

119. Structure Response for Cellulose-Based Hydrogels via Characterization Techniques

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Abstract

Hydrogels are three-dimensional crosslinked polymeric networks capable of imbibing substantial amounts of water or biological fluids and are widely used in biomedical applications, especially in pharmaceutical industry as drug delivery systems. Although their solvent content can be over 99%, hydrogels still retain the appearance and properties of solid materials and the structural response can include a smart response to environmental stimuli (pH, temp, ionic strength, electric field, presence of enzyme etc.) These responses can include shrinkage or swelling. Cellulose-based hydrogels are one of the most commonly used material and extensively investigated due to the widespread availability of cellulose in nature. Cellulose is the most abundant renewable resource on earth is intrinsically degradable. Additionally, the presence of hydroxyl groups results in fascinating structures and properties. Also, cellulose-based hydrogels with specific properties can be obtained by combining it with synthetic or natural polymers. This chapter surveys different characterization for cellulose-hydrogels and the structure response relationship. As such we would describe the techniques involved for characterizing cellulose-based hydrogels and their response in terms of their morphology such as polarized optical microscopy (POM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM); their stability by thermal properties (often with differential scanning calorimetry, DSC), structure response such as Fourier Transform Infrared Spectroscopy (FTIR), Nuclear magnetic resonance (NMR). In addition, we give a focus on measuring the mechanical properties of superabsorbent hydrogels giving examples with cellulose where applicable. Finally, we describe the techniques for analyzing biological techniques and the applications with cellulose.

Keywords: Characterization, Cellulose analysis, Structure-response, Hydrogels, Materials

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1 Introduction

A representative of the kingdoms Plantae, Animalia and the Eubacteria domain synthesize cellulose and is the most abundant product on earth. Although these different sources of cellulose have many varieties, they share many characteristics. Cellulose can be characterized biochemically as polysaccharide with a β -1,4-glycosidic linkage, which is formed by condensation and polymerization of long anhydroglucose chain units [1].

Cellulose is associated with biopolymer due to the many characteristics of benefit to the formation of a hydrogel. Such characteristics include availability in abundance, renewability, biocompatibility, biodegradability, non-toxicity and many more unique features, which are not easily acquired through chemical synthesis [2].

Hydrogels can be defined as three-dimensional crosslinking networks of polymers that can absorb and retain water. Hydrogels have potential applications in several areas, including medical, pharmacology and agriculture areas. Due to hydrogels water retention properties and diffusion system, it can be used as drug delivery systems, microfluidic devices, biosensors, tissue implants and contact lenses [3].

Hydrogels can modify their water retention and release capacity in response to external stimuli such as temperature, pH and ion concentration. To reduce the toxicity or bioinert impact of these external factors, hydrogels are often co-polymerized with various biopolymers, including cellulose which has been widely used and studied [4].

This chapter aims to investigate the mechanisms involved in the characterization of cellulose-based hydrogels, describing the effect in terms of their structure response.

2 Cellulose-based Hydrogels

There are many variations of cellulose that can be used in the preparation of hydrogels, including native cellulose, bacterial cellulose, and cellulose derivatives. The main difference between these variations is in terms of the solubility; the native cellulose presents a major challenge as it is not easily dissolved at common solvents due to its highly extended hydrogen bond structure [5].

The cellulose produced by bacteria, such as the genus *Acetobacter xylinum* or bacterial cellulose, is a natural polymer which consists of a three-dimensional network of polymers that are able to retain up to 99% of its weight in water. Bacteria cellulose have high strength mechanical properties and excellent biocompatibility, these polymers can be used as tissue repair and implants [6].

The production of cellulose-based hydrogels occurs through physical cross-linking, this is possible to achieve due to the large number of hydroxyl groups in cellulose, which can form hydrogen bonds that easily link its chains [5].

The morphology of cellulose-based varieties of hydrogels is complex. Commonly to the geometry includes fibres, films, membranes, sponges and beads [7]. The bacterial cellulose due to its high properties such as mechanical, swelling, crystallinity and biocompatibility has an extended application spectrum and can be used in tissue engineering [8] and implants [9].

The addition of cellulose and its derivatives can tailor its structure for the application intended, among them includes its swelling capacity and drug delivery. These favours its use mainly in the areas of agriculture [10] and horticulture [11]. Among other functions, cellulose-based hydrogels have also been widely used as for drug or protein delivery systems, including different routes of application such as transdermal and oral [1]. In addition, many other applications are currently used cellulose-based hydrogels such as photonic materials responsive to stimuli, which can be tailored to have their functions altered by an external stimuli [12].

