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Historical perspective

Engineering nanocellulose hydrogels for biomedical applications

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ABSTRACT

Nanocellulose hydrogels are highly hydrated porous cellulosic soft materials with good mechanical properties. These cellulose-based gels can be produced from bacterial or plant cellulose nanofibrils, which are hydrophilic, renewable, biodegradable and biocompatible. Nanocellulose, whether fibrils (CNF), crystals (CNC) or bacterial (BNC), has a high aspect ratio and surface area, and can be chemically modified with functional groups or by grafting biomolecules. Cellulose functionalization provides enhanced physical and chemical properties and control of biological interactions, tailoring its hydrogels for specific applications. Here, we critically review nanocellulose hydrogels for biomedical applications. Nanocellulose hydrogels have been demonstrated for 3D cell culture, mimicking the extracellular matrix (ECM) properties with low cytotoxicity. For wound dressing and cartilage repair, nanocellulose gels promote cell regeneration while providing the required mechanical properties for tissue engineering scaffolds. The encapsulation of therapeutics within nanocellulose allows the targeted delivery of drugs. Currently, cellulose crosslinking to peptides and proteins enables a new generation of low cost and renewable smart materials used in diagnostics. Last, the organized mesh of fibres contained in hydrogels drives applications in separation of biomolecules and cells. Nanocellulose hydrogels have emerged as a highly engineerable platform for multiple biomedical applications, providing renewable and performant solutions to life sciences.

Contents

1. Introduction

Hydrogel refers to a class of soft materials formed by a porous three-dimensional network of crosslinked polymer chains which hold large quantities of water (up to 99.9%). They are produced from natural or

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synthetic sources and show unique properties of biocompatibility allied to excellent mechanical properties [1]. Hydrogels can be synthesized to become environment sensitive by altering their structure and properties upon a change of pH, ionic strength, pressure, light, temperature, electric and magnetic fields [2–5]. Hence, these are engineerable materials for multiple technological and industrial applications. Hydrogels are particularly promising in the fields of biology and medicine [6]. Indeed, hydrogels have been extensively explored as scaffolds, contact lenses, wound healing and dressing, hygienic products, drug delivery and diagnostic devices [7–11].

These biomedical applications rely on the ability of hydrogels to mimic the physical and biochemical conditions found in the human body or demanded by biological systems [12,13]. Each application has specific requirements. For example, cells and organoids cultures require a flexible 3D mechanical support coated with adhesion proteins which possesses a sol-gel transition enabling the manipulation of the biological material at different phases [14]. Engineered-cartilage and skin regeneration in gels necessitate the control of mechanical properties (strength, rigidity, elongation) and easy processing into complex shapes [15,16]. Drug delivery systems aim to protect the therapeutic agent until it reaches the target-site which triggers a controlled kinetics of drug release [17]. Diagnostics demand gels to selectively retain a specific marker or biomolecule at very low concentration and the ability to easily communicate the presence or concentration of this analyte [18]. Finally, separation processes require a network of uniform mesh size distribution, the ability to control this mesh size and to functionalize the medium. Therefore, strong mechanical properties and structure stability over a wide range of pH, ionic strength and temperature are essential [19].

However, a few critical factors have hindered the extensive use of hydrogels in the biomedical field. The removal of toxic polyfunctional crosslinking agents when synthesizing hydrogels is time-consuming and residues can remain in the medium [20]. Also, the polymers used are most often derived from non-biodegradable and non-recyclable synthetic origin, challenging its environmental sustainability [21,22]. Hence, there is a need for natural resources which can easily be engineered into biocompatible hydrogels-based products.

Cellulose has emerged as a compelling sustainable, non-toxic and renewable material for water-based gels. While starch and dextran are designed by nature for the storage and slow release of energy, cellulose provides a structural role to plants and bacteria [23–25]. All these polysaccharides have glucose as monomer in common [26–29]. The main difference between them are the molecular weight, the structures and the bonds linking their monomers. These are exclusively α-1,4 glycosidic bonds for amylose, a combination of α-1,4 and α-1,6 for amylopectin with some additional α-1,3 for dextran [24,30]. The cellulose units are solely linked by β-1,4. These different bonds govern the chain structure of the glycosidic polymers (linear or branched chain, head to head *versus* head to tail link) and therefore their hydrogen bonding ability, their organization into hierarchical structures which both affect solubility in water, random coil configuration and mechanical properties of the formed structures [23,31]. While dextran and starch dissolve into polymeric solutions in water, cellulose is insoluble and forms colloidal suspensions. Cellulose gels present a statistical network with meshes of different diameter and rigidity, and different crosslinking-densities [29,32,33]. The biodegradability, non-toxicity, flexibility and biocompatibility of nanocellulose hydrogels have been proven suitable for multiple biomedical proposes.

This article critically reviews the current progress in engineering and applications of nanocellulose hydrogels in the biomedical field. It identifies the unique characteristics offered by cellulose gels interfacing with biomolecules and cells (Fig. 1). The first part of this review highlights the formation of nanocellulose hydrogels and analyses their physical and chemical properties in relevance to biomedicine. The second part focuses on recent findings and highlights critical issues of cellulose-based hydrogels for biomedical purposes. Last, the gaps in knowledge are analysed and the perspectives for future development and their requirements are projected. It is the objective of this review to fully explore the potential of nanocellulose hydrogels as a renewable and performant platform for biomedical applications.

2. Formation of nanocellulose hydrogels

2.1. Types and characteristics of nanocellulose

Nanocellulose was first described by Bengt Rånby in 1951 as bundles of cellulose molecules forming micelles in aqueous colloidal solutions [34]. Over the last decades, many studies have described the preparation and properties of nanocellulose [35–37]. Of special clarity are those of De France, Dufresne and Isogai [31,38,39]. Nanocellulose can be extracted from wood or plants by mechanical, enzymatic or chemical treatment to first remove the residues of lignin and hemicellulose and second to deconstruct the cellulosic hierarchical structure [40]. It is also isolated from bacteria [41]. The term “nanocellulose” broadly refers to 3 distinct types of fibrils: cellulose nanofibers (CNF), cellulose nanocrystals (CNC) and bacterial nanocellulose (BNC), each having different dimensions and properties [42].

TEMPO-mediated oxidation, developed by Isogai, is the most popular method to extract cellulose nanofibers (CNF) from wood pulp; it produces nanofibers of 5–60 nm in diameter and several microns in length. CNC is obtained by strong acid hydrolysis of cellulose pulp, dissolving the amorphous parts of the fibrils while keeping their crystalline domains intact [43]. The extracted CNC are of 5 nm in diameter with length of 20–100 nm. Depending on the type of cellulosic source and the treatment process, the size of both CNC and CNF varies. BNC can be synthesized from different bacteria strains, including *Gluconacetobacter xylinus*, *Agrobacterium*, *Rhizobium*, *Salmonella*, *Aerobacter*, *Escherichia*, *Sarcina Rhodobactor* and *Komagataeibacter*. *Gluconacetobacter xylinus* is the most common bacteria used for BNC production [44,45]. BNC has average diameter of 100 nm and length of micrometres. It is secreted by bacteria as an extracellular product of fermentation while cultivated in a glucose culture media [46]. Washing the cellulosic bacterial product with NaOH (usually 1 M) helps to extract BNC and remove the unwanted proteins residues. This nanocellulose is free of impurities such as lignin and hemicellulose and has a higher crystallinity (80%) than the nanocellulose produced from wood source [47]. Table 1 summarizes the diameter, length and characteristics for each type of nanocellulose [31,38–41]. Independent of its source, all types of nanocellulose fibrils can form hydrogels.

2.2. Mechanisms of hydrogels gellification

Cellulose hydrogels differ from most hydrogels as their constitutive polymer is water insoluble, while most others are water soluble. This means CNF, BNC and CNC form a colloidal suspension in water while other gels form a solution [2,39]. This has two major implications. The first one is that the critical dimension of the constitutive unit of nanocellulose is order of magnitude higher than in other gels. Indeed, nanocellulose are bundles of chains of diameter 5–50 nm and length up to the micron [31,39]. The second implication is that the mode of gel networking differs. Nanocellulose gels network are due to entanglement of fibrils combined with electrostatic stabilization; chemical crosslinking can further contribute to the strengthening of the structure [48].

