 FZ) at speeds of 1400, 2000, 2500 and 3500 rpm for 5 min each. After complete dispersion of CNC, the photo initiator Irgacure 2959 (0.1 g) the crosslinker EGDMA (190 μl) and the oxygen scavenger glucose oxidase (9.5 mg), and glucose (158 mg) were added to the suspension and mixed at 1400 rpm for 5 min in the speed mixer. The same procedure has been adopted for other CNC concentrations, just varying the initial CNC and the water contents.

2.2.2.2. CNC/CNF-based inks. Similar protocol used in the preparation of pure CNC-based inks was used to prepare the CNC/CNF inks. However, prior to addition of NIPAM, photoinitiator, glucose oxidase and glucose, the water dispersion of CNC/CNF was processed two times on a three-roll mill (DSY-200, Bühler, Switzerland) to enhance the dispersion
of CNF within the inks and to avoid clogging of the nozzles while printing.

2.2.3. Functionalization of ε-poly-lysine (EPL)

e-poly-lysine was modified according to the procedure described elsewhere (Zhou et al., 2011). Methacrylic acid – MA (0.63 g, 7.34 mmol) and N-Hydroxy-succinimide – NHS (0.93 g, 8.11 mmol) were dissolved in 10 mL DMF (≥ 99.8 %, VWR) and cooled to 0 °C. N, N′-Dicyclohexylcarbodiimide – DCC (1.51 g, 7.34 mmol) dissolved in 10 mL DMF was added dropwise to the NHS-MA solution over a period of 20 min keeping the temperature at 0 °C. The mixture was stirred for 2 h at 0 °C another 4 h at room temperature. After filtration the filtrate was added to a solution of epsilon-poly-lysine – EPL (20 g, 6.67 mmol) in water/DMF (200 mL: 100 mL) and stirred for 24 h at room temperature. The solvent was then removed with a rotary evaporator and acetone was added to the solid. After filtration, the remaining solid was dissolved in water and the undissolved product was filtrated again. The sample was vacuum-dried over night at 50 °C and purifed to remove contamination of DMF. Next, EPL-MA-powder was re-dissolved in the lowest amount of water possible and acetone was added in excess. After washing twice with acetone, the excess solvent was removed and the remaining solid was dissolved in water. The filtrate was vacuum-dried at 40 °C over night yielding EPL-MA (6.41 g, 2.08 mmol, 28 %) as a white powder with minor amounts of DMF (<1 %) and an unidentifiable solvent.

2.3. Preparation and characterization of composites

2.3.1. 3D printing

Nanocellulose-NIPAM hydrogels were printed using a direct ink writing (DIW) equipment from EnvisionTEC (Bioplotter Manufacturing Series, Germany). The hydrogels were filled in plastic cartridges and extruded through uniform steel nozzles (H. Sigrist & Partner AG) with compressed air at pressures in the range 1.0–3.5 bar, at 10 mm/s and at a fixed temperature of 10 °C. The extrusion needles were 12.7 mm long and exhibited a non-tapered geometry with diameter of 0.41 mm, for comparison of swelling properties and degree of alignment some samples were printed with nozzles of 0.84 mm in diameter. The substrate onto which the materials were printed was kept at 25 °C. The nozzles sizes were chosen considering the rheological properties of the inks aiming at high resolution and high degree of alignment of the nanocelluloses.

After printing, the materials were cured with UV light under nitrogen atmosphere to avoid oxygen inhibition of the polymerization reaction. The printed structure was placed in a customized UV-curing chamber prepared with 5 LEDs (15 W, 75 lm, 400–410 nm wavelength) positioned 10 mm above the sample. The curing time was set to 10 min. Samples were post-cured for 5 min under a 400 W high pressure mercury lamp (DrHoenle, UVA-spot 400/T) at a distance of 10 cm from the lamp.

Swelling experiments were performed on samples of 2.0 cm width, 5.0 cm length while their thickness vary according to the diameter of the needle (e.g. 0.41 or 0.84 mm per layer).

2.4. Microstructural characterization

2.4.1. Transmission electron microscopy (TEM)
The morphology of the CNC was characterized by transmission electron microscopy (TEM, Jeol JEM-2200FS, USA Inc.) using an acceleration voltage of 200 kV. Plasma activated (30 s) carbon-coated grids were used as a support onto which a drop of a 0.02 wt % suspension of the cellulose nanocrystals was deposited and stained with a 2 wt % solution of uranyl acetate for 30 s. The average length and diameter of the CNCs were determined using the measuring tool in Image J.