3 Morphology Analysis

Visual information of hydrogels at the nanoscale is extremely important to understand the morphology such as pore sizes distribution, fibres dimensions, distribution of fillers and or nanoparticles second phases. In addition, it can also provide important information in terms of drug encapsulated into the hydrogel structure, while also can clarify results of different characterization techniques, such as nanoindentation or biology tests.

3.1 Polarized Optical Microscopy (POM)

Light-Sensitive hydrogels have applications in sensors, optical filters, inks, displays and other technologies. Biopolymers can be incorporated into the hydrogels to improve their mechanical properties, as well as stimulate the formation of chiral nematic liquid crystals, which have unique and valuable photon properties, including manipulating the hydrogel response to external stimuli [13].

Tatsumi et al., (2012) used polarized optical microscopy (POM) to observe and characterize the phase of chiral nematic formation during the production of composites comprising poly(2-hydroxyethyl methacrylate) (PHEMA) and cellulose nanocrystallites (CNC), with the objective of evaluate the ability of this biopolymer to control the hydrogel response to external stimuli [14]. In addition, POM also allows to observe any tension or residual stress into the hydrogel structure as observed in Fig. 1. These residual stresses are important parameters to identify points of fracture and the limit of failure of the polymeric materials.

Kelly et al., (2013) detected through POM that after the addition of nanocrystalline cellulose in the monomer of acrylamide and crosslink it, promotes a strong red birefringence due to the intrinsic birefringence of the cellulose nanocrystals to this colour. This is an important characteristic since the polarization time is correlated to the swelling, which allows controlling this capacity according to the light stimulus [12].

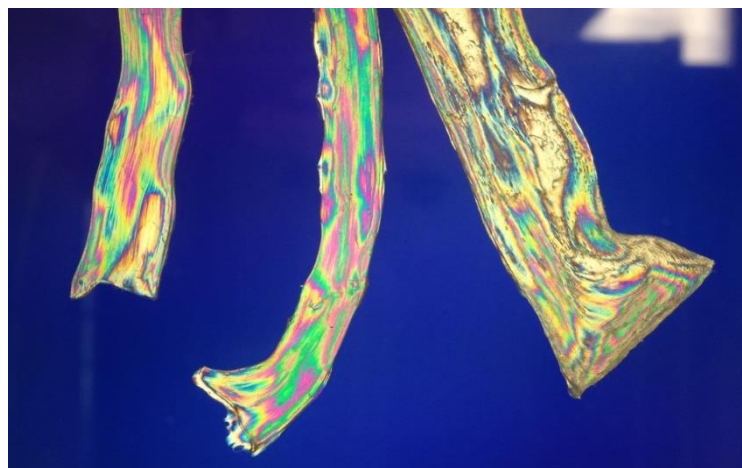


Fig. 1 Polarized Optical microscope from superabsorbent hydrogel composing of PVA+Cellulose. Original artwork.

3.2 Scanning Electron Microscopy (SEM)

Scanning electron microscope (SEM) is a widely used technique when analyzing different formulations of hydrogels since it can provide important data on structural characteristics of these products. In addition, SEM can identify key differences in surface morphology, size, shape and porosity of hydrogels according to the association of polymers used [10].

Demitri et al., (2015) observed through the SEM that adding sodium salt of carboxymethylcellulose (CMCNa) in polyethylene glycol diacrylate (PEGDA700) hydrogels promotes a significant increase in density of the material. Furthermore, the network structure increased the number of pores and consequently the contact area of the hydrogel, favouring the absorption of water [15].

Fig. 2 exhibit a hydrogel with cellulose and it illustrates the porous characteristic of cellulose-based hydrogels. This property, provided by the addition of this biopolymer, brings great advantages for many applications in several areas, especially those in which a greater capacity to retain water is required [16].

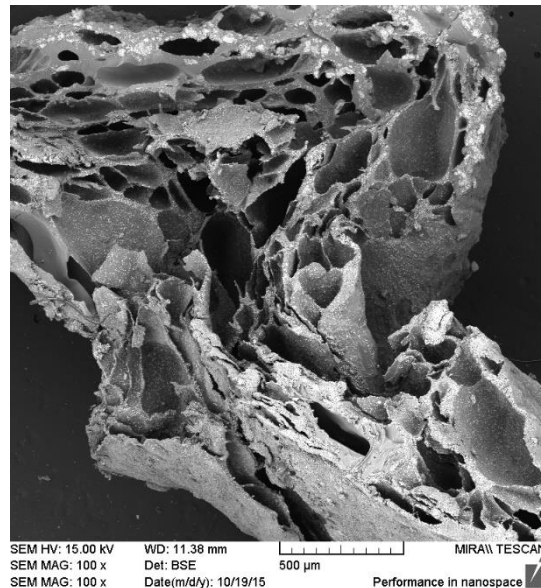


Fig. 2 Internal structure of a superabsorbent hydrogel after dehydration. Original artwork.