Physical interacting forces include the electrostatic, hydrogen bond, chain entanglements, van der Waal, hydrophobic and ionic interactions. These reversible interactions disrupt easily when the interacting force between fibres disintegrates [49]. As example, TEMPO-based modified CNF contains anionic –COOH and CNC produced by sulphuric acid treatment comprises anionic -SO_3 groups, respectively. These charged CNF and CNC can be physically crosslinked by a bonding force characterized by the balance between the electrostatic and van der Waal forces

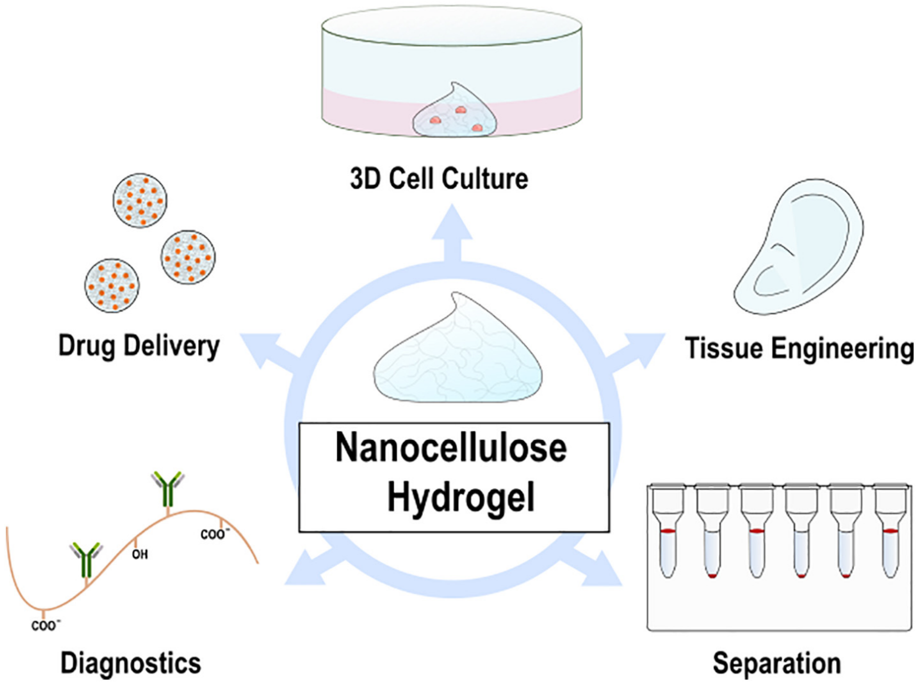

Fig. 1. Nanocellulose hydrogel is biocompatible, biodegradable and show potential for multiple biomedical applications. The five main categories of applications are: 3D cell culture, tissue engineering, drug delivery, diagnostics and separation of biomolecules.

[38,50]. Crosslinkers are also used to produce hydrogels by electrostatic or covalent crosslinking and hydrophobic interactions. Sodium citrate can be applied as electrostatic crosslinker to network cellulose fibres and form hydrogels in an acetic acid suspension at room temperature [51]. Networks of chemically crosslinked fibres are often strong and permanent. These are achieved *via* covalent bond by radical polymerization, chemical reactions, irradiation and enzymatic reactions. Cellulose fibres can be covalently crosslinked with chemical crosslinkers such as epichlorohydrin (ECH), metal ions, citric acid, succinic anhydride and many others [48,52,53].

Both crosslinking methods can originate a well-defined mesh of fibres/chains with a general macrostructure as defined in Fig. 2. Two individual chains are connected at the reticulation point, while a single chain can also connect to itself and form a closed loop. Free terminal segments are named dandling ends. When looking into the microstructure of the hydrogel, different association of fibres can be found. Physical crosslinking of fibres is normally characterized by the formation of helical structures with two or more chains. Similarly, the entanglement of fibres can form crystalline microstructures that varies in complexity and size according to the number of reticulation points and the chain length [49]. These types of associations are commonly found in biopolymer-based hydrogels, which is the case of nanocellulose hydrogels [2,54]. Chemical crosslinking microstructure, in contrast, usually varies in the association between single or multi-type of polymer chains, forming a homogeneous or blend gel, respectively. Independent of the bonding type occurring, the crosslinking density (CD) is responsible for the hydrogel porosity [55]. Different CDs produce variable spaces within the hydrogel network macrostructure; CD reflects changes in the pore size (ε), diffusivity and correlation length. The pore size plays an important role in the diffusion of different molecular entities (proteins or drugs) and gases within the material [56,57]. In addition, the number of inter-connected chains has a direct effect on the swelling ratio of the hydrogel, as well as on the shear modulus and mechanical properties [58,59]. Determining the proper crosslinking method during the

Table 1
Properties of cellulose nanofibres (CNF), nanocrystals (CNC) and bacterial nanocellulose (BNC).

Source	Properties
Cellulose Nanofibres (CNF) *Synonyms: Microfibrillated cellulose, cellulose microfibrils, nanocellulose fibres, nanowiskers*	- Produced by mechanical (high pressure homogenizer), chemical (TEMPO-mediated oxidation), enzymatic treatment - Plant and algae as sources - 5-60 nm diameter and several microns length (high aspect ratio) - Flexible and long fibres able of entanglement - Gel-like characteristics in water - Contains both crystalline and amorphous regions
Cellulose Nanocrystals (CNC) *Synonyms: Nanocellulose crystals, nanocrystalline cellulose, cellulose crystallites*	- Produced by strong acid hydrolysis (H_2SO_4, HCl) followed by ultrasonic treatment -Plant source - 5 nm diameter and 20–100 nm length - Elongated crystalline rodlike shapes - Rigid rods (elastic modulus ~ 100 GPa) - Crystalline: no amorphous regions
Bacterial Nanocellulose (BNC) *Synonyms: Bacterial cellulose, microbial nanocellulose*	- Produced as polymer by bacteria from low-molecular weight carbon sources - 20-100 nm diameter and several microns length in a continuous 3D network of CNF - Form stable hydrogels with up to 99% water - High crystallinity and pure cellulose (no hemicellulose, lignin) - Good mechanical stability

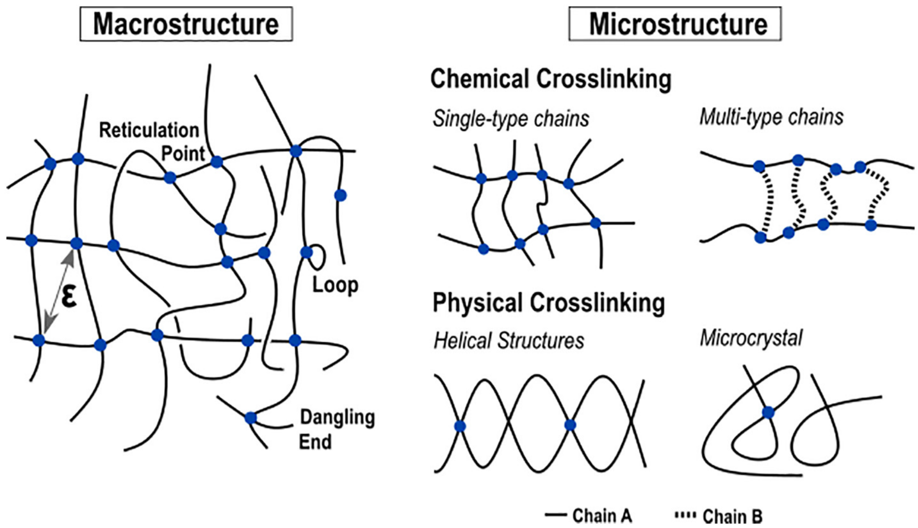

Fig. 2. The hydrogel gel macrostructure is formed by a well-defined mesh, where fibres connect at the reticulation point; the self-connection of fibres forms loops. Free terminal segments are named dandling ends. The pore size (ε) is directly proportional to the fibre concentration and inversely proportional to the crosslinking density. The microstructure of hydrogels is based in organized mesh for those chemically crosslinked and differ by the types of chains/fibres. Helical and microcrystalline microstructures are found in physically crosslinked hydrogels.

hydrogel synthesis dictates the gel structure which governs specific application efficiency.

2.3. Protocols for crosslinking nanocellulose

Dissolving cellulose chains in a solvent to promote crosslinking and the formation of hydrogels is challenging [60]. It is dictated by the balance between entropy and interactions, favouring and opposing solubility, respectively [61]. The ability to break both intra and intermolecular hydrogen bonds is required for an efficient dissolution of cellulose. The hydrophobic interactions are dominant over the cellulosic crystalline structure for solubility due to the polymer amphiphilic nature. This association plays a key role on cellulose solubility [62–64]. Different chemical procedures and modification protocols were developed for the dissolution of cellulose in water or organic solvents [65–67]. Cai et al. studied aqueous solutions of NaOH/Urea and LiOH/Urea to dissolve cellulose at temperatures as low as −10 °C [68]. Both alkali and urea hydrates assisted the cellulose dissolution, with better results for urea at 12% concentration. Once dissolved, a suspension of regenerated cellulose could form an optically transparent hydrogel crosslinked by epichlorohydrin cross-linker (ECH) at −3 °C [69]. Jun Liu et al. relied on cellulose nanofibers (CNF) derived from the TEMPO-oxidised method in conjunction with different types of hemicellulose (galactoglucomannan (GGM), xyloglucan (XG), and xylan) crosslinkers to produce a series of nanocellulose hydrogels [70]. They compared two different methods to prepare hydrogels: pre-sorption and *in situ* sorption of hemicellulose (Fig. 3). In the pre-sorption method, hemicellulose adsorbs onto the CNF network by hot mixing followed by swelling in water at room temperature for 24 h. For the *in situ* sorption method, hemicellulose adsorption and water addition (for swelling) were performed simultaneously to define the hydrogel structures. The pre-sorption method with XG cross-linker showed the highest sorption capacity onto the CNF [71,72].