2.4.2. Scanning electron microscopy (SEM)
The morphological characteristics of CNF were accessed by scanning electron microscopy (FEI Nano SEM 230) using an accelerating voltage of 5 kV and a working distance of 5 mm. A drop of 0.05 wt % CNF solution was deposited on mica support. Samples were coated with 5 nm platinum to avoid surface charge.

2.4.3. Optical microscopy (OM)

Optical microscopy analyses of CNC/CNF-based inks were performed on an Axioplan microscope from Zeiss equipped with cross-polarized filters.

2.4.4. HNMR spectroscopy

The 1H NMR spectra of neat and functionalized ε-poly-lysine were recorded on a Bruker AV III HD 400 MHz wide-bore NMR spectrometer. 40 mg ε-poly-lysine (EPL) or ε-poly-lysine-modified (EPL-MA) was dissolved in 1 mL D2O. The NMR values of both EPL and EPL-MA are shown in the Supplementary Information.

2.5. Physical characterization

2.5.1. Rheology of nanocellulose-NIPAM hydrogel inks

The rheological behavior of the CNC/CNF-based inks were determined using an MCR 302 Anton Paar Rheometer with a 50 mm plate-plate geometry, 0.5 mm of gap and at a constant temperature of 25 °C. Shear sweep tests were performed at shear rates ranging from 0.01 to 1000 s−1. An aqueous solvent was used in all experiments to mitigate drying effects. The parameters for the calculation of the maximal shear stress experienced upon printing is presented in Table S1 (Supplementary Information).

2.5.2. Wide angle X-ray diffraction (WAXD)

Two-dimensional wide-angle X-ray diffraction (2D-WAXD; STOE IPDS-II, 0.71073 Mo Kα radiation source) was used to study the degree of CNC alignment within the printed nanocellulose-hydrogels and the neat NIPAM-hydrogel. The equipment was operated at 40 mA and 50 kV for 30 min using a beam diameter of 0.5 mm in transmission mode. The samples were fixed on the goniometer head and then placed perpendicular to the beam to allow the X-rays to pass only through the specimen. The 2D-WAXD patterns were recorded on an Image Plate Detector System with a 340 mm diameter placed at a distance of 200 mm from the sample. For each sample position a full image was recorded covering a 20 range from 3 to 40°. Azimuthal scans were integrated for the cellulose (200) reflection. The patterns were corrected for air scattering and background by subtracting a no-sample diffraction pattern from the raw data. The degree of orientation and Herman’s order parameter were calculated according to the methods described elsewhere (Siqueira et al., 2017) and depicted in the Supplementary Information (Table S2).

2.6. Mechanical properties of hydrogels

2.6.1. Compression tests

3D printed cubic specimens (1.0 × 1.0 × 1.0 cm) were filled in different directions (e.g. 0°, 45°, 135° and 90°) were prepared using nozzle and line distance of 0.41 mm. Prior to compression tests, the samples were swollen in distilled water for 4 days until no further water uptake could be observed. 3D printed hydrogels were tested using a uniaxial mechanical tester (Zwick Roell - model Z010 Universal Testing System) with a load cell of 200 N. Stress data were recorded at compression rate of 1 mm/min at temperature of 25 °C and relative humidity of 55 %. A pre-load of 0.05 N and 70 % of compression strain were set. A minimum of 5 samples per filling pattern was used to characterize each hydrogel.
2.7. Functional properties characterization

2.7.1. Swelling properties

The swelling capacity of the hydrogels was determined on 1.0 × 1.0 × 1.0 cm³ 3D printed samples at the temperature of 25 °C. With this experiment, we can calculate the equilibrium moisture content (EMC) starting with a fully dried sample of the hydrogel, as follow:

\[
EMC(\%) = \left( \frac{W_s - W_d}{W_d} \right) \times 100
\]

where \( W_s \) is the weight of the swollen hydrogel, and \( W_d \) is the weight of the dry sample. The effect of reversible swelling was investigated by repeating such drying and swelling procedure over several cycles.