3.3 Transmission Electron Microscopy (TEM)

Unlike SEM, which basically characterizes the morphology of the sample surface, TEM collects information about a deeper internal composition of the samples which improves the

understanding of these structure in terms of their morphology, crystallization and magnetic domains., Images obtained with TEM have a very high definition [1].

The use of TEM can characterize and contribute to the understanding of the behaviour of compounds such as cellulose nanocrystals, which are promising alternatives in the manufacture of various products. Through this technique, it is possible to observe its role of induction in the formation of chiral materials during the hydrogenation of prochiral ketones [17]. Li et al., (2017) investigated through TEM on cellulose nanocrystals with carbon-dot hydrogels, a tendency of fibres formation with large dimensions, being able to reach values of the diameter of 5-10 nm and length of 140-260 nm [18]. However, cellulose is also added to the hydrogel structure due to its high water solubility, low cost and high swelling capacity [19]. This further improvement in swelling can help in wound healing to keep a wound moisture environment while also able to target deliver a drug, such as exhibited in Fig. 3 where it is possible to observe silver nanoparticles in between the structure of cellulose hydrogel.

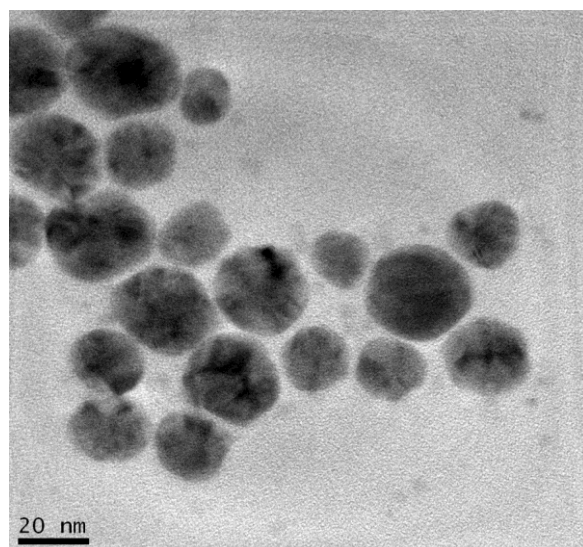


Fig. 3 TEM image of Polyvinyl pyrrolidone (PVP) crosslinked with Carboxymethyl cellulose (CMC) and Silver nanoparticles (AgNP). Original artwork.

4 Swelling Characterization

When a hydrogel is in contact with solvent molecules, the solvent initially penetrates the hydrogel surface. In this case, the unsolvated glassy phase is separated from the rubbery hydrogel region with a boundary move. In other words, swellability is based on ease of

migration of water from surrounding areas into the pre-existing hydrogel chain spaces. The process involves segmental motion of water that results in greater separation distance between these chains. Against the favourable osmotic force, there is an opposite elasticity force, which balances the stretching of the network and prevents its deformation. At the equilibrium where the elasticity and osmotic forces are balanced, there is no additional swelling [20]. Swelling profile of hydrogels can significantly affect the mechanical properties and subsequent cell attachment, migration and neovascularization [21] and, therefore, must be considered when synthesizing the hydrogel scaffold.

One of the most important characterizations of superabsorbent hydrogels is to understand the swelling kinetics since, on these polymers. In effect, preserve the shape is maintained after water absorption and swelling. The swollen gel strength should be high enough to prevent a loosening, mushy or slimy state.

Tests of swelling are one of the easiest techniques to analyze from a hydrogel and the measure occurs by pre-weighting samples which are then immersed into a solution, which can be buffer solutions or distilled water, and after removing the excess surface water of the hydrogel they are measured at various time intervals over a 24-hour period. With these values, it is possible to obtain the swelling percentage; although, with superabsorbent hydrogels, some parameters are added to analyze if these hydrogels hold the values under pressure. A cylindrical solid load is put on dry superabsorbent hydrogels while it can be freely slipped in a glass cylinder, Saline solution (0.9% NaCl) is then added when the liquid level is equal the height of the sintered glass filter. After 60 min, the swollen particles are weighed again, and absorbency under load is calculated based on the final and initial weights.