In a different approach, metal ions can be used as ionic crosslinkers to produce bioinspired composite gels of enhanced toughness and stiffness; thermal and optical properties are also improved [73]. Hydrogelation of carboxylated cellulose fibres (CNF produced by TEMPO oxidation) is achieved with di or trivalent metal cations salts such as:

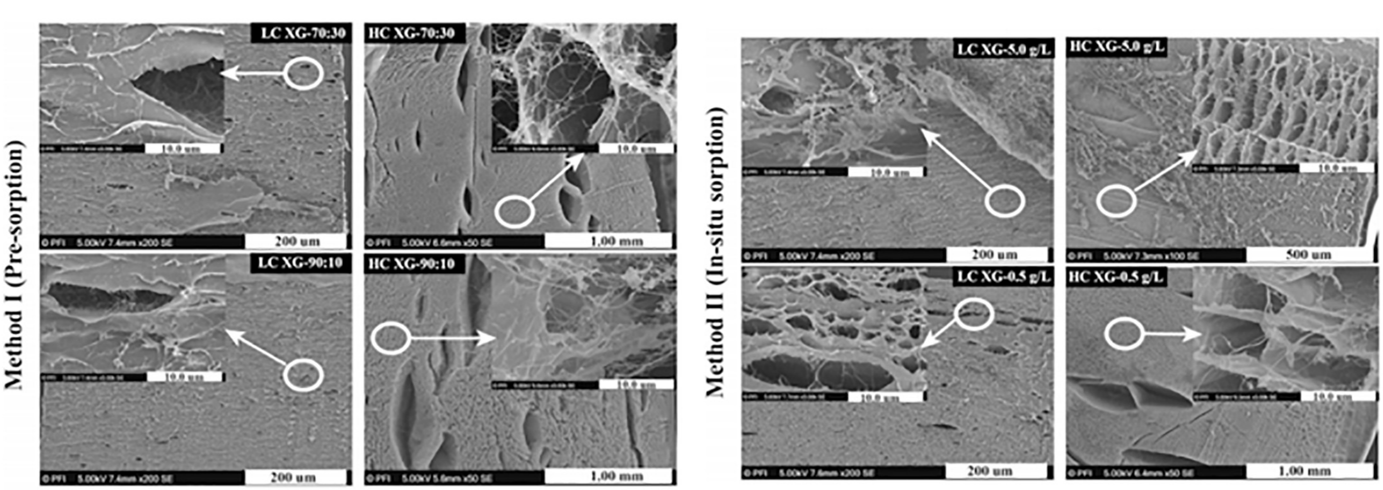

Fig. 3. SEM images and cross section of the freeze-dried nanofiber cellulose -xyloglucan (NFC-XG) composite aerogel using two different method: pre-sorption and *in situ* sorption of hemicellulose. Reproduced with permission from Ref. [70].

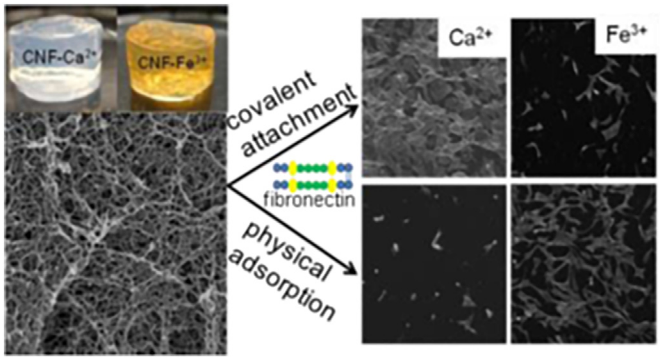

Fig. 4. SEM of nanocellulose hydrogels produced by crosslinking of carboxylated TEMPO-oxidised cellulose fibres with metal cations (Ca^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+} and Fe^{3+}). Reproduced with permission from Ref. [75].

Ca^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+} and Fe^{3+} (Fig. 4). The gelation starts rapidly upon addition of metal ion salts to the CNF aqueous suspension. The cellulose gelation happens by cation-carboxylate interactions screening the repulsive charges on the nanofibrils and also resulting in the formation of gel by ionic cross linking [74,75].

Similarly, Yang et al. produced CNF-polyacrylamide composites hydrogels by forming ionic and covalent bonds with multivalent cations (Ca^{2+}, Zn^{2+}, Al^{3+} and Ce^{3+}) (Fig. 5). Polyacrylamide forms covalent bonds, while metal ions contribute to ionic bonds [76]. The tensile strength and toughness of the hydrogels increased with the type of metal ions with $Zn^{2+} < Ca^{2+} < Al^{3+} < Ce^{3+}$. The strong ionic-covalent bonds resulted in hydrogels with enhanced mechanical properties.

Physically crosslinked cellulose nanofibers (CNF) hydrogels can be formed by increasing the surface charge density of the CNF at a given solid content. The high surface charge density hinders the agglomeration of CNFs in suspension by electrostatic repulsion and triggers entanglement of the CNF into hydrogel structures. Those with a low degree of entanglement remains as nanocellulose suspensions. Mendoza et al. produced nanocellulose hydrogels by physical crosslinking TEMPO-oxidised pulp. The chemical treatment was followed by fibrillation in a high pressure homogenizer with multiple passes depending on the concentration of suspension (Fig. 6A) [54]. Rheology characterization of hydrogels using amplitude, frequency and strain sweep assays was performed to determine the bulk mechanical properties, gelation mechanism and hydrogel flow behaviour [77,78]. Results showed the pH and salt-dependent stability for the gelation of nanocellulose hydrogel. Indeed, controlling pH and salt (concentration and type) tunes the electrostatic repulsion between cellulose fibres, which also affects the network bound water, resulting in stabilization and destabilisation of gel network (Fig. 6B) [2].

Similarly, bacterial nanocellulose (BNC) based hydrogels are formed by a variety of methods [45,79]. Hobzova and collaborators made hydrogels from BNC and poly (2-hydroxyethyl methacrylate) (PHEMA) by *in situ* UV radical polymerization of HEMA monomer in BNC structure [80]. Both BNC and PHEMA are biocompatible and produce hydrogels of tunable mechanical strength. The tensile strength of BNC-PHEMA composite hydrogel with 10 wt% BNC had a tensile strength and Young modulus, 80 and 120 times higher than the PHEMA gel. BNC-chitosan hydrogel composites prepared in EMIM-Ac ionic liquid effectively supported enzyme immobilization with higher adsorption capacity and catalytic activity than the microcrystalline-chitosan hydrogels [81]. Muller et al. performed static cultivation of *Komagataeibacter xylinus* bacteria to produce BNC hydrogels which were loaded with Bovine Serum Albumin (BSA) protein using the high speed vortex method [45]. The vortex method introduced 8% of BSA protein into the BNC hydrogel in 10 min; this is much faster than the conventional adsorption loading method requiring 24 h to incorporate the same amount of protein (Fig. 7).

These few examples demonstrate the variety of protocols available for the synthesis of nanocellulose hydrogels. Basically, the hydrogel preparation determines the network structure, with the size of the mesh determining the pore size distribution. Both structure and shape are affected by the density of crosslinking sites, controlled with the length and concentration of fibres, and the concentration and uniformity of distribution of crosslinking points. Normally translucid, the hydrogel appearance becomes turbid at higher solids content, or colourful with the presence of additives such as ions, proteins, polymers and copolymers. Choosing the most appropriate technique for processing nanocellulose hydrogel is guided by the final application aimed and its requirements.

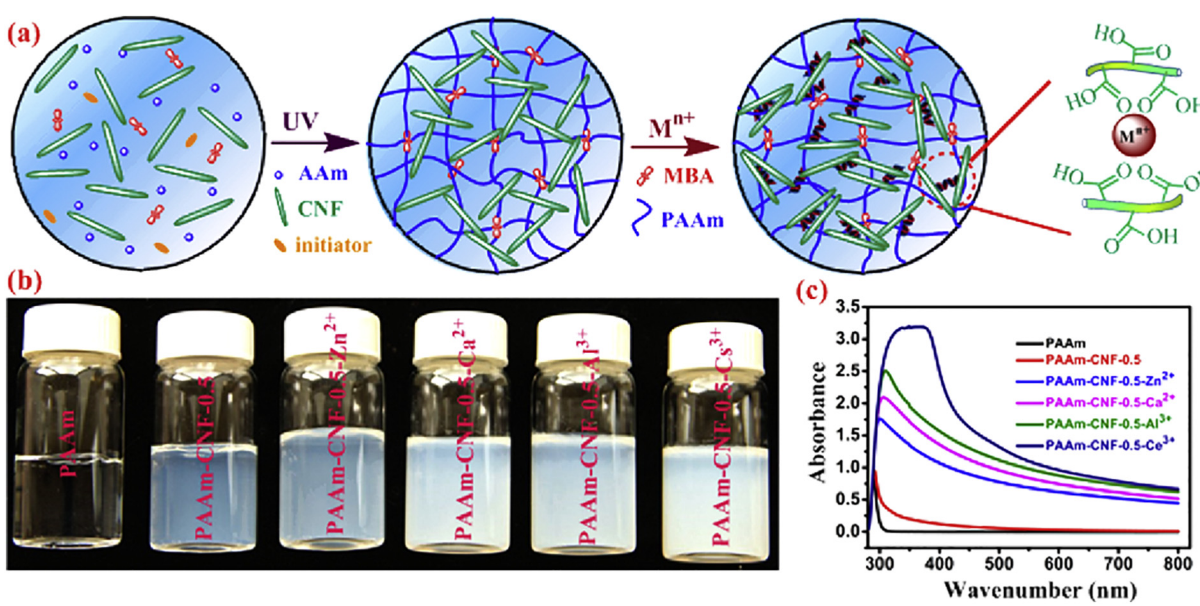

Fig. 5. (a) Schematic illustration of ionic hydrogel synthetic process that includes the *in-situ* polymerization to form composite hydrogels followed by immersion in a cation solution to produce ionic coordination. (b) Ionic coordination leads to CNF aggregation in ionic gels of increased opacity and (C) corresponding absorbance in UV/vis spectra. Reproduced with permission from Ref. [76].