2.7.2. Antimicrobial properties

The activity of the modified hydrogels against bacteria *S. aureus*, *S. arlettae*, *E. coli* and *P. fluorescens* was evaluated by adapting the procedure developed by Schubert, Engel, Thony-Meyer, Schwarze, and Ihssen (2012), as follow. First, 3D printed samples (1.0 × 1.0 × 1.0 cm³) of modified hydrogels containing EPL-MA in two different concentrations (1 and 2.5 wt%) and the control without EPL-MA were prepared. The printing conditions were set as follow: pressure of 1.5 × 10⁵ Pa, at 10 mm/s, nozzle offset of 0.32 mm and nozzle diameter of 0.41 mm.

After curing, the samples were thoroughly washed with distilled water using dialysis membrane over a period of 5 days. A minimum of 15 samples per group was used to characterize each hydrogel. After, excess liquid was removed by placing the samples on sterile paper towels and the hydrogel surface was inoculated with 40 μl of either a gram-positive or -negative bacterial suspension diluted to an optical density of 600 nm – negative bacterial suspension diluted to an optical density of 600 nm. The inoculated hydrogel surface was then placed on an agar plate. The growth of bacteria for 20–28 hours at optimal conditions on agar plates was visually determined. Growth was determined as follows: 0: no growth, 1: weak growth, 2: intermediate growth, 3: strong growth and 4: contact area completely grown.

3. Results and discussion

3.1. General overview of ink preparation, printing and functionalization of hydrogels

The manufacturing of complex-shaped NIPAM-based hydrogels with high loadings of cellulose nanocrystals (CNC) was carried out using three main steps: A) assuring the homogeneous dispersion of the ink components using planetary and mechanical mixing procedures, B) 3D printing of cellulose scaffold with textured cellular architecture by highly aligning the anisotropic CNCs upon printing and C) UV curing of the printed scaffold. These steps are illustrated in Fig. 1 and described in more detail below.

The dispersion of high loadings of nanoparticles in either aqueous or non-polar solvents is, in general, non-trivial and requires laborious work. Nevertheless, a good dispersion of the ink components, especially the nanocelluloses, is a crucial step to successfully print such materials using the direct ink writing (DIW) technique (Fig. 1A). The absence of aggregates (corresponding to a good dispersion of ink components), was evaluated using cross-polarized light microscopy. The resulting optical microscopy images of nanocellulose-based inks are presented in Figs. S1 and S2 (Supplementary Information). The DIW technique consists in the extrusion of a fluid through a nozzle and deposition onto a substrate as depicted in Fig. 1B. After the printing step, the sample undergoes a post-polymerization step to ensure and tune its mechanical properties (Fig. 1C). The polymerization of CNC-based hydrogels is achieved by UV-curing the printed parts under nitrogen (N₂) atmosphere to reduce the oxygen inhibition of the monomer (NIPAM). In Fig. 1D(I–V) we schematically represent the fabrication explored in this work from the ink preparation to the realization of aligned CNC 3D printed PNIPAM hydrogel with controllable shape-changes, mechanical and functional properties.

All steps of the ink preparation, alignment of anisotropic nanoparticles, swelling and final properties of the printed parts are discussed in the following sections.

Fig. 1. Schematic illustration of the steps involved in the synthesis and 3D printing of functional cellulose-based hydrogels and the testing of their properties. A) Ink formulation. B) Direct ink writing of cellulose-based polymer ink and effect of extrusion on the alignment of cellulose nanocrystals during the flow of the ink within the nozzle. C) Post-treatment to cure the printed structure in functional parts. D – I–V) characteristics and properties evaluated for the inks and final hydrogels for functional applications.
3.2. Ink properties and rheological characterization