The work performed by Cipriano, B. H. et al., (2014) exhibited a superabsorbent hydrogel (Fig. 4) with a gel based on N,N- dimethylacrylamide (DMAA) with sodium acrylate (SA) and potassium persulfate (KPS) that can swell up to 10 times its size when immersed in water and is robust enough that it can be held in one's hands [22].

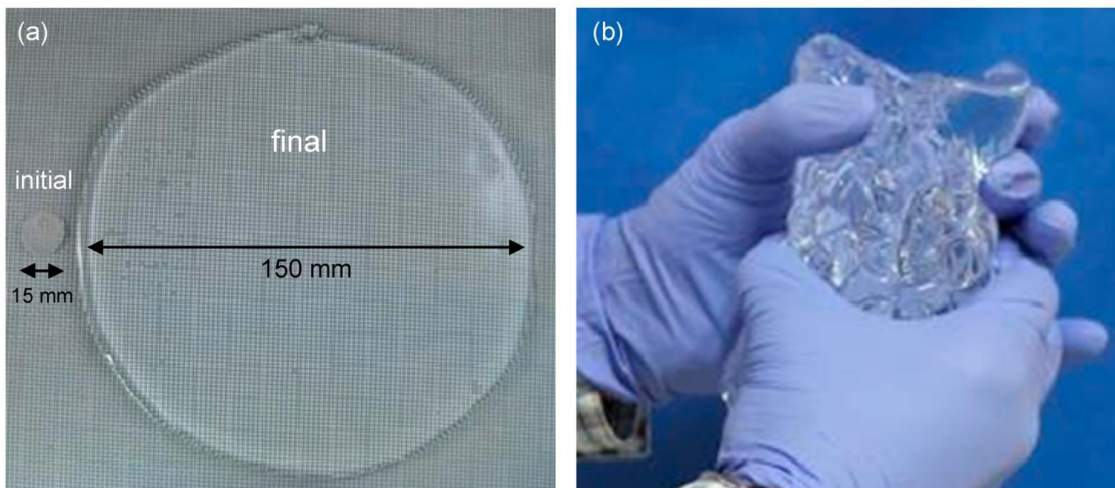


Fig. 4 (a) DMMA-SA+KPS hydrogels before and after the swelling tests; after swelling equilibrium (b) the hydrogel can withstand the pressure of the hands and is malleable. Reprinted with permission from [22]. Copyright © 2017, ACS.

5 Structure Analysis

To understand many features that hydrogel possess, it is necessary to analyze its structure, and for that, many characterizations techniques are available with key differences and advantages' depending as to what is to be understood from the hydrogel. The most important characterization techniques for cellulose hydrogels are described in Table 1.

Table 1 Structure characterization techniques for cellulose hydrogels.

Equipment	Basic principle	Key analysis
Nuclear magnetic resonance (NMR)	Measurement of absorption of radiofrequency radiation by a nucleus in a strong magnetic field. Spectroscopy of nuclear spin states.	Obtain molecular organization, interactions and mobility of gels constituents.
Fourier Transform Infrared Spectroscopy (FTIR)	Measurement of absorption of infrared spectrum via emission of a solid, liquid or gas.	Obtain chemical structure of the hydrogels, such as chemical bonds.
Ultraviolet-visible spectroscopy (UV-Vis)	Measurement spectroscopy in the ultraviolet-visible spectral region.	Quantitative determination of different analytes and biological macromolecules
Circular dichroism (CD)	Absorption difference between the left and right circularly polarised light which occurs if a molecule possesses one or more chiral chromophore	Obtain information about chiral molecules and useful for analysing secondary structures of macromolecules.
Fluorescence spectroscopy (FS)	Measures the molecular absorption of a light energy at one predetermined wavelength.	Quantitative determination of different analytes with accurate results.

5.1 Nuclear Magnetic Resonance (NMR)

The Nuclear magnetic resonance (NMR) technique analyzes magnetic fields through specific resonances frequencies that are emitted and reabsorbed depending on sample field strength and magnetic properties, which can characterize complex systems of polymers or isolated biopolymers of plants [2].

The NMR spectra supplement important information for the characterization of hydrogels, it is through the changes observed in the lines of this spectrum that one can suggest if changes occurred in the chemical structure of these polymers, often using external stimuli

hydrogels, to investigate the continuity on the chemical integrity or if some chemical reaction occurs between the polymeric chains [18].

For cellulose-based hydrogels, NMR indicates, in addition to other properties, good chemical stability since no reactions are observed between the most common hydrogels even when exposed to temperature ranges of 150 to 190 °C. This is concluded by the results at the end of the analysis, where samples have the same chemical structure when compared with the beginning [23].