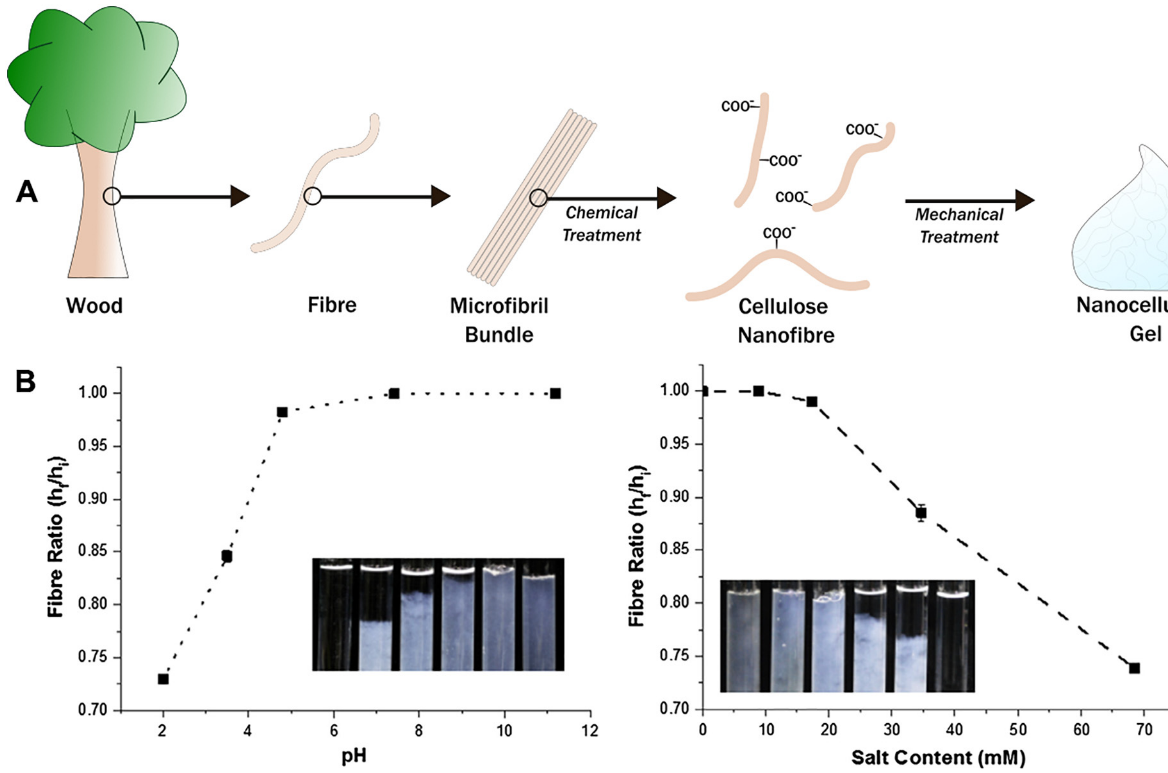

Fig. 6. (A) Representative process to produce nanocellulose gels by physical electrostatic repulsion. Cellulose fibres are obtained from wood as bundle of microfibrils. TEMPO-mediated oxidation allows to individualize cellulose nanofibres by converting hydroxyl into carboxylate groups. High shear mechanical treatments (such as high-pressure homogenization) transforms the nanocellulose into a hydrogel. (B) Effects of pH and salt content over colloidal stability of TEMPO-oxidised cellulose nanofibre gels. Reproduced with permission from Ref. [2].

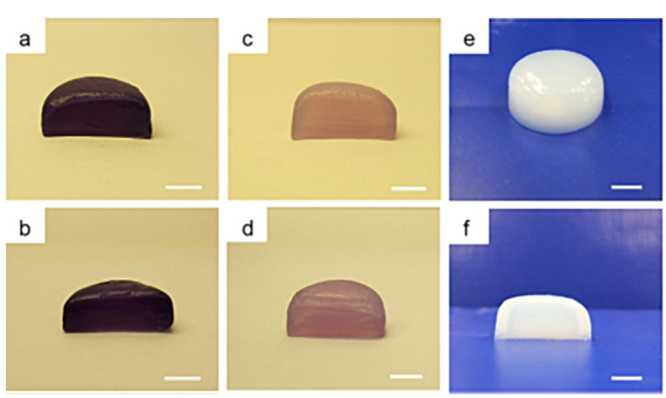

Fig. 7. Visualization of Bovine Serum Albumin (BSA) distribution in bacterial nanocellulose (BNC) (cross sections) by staining with BCA assay reagent. BNC loaded by adsorption (a) and high-speed technique (b), corresponding unloaded controls incubated only in buffer without BSA (adsorption, c; vortex treatment, d), and untreated, unstained BNC (e and f) (scale bars, 5 mm). Reproduced with permission from Ref. [45].

2.4. Hydrogel properties for biomedical applications

Non-toxic, biodegradable and biocompatible, cellulose-based hydrogels also have a high water content (99.9 to 95 wt%), good thermal and mechanical properties [82]. Nanocellulose shows a good swelling ability due to the osmotic effect, also referred to as counterion entropy. Briefly, charged polymer chains in aqueous medium must be surrounded by counterions to reach electrostatic neutrality. The swelling of cellulose fibres is dictated by the mobility of ions from the external solution diffusing toward the centre of the gel [83]. This capacity to absorb water allows the control of the pore size and its distribution within the local gel structure; this also affects gel mechanical properties such as flexibility and stiffness [31]. Like other hydrogels, this aqueous microenvironment can be tuned to a certain pH or ionic strength while maintaining the network structure and colloidal stability [84,85]. Additives (salts, sugars and amino acids) can be dissolved into the gel to provide a balanced osmolality and regulate water flow within the medium [70]. Last, the cellulose backbone can be modified to immobilize biomolecules (enzymes, antibodies and peptides) or functionalized with groups such as methyl, carboxylate or amine [18,86–88].

Many studies have demonstrated the suitability of nanocellulose hydrogels to biomedical applications. Engineering hydrogels for drug delivery and tissue engineering, for example, demands the strict control of structural aspects [89]. The hydrogel network structure requires a minimum rigidity and strength, mainly when sustaining particles such as cells, proteins or nanoparticles. Chemical modification to covalently crosslink nanocellulose fibres enhances the mechanical properties of the gel [90,91]. Prakobna and Kasinee reported the addition of galactoglucomannan (GGM) in CNF to significantly increase the Young's Modulus of CNF hydrogel from 4.1 MPa to 28.8 MPa for CNF/GGM 13.4 wt%. This process also increased tensile strength from 0.11 MPa (CNF) to 0.65 MPa (CNF/GGM) [92]. McKee et al. showed the storage modulus (G') to be tuneable over an order of magnitude by adding different concentrations of CNC to a methylcellulose (MC) network. The hydrogel displayed a large range of elastic modulus (G') varying from 110 to 900 Pa for concentration of CNC/MC = 3.5/1.0 wt%; this is due to physical cross linking [93] (Fig. 8). Lastly, Yang et al. showed that incorporating metal cations (Ce^{3+}) to carboxylated cellulose nanofibres produced stiff CNF-polyacrylamide hydrogels with Young's modulus of 257 kPa and fracture toughness of 386 kJ/m^3; this is 8.5 times higher than for pristine gels [76].

Controlling gel pores or mesh size between the crosslinked cellulose fibres network enables to engineer biomolecules diffusion within the matrix [60,87] (Fig. 9). When the mesh size is smaller than the

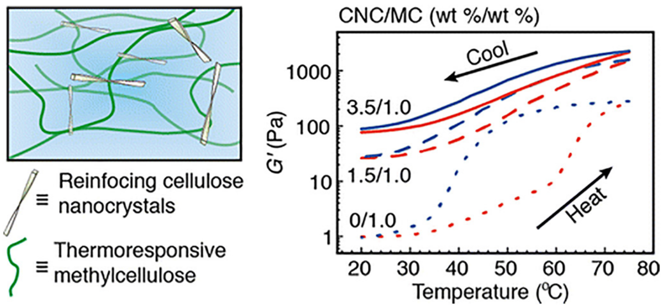

Fig. 8. Thermo-responsive nanocellulose hydrogels produced by mixing cellulose nanocrystals (CNC) with methylcellulose at 60 °C. Reproduced with permission from Ref. [93].

biomolecule's diameter, the hydrogel blocks the diffusion of biomolecules by filtration and *vice versa*. Li and Mooney reviewed the design of novel hydrogels for controlled drug delivery depending on different mesh sizes [17]. Johns et al. studied the affinity and migration of carbohydrate-binding module (CBM) in cellulose hydrogels. They found the different types of cellulose and the regeneration process to affect the diffusion of biomolecules. They reported a CBM diffusion coefficient of 1.07 $\mu m^2\ min^{-1}$ in cellulose hydrogel obtained by the regeneration of bacterial cellulose in methanol. When regenerated in water, the diffusion coefficient within the hydrogel was 50% lower [94]. Patricia Lopez-Sanchez et al. investigated the diffusion of FITC labelled dextran and labelled pectin methyl esterase (PME) in cellulose-based hydrogels (cellulose, cellulose–arabinoxylan, cellulose–xyloglucan). They reported the molecules mobility in the cellulose–xyloglucan to be lower than in the other cellulose hydrogels because of variations in the local gel structure [88].