The fabrication procedure of our nanocellulose-NIPAM hydrogel inks is simple and easy to control. The hydrogel inks consist of rod-like stiff cellulose nanocrystals (CNCs), or a combination of these with flexible cellulose nanofibers (CNF), suspended in an aqueous solution of N,N-isopropylacrylamide (NIPAM), a crosslinker ethyleneglycol-dimethylacrylate (EGDMA) and a photoinitiator (Irgacure 2959), allowing the system to be polymerized after printing (Fig. 2A). To achieve antibacterial properties in the hydrogel network, we functionalized dymethylacrylate (EGDMA) and a photoinitiator (Irgacure 2959), isopropylacrylamide (NIPAM), a crosslinker ethyleneglycol-cellulose nanofibers (CNF), suspended in an aqueous solution of cellulose nanocrystals (CNCs), or a combination of these with flexible nanocrystals that were used, and the rheological behaviour of the resulting CNC-PNIPAM hydrogels: A) CNC-PNIPAM hydrogels not modified with ε-polylysine (EPL) with methacrylic acid (MA) according to the procedure developed by Zhou et al. (2011) and then added it to the hydrogel ink prior to photopolymerisation (Fig. 2B). Glucose and glucose oxidase were added to the system as oxygen scavengers to ensure a sufficient UV curing under controlled nitrogen atmosphere. As a first demonstration, a simple cubic structure has been printed with the nanocellulose-NIPAM hydrogel ink (Fig. 2). This structure is composed of 32 layers of 320 μm and demonstrates the precise fabrication of multi-layered objects, with proper adhesion between the printed layers without delamination of the filaments.

In previous work, we have demonstrated that the rheological properties of similar inks (including shear-thinning behavior, rapid elastic recovery, well-defined yield stress and elastic modulus) are the most important parameters to ensure high shape fidelity, with no distortion of single printed filaments, in the DIW process (Siqueira et al., 2017). Since our main goal in this work is to achieve a high degree of alignment of the nanocelluloses within the printed parts, we have formulated and printed CNC-NIPAM hydrogels with high CNC or CNC/CNF loadings of up to 35 wt%. The CNCs have an average length of 115 nm with a diameter of 7.5 nm, and thus possess an aspect ratio, s, of about 15 (Fig. 2D). The morphological characteristics of TEMPO-CNFs, in particular their high aspect ratio and the resulting entangled network structure, can be seen in Fig. S3 (Supplementary Information). Both types of nanocellulose particles (CNC and CNF) play crucial roles in modifying the rheology of the inks and as anisotropic reinforcements in the final printed hydrogels. In our study, we optimized the ink properties to maximize the degree of orientation of cellulose nanoparticles within the printed structures. The flow-induced orientation of CNCs and CNFs is only possible if the applied stress during printing exceeds the yield stress of the ink (i.e. the differential-flow regime) (Siqueira et al., 2017). We evaluated the rheological properties of inks with 15–35 wt% of CNCs, and also developed inks containing 14 wt% CNC and 1 wt% CNF and inks with 19 wt% CNC and 1 wt% CNF. The rheological properties of the inks containing different amounts of CNC/CNF particles are presented in Fig. S4A–D (Supplementary Information); all of these inks are 3D printable, illustrating the versatility of the formulations and printing method. However, we decided to focus on three ink formulations: containing 20 wt% or 25 wt% CNCs only, or 14 wt% CNC and 1 wt% CNF. These formulations were chosen because they had the best combination of rheological properties for ease of printing with the best observed alignment of the nanoreinforcements.

The rheological behavior of these CNC-NIPAM hydrogel inks is shown in Fig. 2E and F. The pure NIPAM ink (0 wt% CNC) exhibits a constant viscosity (η) of 1.3 × 10⁻³ Pa.s at shear rates higher than 10 s⁻¹ (Fig. 2e). This means that the pure NIPAM ink would freely flow through the nozzles at modest pressures but it does not possess the ability to support itself after being extruded from the printing needle. The addition of nanocelluloses allows to transform the pure NIPAM ink into a viscoelastic fluid (gel-like material) ready to print. In contrast with the pure NIPAM ink, the CNC-NIPAM inks containing 20 and 25 wt% CNC possess viscosities that decrease several orders of magnitude as the shear rate increases from 0.001 to 50 s⁻¹ (Fig. 2E). Because of their high shear thinning behavior, these inks exhibit viscosities ranging from 8.92 to 18.20 Pa.s at shear rate of 50 s⁻¹, which is a typical value applied during DIW process.