From the biological perspective, a low level of toxicity is a key property required of hydrogels before allowing its safe application in biomedicine. Proof of minimal toxic effects is crucial to enable the translation of gels research to medical clinical assays [59,95,96]. Hydrogels, as others cellulose-based materials, are often claimed to be fully biocompatible by range of studies. The synthesis of hydrogels usually includes a last washing step for the removal of reactants potentially harmful to cells and tissues [97]. If required, sterilization by UV irradiation and ozone gas can be used to minimize the occurrence of contamination by microorganisms such as bacteria and fungi [98,99]. Independent of these strategies, the level of toxicity remains under constant investigation. So far, no significant toxic effects of nanocellulose on cells and organs has been reported [86,90,100,101]. CNF, CNC and BNC-based hydrogels are all classified as non-carcinogenic and non-cytotoxic in an extensive review leaded by Lind and Dufresne [86]. From its physical, chemical and biological properties, nanocellulose hydrogels stands as a versatile biomaterial for a range of finalities in life sciences.

3. Biomedical applications of nanocellulose hydrogels

3.1. Key concepts in biomedical sciences

Understanding the interaction between hydrogels and biological material requires a few key concepts. The cell is the elementary unit of living beings. In general, it is constituted of a fluid membrane, specialized organelles and genetic material [102,103]. Cells undergo differentiation and are the origin of specialized and highly organized tissues. The extracellular matrix (ECM) is responsible for providing the conditions for the survival and proliferation of all the different types of cells [104]. This hydrated porous matrix is typically made of structural proteins (laminin, collagen, fibronectin), growth factors, polysaccharides and glycosaminoglycans (GAGs). It is also the ECM role to offer the mechanical support to sustain the cell growth whilst enabling diffusion of nutrients, transfer of metabolic waste and transport of gases [105]. Advances in medicine, engineering and biology allow to mimic *in vitro* the physical and chemical conditions of the human body [14,106]. Cells are currently cultured as bi-dimensional layers as well as over three-dimensional scaffolds. Fragments of tissues can also be cultivated in the laboratory and behave similarly to developed organs; these are named organoids [107]. Similarly, heterogeneous cell aggregates lacking organizational structure which form primitive tumour tissues are referred to as spheroids [108]. For the 3D growth of cells, organoids and spheroids, a biomaterial able to resemble all the ECM properties is a must. Scaffolds are also required for engineered tissues and regenerative dressings, diving its performance (Fig. 10) [109]. Similar to cells, biomolecules - mainly proteins - also demand appropriate conditions for storage and manipulation. Temperature, pH and ionic strength are the most relevant parameters that affect the protein activity and longevity [110]. These conditions are also essential for the optimal pharmacokinetic of drugs when within the human body [111]. Therefore, they must be controlled when separating biomolecules, as well as when interacting with tissues and substrates. In this section, we introduce and review the state of art development for nanocellulose hydrogels

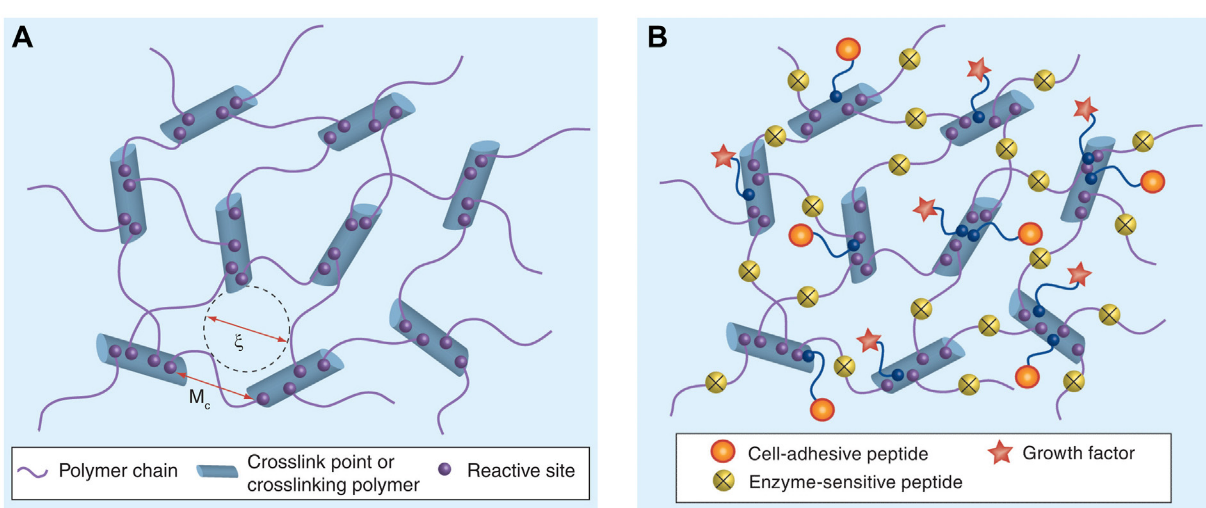

Fig. 9. Representation of a general and functionalized hydrogels structure. (a) Polymeric chains are linked together by an agent, where ξ indicates the distance between two chains, ranging from a few to hundreds of micrometres. (b) A series of peptides, including adhesion molecules, enzymes and growth factors, can be incorporated into the network to enhance biocompatibility between cells and the matrix as well as trigger molecular responses to drive cell differentiation into tissues and organs. Reproduced with permission from Ref. [87].

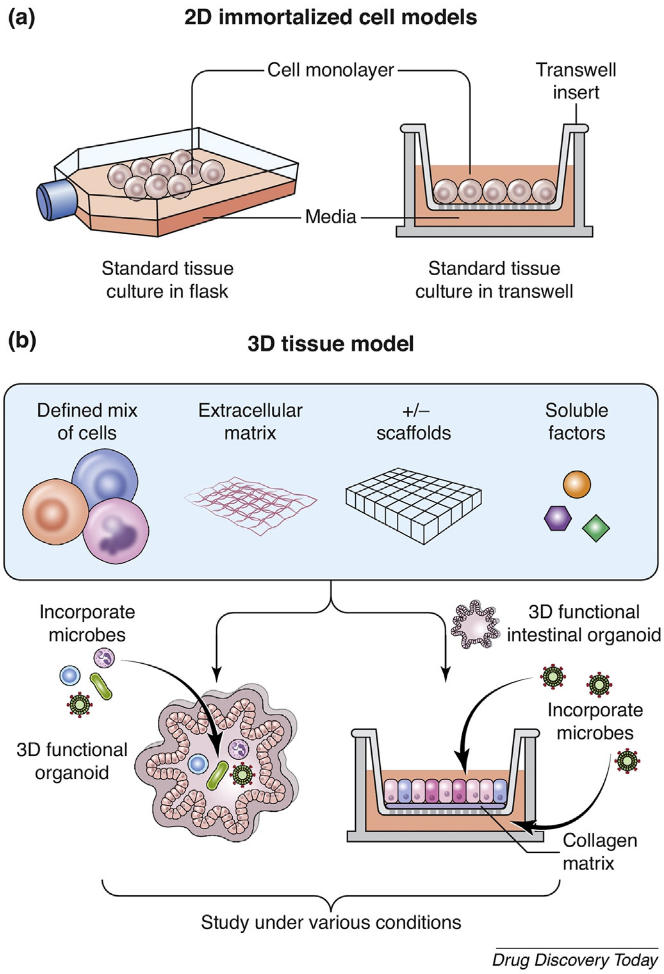

Fig. 10. Schematic representation of cells, organoids and extracellular matrix (ECM) in a (a) traditional 2D and (b) 3D cell culture used for multiple biomedical applications. Reproduced with permission from Ref. [112].

applied to 3D cell culture, tissue engineering, drug delivery, diagnostics and separation.

3.2. 3D cell culture

A 3D matrix is required for the culture of some cell lines such as the stem and tumour cells *in vitro* [109,113]. Gelatinous and flexible, the three-dimensional structure is required to sustain cell growth, reproducing the *niche* found *in vivo*. Different from tissue engineering however, this application does not only aim for the repair, regeneration or replacement of an organ or tissue. 3D cell culture also provides a robust model to investigate cancer progression, drug screening and developmental biology [114,115]. A major effort has focused on developing a synthetic matrix able to fully mimic the extracellular matrix (ECM) properties found *in vivo*. *Matrigel*™ is the most popular commercial ECM-like material for 3D cell cultures. Secreted by Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, this gel is made of laminin, collagen IV as well as proteoglycans [116]. Several reports have showed *Matrigel™* to provide the desired mechanical and chemical conditions for cell growth *in vitro* [116–119]. Due to its bio-origin source, *Matrigel™* might carry non-identifiable pathogens and antigens [117]. Similarly, a high level of multiple growth factor has been reported in *Matrigel™* [120]. The effect of the trace elements over each type of cell can vary from moderate -inducing cell differentiation or quiescence- to extreme, leading to cell proliferation or death [119,121]. This trade biocontamination represents a major drawback of *Matrigel™* for clinical studies and has driven the strong and growing demand for a *Matrigel™* replacement. Last, *Matrigel™* has remained prohibitively expensive with little hope of significant price decrease because of its difficulty in producing and trade mark exclusivity. There is a strong incentive in developing cheap Matrigel alternatives. Using decellularized tissues to isolate and reuse animal ECM has been explored as alternative. Although certain success was achieved, this technique is time consuming, expensive, and requires a series of pre and post treatments [122–124]. Moreover, similarly to *Matrigel™*, remaining biomolecules might persist after decellularization and prevent cell proliferation and/or colonization [125,126]. To palliate these issues, a few studies have aimed at developing a synthetic ECM-like materials able to provide identical conditions to those found *in vivo*.