Fig. 2. Conceptual illustration of the hydrogels with nano-structured architectures used in this work, also showing the morphology of the wood pulp cellulose nanocrystals that were used, and the rheological behaviour of the resulting CNC-PNIPAM hydrogels: A) CNC-PNIPAM hydrogels not modified with ε-polylysine (EPL) and B) CNC-PNIPAM hydrogels modified with ε-polylysine (EPL-MA). C) 3D printed cubic structure of CNC-PNIPAM hydrogel loaded with 20 wt% of CNCs (1cm³). D) Transmission electron image of anisotropic CNC particles (scale bar: 100 nm). e) Steady-shear and f) oscillatory rheological measurements (frequency (1 Hz) for the PNIPAM-hydrogels with varied solid loading (20 and 25 wt% CNC).
To assess the viscoelastic properties of the CNC-NIPAM hydrogel inks, oscillatory measurements at low strains were carried out (Fig. 2F). These experiments showed that the selected inks (20 or 25 wt% CNCs) mainly exhibit elastic behavior at low shear rates (\(G' > G''\)) and a well-defined dynamic yield stress \(\tau_y\), at the crossover point between \(G'\) and \(G''\). The dynamic yield stress varies from 425 to 867 Pa for the inks containing 20 and 25 wt% CNCs, respectively. Similar behaviors on the rheological profiles were observed for the inks containing different CNC contents or for the ones possessing 1 wt% of CNFs in their formulations (see Fig. S4A–D in Supplementary Information).

3.3. Printed-induced and quantified nanocellulose alignment

To investigate the effects of flow-induced orientation we printed 3D and 2D patterns (Fig. 3A and B) using the developed inks and observed how these shapes changed when the hydrogels were allowed to swell in water.

A quantification of the degree of alignment of the CNCs is necessary to allow a reproducible tailoring of the (post-hydration) 3D structure of printed objects with anisotropic actuation, and we used wide-angle X-ray scattering to do this. In previous work on inks containing high nanocellulose contents (Hausmann et al., 2018; Siqueira et al., 2017) we determined the parameters for differential and plug flow regimes as a function of the CNC concentration in gel-like inks and showed that flow-induced CNC alignment is only possible if the applied stress exceeds the yield stress of the inks. Fig. 3B illustrates our ability to control the printing process and shows that a high degree of CNC alignment within the printed filaments can be obtained as long as an appropriate combination of needle diameter and nanocellulose concentration is chosen. (The swelling behaviour of hydrogels for other nanocellulose concentrations and needle diameters is shown in Supplementary Information Fig. S5).

To investigate the flow-induced orientation of anisotropic CNC particles in the printed hydrogels, we carried out 2D wide-angle X-ray scattering (2D-WAXS) measurements of the nanocellulose-NIPAM hydrogels and the pure matrix (Fig. 3C–E, Table S2 and Fig. S6-Supplementary Information). In agreement with our previous studies (Hausmann et al., 2018), the results clearly show more pronounced CNC alignment for the hydrogels (20 wt% CNC) printed with the 410 \(\mu\)m nozzle \((\pi = 86\%)\) as compared to the ones printed with 840 \(\mu\)m \((\pi = 79\%)\) indicated by the full width at half maximum (FWHM) values (Fig. 3D). The pure NIPAM matrix shows no preferential orientation, whereas the printed CNC hydrogels show preferred orientation of CNCs along a printed filament, regardless of the nozzle diameters.

The ink rheology combined with this high degree of alignment allows the printing of 3D structures with intricate architectures, including free-standing components with angles of up to 45\(^\circ\), without the need for rheological modifiers others than the nanocelluloses themselves (Fig. 3A).

Nanocelluloses are able to constrain the swelling and/or shrinkage of the NIPAM structures in the direction of reinforcement, similarly to those observed in biological tissues such as in pine cones (Dawson,