Cellulose-based hydrogels have recently been explored as scaffolds for 3D cell culture.

3D culture of mesenchymal stem cells (MSC), human pluripotent stem cells (hPSC), hepatic tumour cells have all been successful with nanocellulose gels [127–130]. MSCs cultivated for 21 days maintain rates of cell viability of nearly 90% in cellulose matrix of solids content as low as 0.2 wt%. MSCs were evenly distributed across the gel and properly directed differentiation into osteogenic cells [127]. Similarly, hPSCs grown *in vitro* for 26 days showed no decrease of pluripotency during this period. When induced to differentiation, hPSCs successfully originated spheroids [129]. Hepatic tumour cells lines (HepG2 and HepaGR) were also cultured in 0.1–1 wt% nanocellulose hydrogels (Fig. 11). Cell viability was not affected by the gel over 14 days of culture, although proliferation was considerably slower when compared to those cultured in animal-derived ECM. The formation of spheroids of diameter 40 μm was noticed [128]. Despite these achievements, serious limitations remain. First, the recovery of cells has so far been demonstrated only by relying on cellulase-mediated digestion. This process is not only time consuming but it requires incubation at 37 °C for up to 24 h [129,130]. Most importantly, the residual enzymes represent a significant source of protein contamination- unacceptable for many applications. The best protocols rely on thermo-responsive hydrogels, with a sol-gel transition occurring at the lower temperatures [116]. The effects over the stem cells fate and their consequences -such as changes in gene expression or loss in membrane integrity- are not yet fully understood. Indeed, no study has performed a genetic screen to assess potential changes on DNA expression post-culture [127,128,130]. In addition, primary cultures of tumour cells show a poor performance when compared to established cell lines also cultivated in pure cellulose-based hydrogels [130]. Since tumour cells are known for carrying a severe genetic instability, the outcomes obtained are inconclusive [131].

3.3. Tissue engineering

Tissue engineering is a novel and expanding field of medical sciences. The combination of cells, biomaterials and growth factors (mainly signalling polypeptides) produces engineered-organs and tissues for partial or complete replacement of damaged structures in the human body [106,113]. Regeneration of injured tissues is also a target for faster and less painful bone/cartilage reconstitution, as well as for healing skin wounds [8,132–134]. Similarly to 3D cell cultures, engineering tissues also demand an ECM-like microenvironment. In this case, however, scaffolds must provide enhanced mechanical properties to sustain cell proliferation for more than 4 weeks and support its differentiation into specialized structured tissues. A further requirement is for the porous gel structure to allow gases transport and promote vascularization [135–137]. When sufficiently developed, the growth tissue can be incorporated into the patient's organism to trigger a minimal inflammatory response. Finally, it is desirable for the scaffold to naturally degrade into the body fluids with time, preventing surgical intervention for its removal [136]. Wound dressing materials, in contrast, aim at providing a porous network able of swelling; this requires a specific elasticity and the ability to retain moisture and pH over time [8]. The control of

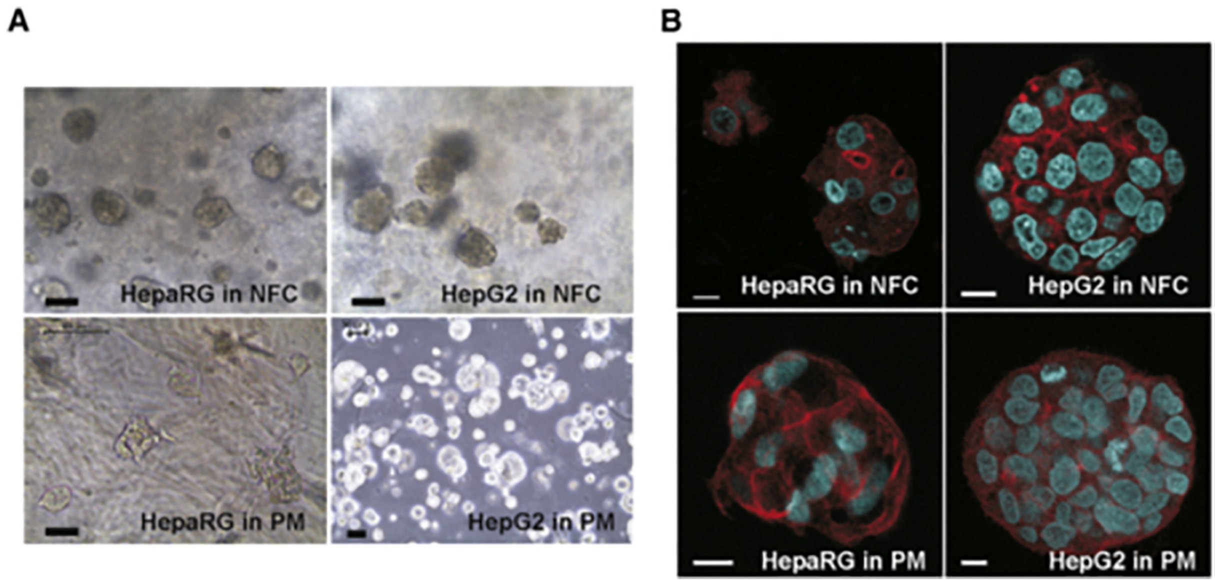

Fig. 11. (a) Phase contrast microscopy of HepaRG and HepG2 cells and (b) Confocal microscopy of spheroids cultured in nanocellulose hydrogel and Purematrix™ (PM), a peptide-based gel. Reproduced with permission from Ref. [128].

gel thickness and density is important to adjust material properties accordingly to the specific organ requirements. Due to the high incidence of infections, it is beneficial for dressings to possess antibacterial attributes to minimize bacterial colonization along the healing process [138,139]. Nanocellulose hydrogels have been extensively explored for scaffold and wound dressing applications [16,28,70,140–147]. However, to match all the requirements discussed, cellulose-based gels are often combined to other materials and technologies to produce efficient scaffolds and bioactive wound dressings.

3D bioprinting is developing into a powerful tool for the production of highly structured tissue engineering scaffolds [148–150]. This technology allows to dispense hydrogels in three dimensions with precision and increasing resolution. Cells are encapsulated within the printed material gel in homogeneous density and quantity. This contrasts with the traditional two-step protocols, wherein cells are inoculated into premade scaffolds. In this case, cell distribution is often heterogeneous and the biomaterial might not be fully colonized [149]. Nanocellulose-alginate based hydrogels were successfully 3D printed as human cartilage. Many studies have reported induced pluripotent stem cells (iPSCs) and human chondrocytes printed together with the hydrogel matrix, constituting a bioink [144–147]. Ngueyen et al. reported iPSCs maintained their pluripotency during printing and proliferated through the scaffold afterwards. Further cell differentiation was detected and the formation of cartilaginous tissue by expression of collagen II was identified after 5 weeks [144]. Markstedt et al. cultivated human chondrocytes within an ear-like structure (Fig. 12). Cell viability was 86% after 7 days; cell differentiation into the targeted tissue was then observed [147]. Similarly, human auricular cartilage was obtained from cells cultured for 28 days inside a 3D printed scaffold. High cell density and viability of 98% are reported. At the end of the culturing period, 75% of cells were still viable and the expression of ECM proteins increased by 20% [146]. These three studies have relied on nanocellulose properties such as viscosity, shear thinning and low shear rates to achieve good printability. Alginate acts as a crosslinker to increase gel viscosity. It improves the scaffold structure and guarantees shape to be maintained during the process [145]. So far, there has been no reference of pure nanocellulose gels used for 3D bioprinting of tissue and organs.

Application of nanocellulose based-gels to wound healing and skin regeneration was reported in multiple studies in recent years [84,141–143,151]. A clinical trial reported microporous network structure and swelling properties of this hydrogel allowed skin regeneration for burn victims within 16 days on average. Nanocellulose hydrogels did neither trigger allergic nor inflammatory responses. Cellulose offers a hydrated layer over the wounds thanks to its high OH concentration. *In vitro* tests also demonstrated the absence of necrosis in wounds treated with the gel. Modified nanocellulose gels containing different antibiotics agents, such as polyhexamethylene biguanide and tetracycline hydrochloride, are mentioned as examples of bioactive wound dressing. In both studies, the presence of antibiotics prevented colonization of bacterial strips, especially *Staphylococcus aureus* and *Escherichia coli* [140,143]. Similar antibacterial effect was reported for gels containing carboxymethyl cellulose fibres in a study by Chinga-Carrasco and Syverud.

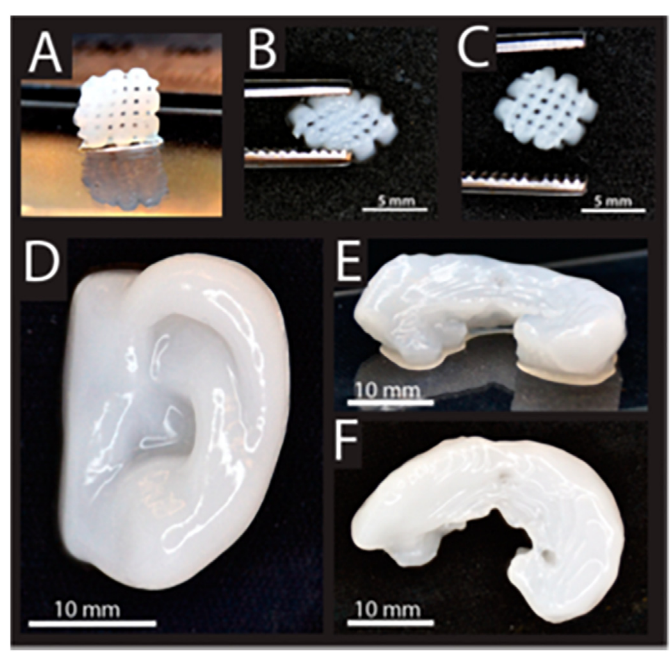

Fig. 12. (a) Model 3D printed nanocellulose—alginate scaffolds. (b) The scaffold deforms when under mechanical force, but (c) then fully recovers its original shape. (d) Bioprinted human ear and (e and f) sheep meniscus scaffolds. Reproduced with permission from Ref. [147].

pH-responsive cellulose gels also demonstrated the controlled release of drugs in wound treatments, completing the healing process in 10 days [84]. Apart from the successful cases reported, there is no comprehensive information on infection in *in vivo* cellulose gels. This is a concern as the emergence of bacterial resistance against antibiotics remains an obstacle in wound treatments [152]. Therefore, continuous validation of antimicrobial substances in cellulose gel is required prior to achieving medical certification.

3.4. Drug delivery

Drug delivery systems refer to a series of bioengineered technologies for the improved transport of therapeutic agents to a target site, *i.e.* tissues and organs. Such system comprises both carrier vesicles and coating-treatments for the specific and controlled-release of medicines and biomolecules [111]. These modifications are mainly designed to enhance pharmacokinetics and optimize the biodistribution of substances in the human body. Micelles are the most common method explored in drug delivery; they provide either a hydrophobic or a hydrophilic environment which enhances the solubility of drugs immersed in biological fluids [153]. The encapsulation of drugs within synthetic or natural shells, in contrast, offers protection against premature degradation, as well as a reduction in side effects against non-targeted tissues [154]. A commonality for both methods is that the nanocellulose hydrogel (as micelle or encapsulation) must be sufficiently stable for a certain period of time and not be affected by variations in pH, ionic strength and temperature before reaching the target site in the body or organ. These variables, however, are essential for the controlled release and dissolution of drugs whilst preventing undesired injury to untargeted organs [96]. Finally, direct chemical modifications of the drugs surface can induce high affinity between ligand and receptor and improve specificity [154]. Biopolymers have been extensively studied as materials for drug delivery systems because of their biodegradability, biocompatibility, low toxicity and high availability [155–157]. Among the different types of cellulose, the bacterial nanocellulose has emerged as the most promising and suitable biomaterial [158]. This is because BNC is produced as an ultrafine three-dimensional nanostructure of high tensile strength and elastic modulus [44,47]. BNC-based hydrogels are highly hydrated while maintaining their network structure. Several studies reported that once functionalized for thermo and pH-sensitive responsiveness, bacterial nanocellulose hydrogels can be applied to the sustained and controlled targeted-release of drugs, antibodies and enzymes. However, Bacterial nanocellulose (BNC) will remain significantly more expensive to produce than cellulose nanofibers (CNF) and cellulose nanocrystals (CNC) in the foreseeable future.

Anin and collaborators investigated the synthesis and characterization of a bacterial nanocellulose-acrylic acid (AA) hydrogel for drug delivery. Acrylic acid monomers were grafted onto cellulose fibres and the gelification activated and accelerated by electron-beam irradiation. This functionalization allowed the hydrogel to become both thermo and pH-responsive [159]. Evaluation of water absorption showed that BNC-AA hydrogel reached its maximum swelling at pH 7, even after 48 h. At pH 10, the equilibrium was achieved in 24 h. This mechanism relies on the hydrophilicity of the carboxylic groups of the hydrogel structure. Image analysis showed that the gel pore size was dependent upon the AA content and the irradiation dose in a proportional manner. In addition, the gel porosity directly affected the kinetics of drug releasing (Fig. 13). Badshah et al. evaluated BNC matrixes as drug carrier for two drugs: famotidine and tizanidine - the first drug is poorly and the second highly water soluble, respectively. Both drugs were uniformly loaded and distributed into the matrixes. The effectiveness of drug loading reached levels as high as 68% upon nanocellulose hydrogel delivery. Approximately 80% of tizanidine (hydrophobic) was released by the cellulose matrix within 15 min, while commercial tablets took at least 3 times longer. For tizanidine (hydrophilic), both delivery systems reached 80% of release in 15 min [160]. Finally, Treesuppharat et al. developed a hydrogel composite synthesized by copolymerization of BNC with gelatin. Both size and shape of BNC chains were uniform and the hydrogel presented thermal stability, chemical resistance and desired mechanical properties. The rate of the hydrogel swelling was estimated to range from 400 to 600% [161]. A few additional studies stated the suitability of nanocellulose hydrogels applied to drug delivery. However, as pointed out by Zelikin et al., further development and progress in a few key points are still required. The most relevant is the absence of hydrogels able to simultaneously release distinct drugs at different rates. It would be particularly advantageous for multidrug treatments against diseases like cancer [154]. The functionalization of nanocellulose for the efficient responsiveness to specific conditions might supply the needs for “smart” materials to drug delivery systems. Moreover, the low cost and sustainability of nanocellulose enhance its attractiveness from a financial perspective.

3.5. Diagnostics

Diagnostic devices are essential tools in modern medicine for the detection of existing viral or microbial agents, measuring vital signals, as well as determining physiological properties of the human body [162]. Diagnostics tests are expected to be user-friendly, low cost, robust and to be sensitive and accurate [163,164]. These characteristics are critical for devices used in remote areas, where facilities and expertise for complex analysis are simply inexistent. Similarly, there is a huge demand for this type of medical technology in poor countries, to improve public health services in spite of financial limitations [165–167]. Developed countries have also realised the health benefits of regular and wide spread testing thanks to diagnostics that cost a fraction of the current technologies. In this context, paper-based diagnostics have been explored over the last two decades. Literature lists many successful examples including blood typing, HIV and dengue tests. A bioactive paper able to detect biomolecules related to cancer was recently reported [168–170]. Hydrogels-based diagnostics, in contrast to paper tests, have barely been explored, although many advantages are foreseen [11,18]. First, the strict control of pH and ionic strength in gels can significantly optimize the activity of enzymes and extend their reactivity [171]. Second, the fibre network can be functionalized with proteins and other biomolecules, enhancing interactions between the molecules or elements tested.

A few studies have evaluated cellulose-based gels as biosensors; those available have mainly focused on proof of concepts [171,172]. Isobe et al. demonstrated the suitability of protein immobilization for biosensors by introducing aldehyde groups on the fibres surface. Model enzymes were covalently bonded to cellulose by Schiff base formation; activity triggered a colour change in the gel. The rate of crosslinking was proportional to the aldehyde content. The saturation point was reached at levels as low as 15 ng of enzyme/g of cellulose as the enzyme amplified the detection signal in a manner similar to ELISA tests (Fig. 14) [171]. In a similar approach, fluorescent CdSe/ZnS nanoparticles were physically adsorbed into cellulose hydrogels by Chang et al. Once irradiated by UV light, the gels emitted fluorescence and acquired different colours depending on the nanoparticle size. No changes were observed in the mechanical strength of the gel after nanoparticle excitation and radiation release. The water retention was not affected, maintaining its levels around 97% [172]. Both studies focused on the proof of concept without showing any specific application for these findings. However, functionalized nanocellulose gels are clearly highlighted as promising materials for a new generation of diagnostic devices.