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**Fig. 3.** CNC orientation within 3D printed NIPAM hydrogels. Simple 3D printed A) Complex and angled honeycomb 3D printed structure using nanocellulose-NIPAM hydrogel (20 wt% CNC). B) Bilayer strips of CNC-NIPAM hydrogel. C) 2D-WAXS patterns of pure NIPAM matrix and 3D printed CNC-NIPAM hydrogels (20 wt%) using 840 \(\mu\)m and 410 \(\mu\)m diameter nozzles respectively. D) Normalized 2D-WAXS azimuthal intensity distributions of the equatorial reflection (200) of 3D printed CNC-NIPAM hydrogel (20 wt% CNC) focused on the axial direction of the printed filaments for printed structures with 840 \(\mu\)m and 410 \(\mu\)m diameter nozzles. The inset image in D) shows the 3D printed grid and the location of the X-ray beam spot where the scattering measurements were performed. E) Dependence of hydrogel swelling behaviour as a function of the degree of orientation and printing nozzle diameters.
nature, especially in plants, the basic mechanism underlying the actuation by the swelling or shrinking (shape-changing) of cell walls is the orientation of cellulose fibrils in a swellable natural polymer matrix (Burgert & Fratzl, 2009; Erb et al., 2013). We investigated the dependence of the anisotropic swelling of composites upon the nozzle diameter used when printing (410–840 μm) (Fig. 3B). In nature, especially in plants, the basic mechanism underlying the actuation by the swelling or shrinking (shape-changing) of cell walls is achieved by the orientation of cellulose fibrils in a swellable natural polymer matrix (Burgert & Fratzl, 2009; Erb et al., 2013). We investigated the dependence of the anisotropic swelling of composites upon the nozzle diameter used when printing (410–840 μm) (see Fig. 3E), the CNC concentration (20 or 25 wt%) or nanocellulose morphology (CNC and CNF) (see Supplementary Information Fig. S5). Both shear and extensional flows impose orientation of anisotropic particles in fluids (Håkansson et al., 2014; Nessei, Rock, Wang, Kessler, & Gozen, 2017). Considering only shear stresses, the use of the smaller nozzle diameter (410 μm) results in higher shear forces from the walls of the nozzle than larger diameter nozzles. As a result of this, a formulation containing 20 wt% CNC extruded through a needle of 410 μm in diameter requires a pressure of about 1.5 bar to induce alignment of the cellulose nanoparticles. However, when the larger needle diameter of 840 μm is used with the same ink formulation, only 0.3 bar is necessary to enable the extrusion of the 20 wt% CNC ink. Consequently, as the shear forces on the wall of the nozzles are lower, the cellulose nanoparticles’ degree of alignment and anisotropic swelling for the composites printed with 840 μm nozzles are inferior than the ones printed with the 410 μm nozzles. The higher anisotropic swelling effect found for the ink containing 1 wt % of CNF and 14 wt% CNCs is ascribed to the physical interactions between the nanofibers. Such interactions, named entanglements, contribute to an even higher reduction of swelling along their orientation direction (Hausmann et al., 2018).

3.4. Soft actuation of printed bilayer structures

To assess the anisotropic swelling properties of the CNC-NIPAM hydrogels, we printed bilayer strips with at least two different orientations of filaments and observed their actuation over time as the inks were hydrated. These experiments confirmed that the anisotropic swelling of the nanocellulose-NIPAM hydrogels could lead to a macroscopic programmable change in shape of the synthetic printed structures.

To compensate the changes in rheology due to the presence of CNF, we reduced the CNC concentration in the printed materials (Fig. 4A) to 14 wt% (overall 15 wt% of nanocellulose is present in the inks) aiming for maximal swelling actuation and shape changes. Akin to the cellulose fibrils microreinforcements in plant cell walls, in our system, shape motion occurs because the nanocelluloses do not swell in their axial direction (Burgert & Fratzl, 2009a, 2009b). On the contrary, swelling will occur preferentially in the orthogonal direction to the nanocellulose orientation within the printed filaments, which result in a highly anisotropic deformation of the structure upon water uptake. Therefore, the programmable shape change in our system is achieved due to the orientation of stiff nanocellulose reinforcements within the hydrogels. These aligned nanocelluloses create internal stresses when the structures swell which can only be reduced by undergoing a deformation (Erb et al., 2013; Le Ferrand et al., 2016). The transformation of the printed bilayer from a flat to twist/bended or curled/bended configuration follows the programmable designed direction. However the final twisted, curled, or bended architectures of the swelled bilayer structures are governed by the nanocellulose orientation in the upper layer as it is less affected by misalignment, as proved by 2D-WAXS measurements.

![Fig. 4. 3D printed structures of nanocellulose-NIPAM hydrogels with swelling and anisotropic actuation behaviours. A) 3D printed bilayer structures of NIPAM hydrogels with 14 wt% CNC and 1 % CNF after swelling in water. The schemes in the right and top show printing patterns (0/90° and 45/135°). The lines drawn on the top of printed structures indicate better the printing pattern and bending according to predictions. B) Evolution of water uptake of nanocellulose-based NIPAM hydrogels on 9.2 × 9.2 × 10 mm samples. Error bars show standard deviation (n = 5). C) Combination of two bilayer strips produced by 3D printing of nanocellulose-NIPAM hydrogels (20 wt% CNC) leading to synthetic architectures that twist. On the left is the scheme of the printing pattern (45/135° filling) and on the left side, pictures of the evolution of the anisotropic actuation of the printed structures.](image-url)
(Fig. 4A and Table S1). This is in contrast to the first printed layer which has a lower degree of orientation of nanocelluloses due to the fact that the filament is squeezed closer to the glass substrate to ensure proper adhesion between this layer and the substrate. This extra pressure was obtained by using a smaller nozzle offset for this layer than in the second layer.