3.6. Separation

The ability to separate biomolecules, ions or even cells in a suspension has a considerable value for biomedical research, biopharmacology processing and medical tests. Electrophoresis and chromatography are

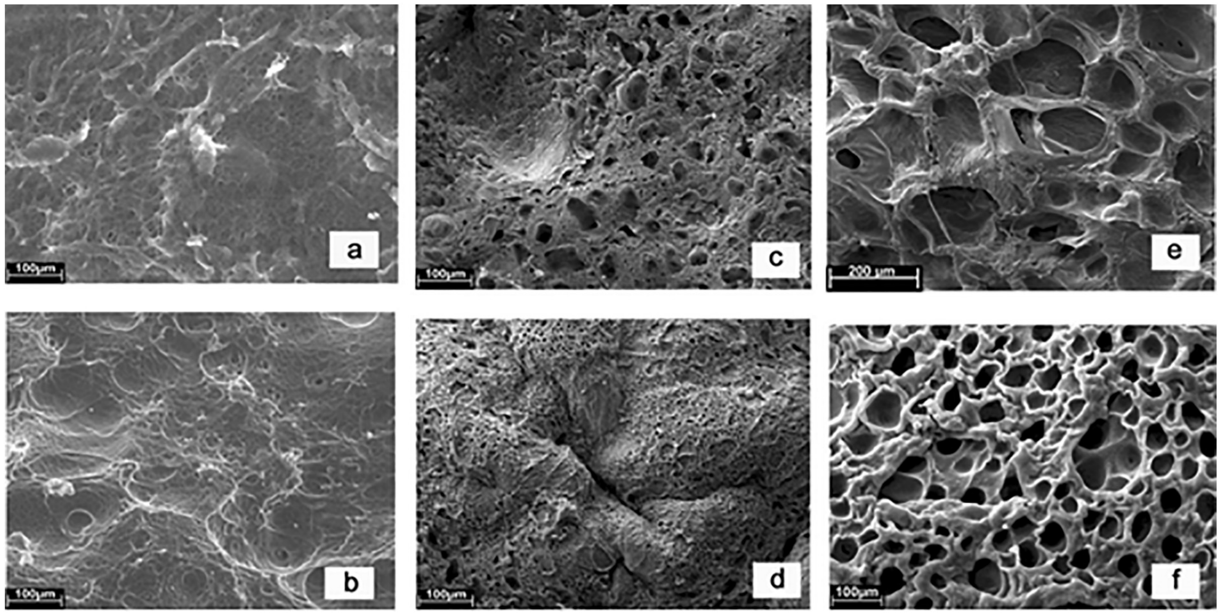

Fig. 13. Scanning electron microscopy images of thermo- and pH-responsive poly(acrylic) acid-bacterial nanocellulose hydrogel applied to drug delivery. Reproduced with permission from Ref. [159].

two of the most widely used and dependable analytical methods. The first one separates proteins and nucleic acids according to their molecular weight and electrical charge. Samples migrate through the gel pores under electrical current and form bands of size proportional to the concentration of each polypeptide or DNA fragment to analyse [173,174]. Chromatography is based on the elution of a mixture through a gel column and its separation into individual fractions for further analysis and use. The affinity between sample and column - also referred to as stationary and mobile phase, respectively - dictates the velocity and time for purification [175,176]. Lastly, the separation of individual cells from aggregates is the basic principle of operation for the gel card method, now the traditional technique for blood typing tests. Briefly, the identification of existing antigens on red blood cells (RBCs) allows to classify them in a specific blood group. For that, RBCs and antibodies are let to interact on the top of the gel column and then centrifuged. Agglutinated RBCs represent a positive result visualized by the blood agglutinate trapped at the upper region of the gel. Negative results are formed by individual RBCs located at the bottom [177]. Usually, the gel column used for all these applications are made of synthetic polymers, mostly dextran-polyacrylamide, silica beads, and polysaccharides such as agarose [178,179]. Although efficient, most of these materials poorly biodegradable, are not renewable and difficult to chemically functionalize.

Thanks to its abundancy, low toxicity and biocompatibility, cellulose has been investigated as gel for separation. In the early 80's, Kuga demonstrated cellulose microfibril gel for chromatography. Pore size was controlled with the concentration of cellulose, varying from hundreds to thousands of nanometre. Compared to agarose and dextran, the cellulose gel presented enhanced rigidity, the absence of solute adsorption and excellent thermal stability [180]. Oxidised cellulose gels were also successfully developed by Kim and Kuga for cationic ion-exchange chromatography. Gels were tested for their reactivity with different amines at pH ranging from 2.5 to 5.5. The material had the desired mechanical strength and colloidal stability, behaving similarly to the conventional carboxylated cellulose gel [181]. Last, Velander et al. developed improved cellulose and lignocellulose beads for bioaffinity chromatography. Cellulose was dissolved in dimethylacetamide-lithium chloride solvent systems (DMAc/LiCl) beaded into a non-solvent (ethanol/water mixture) and washed in water, producing strong and porous beads of 0.3 mm diameter. Antibodies, enzymes and blood proteins were successfully isolated from complex mixtures. The material presented excellent mechanical strength and lower production costs compared to agarose and dextran beads [182]. For electrophoresis, Deshmuhk et al. compared cellulose acetate and polyacrylamide gels. Cellulose-based gels properly separated milk proteins (casein and lactoglobulin), although less accurately than the current synthetic materials [183]. These miscellaneous reports are the only documentation found on nanocellulose hydrogels for chromatography or electrophoresis. With a high aspect ratio, large surface area and porous network structure, nanocellulose shows potential as a new material for analytical separation. Nanocellulose hydrogels have been investigated for blood typing tests. As shown in Fig. 15, cellulose nanofibres can reproducibly immobilize agglutinated red blood cells (RBC). In contrast, individual red cells are centrifuged to the bottom without undergoing lysis. These results are very similar to those from dextran-based gel cards. Nanocellulose-based hydrogels is a suitable media for clear and well-defined blood typing diagnostics.

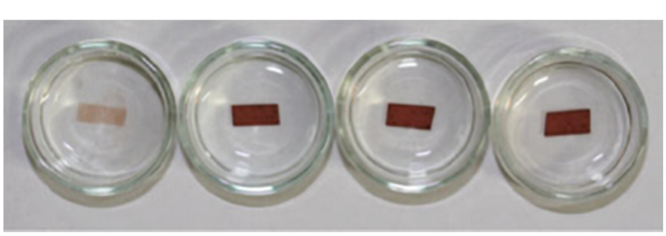

Fig. 14. Models of cellulose hydrogel containing immobilized horseradish peroxidase enzyme. Colour intensity is proportional to the concentration of enzymes crosslinked with the cellulose aldehyde groups. Reproduced with permission from Ref. [171].

4. Perspectives

Nanocellulose hydrogels are a new class of engineerable, renewable, biodegradable and non-toxic materials. Cellulose is a highly hierarchical material with structures of dimensions ranging from the nanometre to the millimetre. Simple methods, such as the homogenization at high intensity to shear carboxylated pulp or microfibre in aqueous suspensions at low concentrations (0.1% to 5 wt%) form hydrogels. These gels are highly hydrated (99.9 to 95% water) and form a three-dimensional

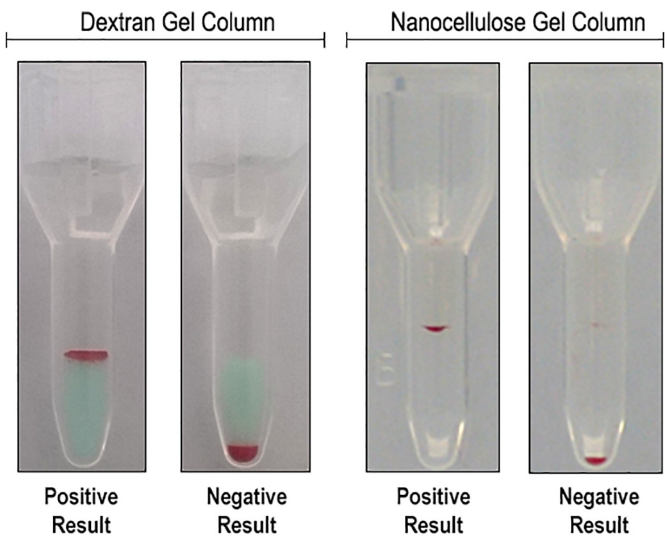

Fig. 15. Example of gel card for blood typing tests. Red Blood Cells and antibodies react and are then centrifuged. Agglutinated cells are immobilized at the top of the gel column, while individual cells pellet at the bottom. Nanocellulose gels present clear and well-defined diagnostic results, similar to those obtained from the commercial dextran-based gels.

porous network of good mechanical properties. They are ideal materials for many types of biomedical applications including 3D cell culture, tissue engineering, diagnostics, drug delivery and separation. Five main gaps in knowledge have emerged from this review. First, nanocellulose hydrogels are reported to be biocompatible scaffolds for 3D cell culture. However, the hydrogel removal at the end of the cell culture still depends on an enzymatic digestion, without proper understanding of the long-term effects of enzymes on cells. Second, there are no reports of human organoids cultivated in nanocellulose hydrogels. Literature has been restricted to tumour cell lines and limited types of stem cells. Third, nanocellulose hydrogels are described as beneficial for engineered-cartilage and wound dressing. However, 3D bioprinting of gels is efficient only when combined to other biopolymers. Similarly, cellulose dressings demand antibiotics or additives to properly accelerate regeneration and prevent infections. With regards to drug delivery systems, the demand for smart controlled-release of therapeutics stands as the next step for the pharmaceutical industry. The easy functionalization and low cost of nanocellulose makes it a good candidate for this propose. For diagnostics, cellulose paper-based test was developed to detect antigens, virus and microorganisms. Recently, cellulose hydrogels have been explored for the same finalities. The control of chemical properties of the cellulose backbone could provide enhanced biosensing capability. However, this field has remained mostly unknown. Last, the separation of biomolecules and cells through cellulose gel column has been investigated since the 80's. Novel hydrogels relying on structured nanofibres/nanocrystrals can replace synthetic materials, possibly with better performance for the optimal purification of analytes. In conclusion, nanocellulose hydrogels have emerged as an exceptional platform to offer diagnostic performance combined with environment-friendly, low-cost and ease of processing for biomedical applications. However, the full interactions mechanisms of nanocellulose with biomolecules and its long-term effects on biomedicine and the body as a whole still require a better and more fundamental knowledge.

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