Time-dependent swelling tests were conducted to quantify the maximal swelling capability of the hydrogels prior to mechanical testing and actuation performance (Fig. 4B). The 3D printed cuboids (9.2 × 9.2 × 10 mm) produced with 0° filling pattern showed the highest swelling rate in the beginning of the tests due to the high osmotic pressure between water and the dried hydrogel. The equilibrium moisture content is reached after 4 days when the osmotic pressure is equal to the retractive forces of the stretching polymer chains (Buenger, Topuz, & Groll, 2012). The reversible swelling allows the generation of composites with shape-memory characteristics after drying cycles of printed hydrogels in oven at 60 °C (Video S1 and Fig. S7 Supplementary Information).

The twisting transformation is generally not possible in simple synthetic bilayer materials. To achieve such chiral twisting motions, the reinforcing elements should be oriented with an angle of 45° or −45° from the first to second layer (Erb et al., 2013). We investigated the twisting motion on NIPAM-hydrogels reinforced with 20 wt% CNCs (Fig. 4C and video S2–Supplementary Information) to evaluate if such shape-morphing would also be possible with pure CNC-NIPAM-based inks. For this test we kept the printing pressures and offset constant to avoid or minimize possible misalignment of CNCs in the first printed layer due to variations of printing parameters. The results show that the two layers attempt to expand in perpendicular direction during hydration thus resulting in helically twisting motion, similar to the natural response found in plants as Bauhinia variegate (seedpod of orchid trees) and climbing plants coil tendrils (Erb et al., 2013; Studart & Erb, 2014).

3.5. Structural characterisation of printed materials

Control over the orientation of nanocellulose particles enables tailoring of mechanical properties of 3D printed hydrogels in specific directions (Fig. 5A–C). We investigated the effect of cellulose nanocrystals alignment on the mechanical behaviour of neat NIPAM and CNC-NIPAM hydrogels by measuring the compressive mechanical properties of specimens containing CNCs aligned in the longitudinal or transverse direction relative to the applied load Fig. 5A–C). While the hydrogel matrix alone has a soft and stretchable behaviour with a
Young's modulus of only about 70 Pa (Fig. 5A), the reinforced sample containing 20 wt% CNCs tested in the transverse direction had on average a Young's modulus of 13 (0° filling) to 16.6 kPa (for samples filled at 45/135° and 0/90°). This corresponds to an increase of the Young's modulus by a factor of 236 compared to the pure matrix. Likewise, the reinforcing effect of CNCs on the mechanical properties of NIPAM hydrogels is even more remarkable when comparing the properties of the pure hydrogel matrix with the composites reinforced with 25 wt% CNCs, regardless of the filling pattern. The Young's modulus of composites loaded with 25 wt% CNCs, tested in the transverse direction with filling patterns of 0°/90° and 45/135° (Fig. 5B), is closer to 3-orders of magnitude (650x) higher than that of the pure hydrogel matrix. However, the Young's modulus remains nearly the same for the 20 wt% CNC-NIPAM hydrogels tested in the longitudinal and transverse directions. The increase in the elastic modulus with increased CNC concentrations (from 20 to 25 wt%) is accompanied by a decrease of at least 13% in the strain at rupture.

The average ultimate stress properties of the composites clearly reveal a significant influence of the testing direction relative to the orientation of the CNCs within the 3D printed filaments (Fig. 5C). Such an effect is observed in composites reinforced with 20 wt% CNCs and it is clear for all samples regardless of the filling pattern. However, the enhanced mechanical properties of the composites, in the longitudinal direction, become more pronounced for the samples tested at 0° filling pattern due to the orientation of CNCs. Hence, in such condition, we likely maximize the CNCs orientation with the probe direction. These results illustrate our capability to precisely control the CNC orientations and, therefore, the mechanical properties of the hydrogels by designing inks with varied CNC loads and controlling the printing fillings and parameters as needle sizes, pressure and speed.

3.6. Extended hydrogel functionalities

Combining natural antimicrobial peptides, such as ε-polylysine, with the 20 wt% CNC-NIPAM hydrogels would allow to broaden the spectrum of applications of our complex-shaped and textured materials (Fig. 6A). To accomplish this, we functionalized ε-polylysine (NMR spectrum Fig. S8-Supplementary Information) with methacrylic acid. The success of this chemical modification, as shown by nuclear magnetic resonance spectroscopy (NMR), is demonstrated in Fig. S9 (Supplementary Information). Antimicrobial properties of 3D printed materials were achieved for contents of EPL-MA in the hydrogels varying from 1 to 2.5 wt%. Recognized more than 30 years ago as antimicrobial agent, the mechanism responsible for the antimicrobial activity of EPL is not completely understood (Hyldgaard et al., 2014). However, it has been suggested that such cationic polypeptide interacts with negatively charged cell surface by ionic adsorption followed by microbial cell membrane interaction, membrane disruption and ultimately cell lysis (Salomé Veiga & Schneider, 2013). Significant reduction of bacteria growth compared to the control (Fig. 6B) was determined with the Kruskal-Wallis test and Mann-Whitney U test for pairwise comparison. A significant deviation (P < 0.05) from the control (no EPL-MA) was identified in all the samples where 1 or 2.5 wt% EPL-MA were added (Fig. 6B II and III). The quantitative results presented in Fig. 6C also indicate strong and significant reductions of both, gram positive and gram negative, bacterial growth in the hydrogels prepared with EPL-MA when compared to the biofilm formation in the control. This study reveals the effectiveness of the antimicrobial properties added to the final 3D printed nanocellulose hydrogels given by EPL-MA, but other more clinically relevant or specific antimicrobial agents could also be considered.

To understand the effect of EPL on the final properties of the NIPAM hydrogels we prepared inks containing 1 or 2.5 wt% EPL and measured their rheological properties (steady-shear and oscillatory at the...
frequency of 1 Hz) and compared these results with the ones of the ink without EPL. The results (Fig. S12A - Supplementary Information) indicate that all inks presented shear thinning, in which their viscosities decrease by several orders of magnitude as the shear rate increases from 0.001 to 50 s⁻¹ (typically applied during DIW). It also is noticed that the viscosities of the inks containing EPL are in the same range as the one without EPL. The amplitude sweep tests (Fig. S12B - Supplementary Information) show that all inks present G' > G'', and dynamic yield stress in the order of a few hundred Pa indicating that all of them can be considered in the range of printable inks without the need for applying prohibitive high printing pressures. Nevertheless G' and G'' are significantly higher for the inks containing EPL thus indicating possible differences in their final mechanical properties after polymerization, however such properties were not investigated in the present work. EPL-MA has the possibility to crosslink which may also lead to increased mechanical properties of the hydrogels.

4. Conclusions

In summary, complex shape morphing nanocellulose-based composites have been produced through direct ink writing 3D printing. Alignment of high aspect ratio nanocellulose particles along the ink flow direction occurs as a result of the shear and extensional forces in the print nozzle, giving rise to anisotropic mechanical properties and swelling behavior of the printed structures. The ability to produce hydrogel based 3D printing inks in which both the nanocellulose content (up to 35 wt%) and morphology (cellulose nanocrystals and/or cellulose nanofibers) can be varied allows to tune the mechanical properties of the printed structures along specific directions. Because of the high degree of nanocellulose alignment upon printing, hydrogel structures with complex architectures (angles and texture) and programmable self-shape actuation can be fabricated with these new inks. This is an elegant method to synthetically create structures that can, upon hydration, bend or twist — resemble the mechanism in plants which use the orientation of cellulose fibrils. The simplicity of the synthesis and printing procedures demonstrated here mean that this approach has great potential to be extended to similar materials such as hydrogels used for wound healing. The antimicrobial properties provided by functionalization of the hydrogels with modified ε-polylysine highlights the potential use of this and related AMPS in biomedical applications of composite hydrogels.

Author contributions

Experiments were designed and coordinated by G.S., T.Z., G.N. and O.F., and were conducted by O.F. and M.H. The X-Ray analysis was performed by A.N. Figure graphic designs were prepared by M.H. and O.F. Antimicrobial tests were carried out by O.F. and M.S. G.S. wrote the manuscript with input from all coauthors. All authors reviewed and commented on the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2021.117716.

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