Through NMR it is possible to analyze the interference of the association of other polymers, such as cellulose, on the molecular mobility of hydrogels. The addition of cellulose nanowhiskers into gelatin promotes an increase in the stiffness, to the same extent that it decreases its mobility [24].

NMR can also be used to verify the integrity and structural behaviour of hydrogels during some external stimuli. The addition of cellulose nanocrystals in the carbon-dot hydrogel formulation promoted a considerable thermal resistance to the product, typical cellulose bands are observed in samples subjected to hydrothermal carbonization (up to 240 °C) [18].

5.2 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a technique which has been commonly used for characterization of hydrogels. This technique provides the absorption peaks corresponding to frequencies of vibrations between the bonds of atoms. Furthermore, a unique spectrum is created for each material, therefore enabling accurate identification of the materials and characterization.

The use of FTIR for characterization of hydrogels consists of investigating and evaluating the chemical structural surface of the functional polymer groups. This effective technique obtains the spectra of absorption, emission and photoconductivity of materials in any physical state, giving relevant information on the molecular structure of polymers [16].

Studies performed via incorporation of cellulose nanocrystals on carbon-dot hydrogels exhibited thermal resistance, which provided similar bands in FTIR spectra between the hydrogels before and after the hydrothermal treatment (180-240 °C). In addition, the hydrogel

maintained the main functional groups of the cellulose, without altering the main structure. However, an alteration was noticed when the hydrothermal treatment was raised to 260 °C, which carried the hydrogel to a complete hydrothermal carbonization [18].

It is also possible with the FTIR analysis to evaluate the interaction between different compounds and polymers in between the hydrogels. For example, the association of cellulose hydrogel with hydroxyapatite for application in biomedical area is possible to identify through peaks in FTIR spectra, and it can confirm the interaction between these two materials, suggesting an association capacity of cellulose [25].

5.3 Ultraviolet-visible Spectroscopy (UV-Vis)

The evaluation of hydrogels on ultraviolet light is an interesting alternative to evaluate drug delivery mechanisms. UV-Vis occurs by passing a light through a specific region of the degraded or diluted polymer solution or drug. The light absorbed by a molecule of the compound can go from its ground state to an electronically excited state and this information is stored by a detector which quantifies these parameters. For the majority of conjugated molecules, its photons absorption energy fall within the range of near UV and visible light [26].

Characteristics of cellulose-micelles or dendrimers can be evaluated using UV-Vis, sharing important information such as density values and hydrogel efficiency of drug loading. [26]. In addition, it can also hold important information in terms of concentration of nanoparticles incorporated into the hydrogel material.

Raghavendra et al., (2013) utilized gum acacia (GA) and gaur gum (GG) in various concentrations to incorporate and form silver nanoparticles from silver nitrate (AgNO_3). Confirmation of silver nanoparticles formation was obtained via UV-Vis and the process of formation Ag^+ ions to silver nanoparticles is due to the reduction action of functional groups present in GA and GG, where the pendent hydroxyl groups were involved in the reduction factor. Increasing the molecular weight had to obtain more stable silver nanoparticles [REFS]. It is understood that there is a relationship between the wavelength emission of a polymer or hydrogel with its structural characteristics, that emission can still be influenced by the environment or not. The nanocellulose-based hydrogels can stabilize other compounds when associated, preventing their aggregation, this capacity is related to the carboxylic groups of

the cellulose, the result of which is a higher molecular organization, consequently causing a larger wavelength emission [27].

5.4 Circular Dichroism (CD)

Circular dichroism is a spectroscopy technique which characterizes the structure of polymers using the variation of polarized light absorption. Cellulose-based hydrogels tend to demonstrate a strong positive ellipticity, arising from the direction of reflection from left-handed circularly polarized light in chiral nematic phase [12].

The circular dichroism on hydrogels evidences the organization or molecular disorganization on the structure. These evaluations can still be performed on different conditions, where the molecular organization's response to external stimuli is observed, and it is possible to perceive a variation of the molecular order according to the temperature [12]. Hydrogels based on cellulose tends to present a high molecular organization.

5.5 Fluorescence Spectroscopy (FS)

Fluorescence spectroscopy (FS) is a technique that consists of exposing the sample to a beam of light, most often ultraviolet light or visible light, causing an excitation of the molecules of the polymers and once stimulated, reaction occurs, and FS is done by measuring the light emitted from a sample after absorbing light at a higher energy than it is emitting [18].

Hydrogels based on nanocrystals cellulose and acrylamide demonstrate excellent photoluminescence properties by showing a direct relationship between emission wavelength and maximum intensity with excitation wavelength. This characteristic of cellulose nanocrystals based hydrogels can be related to the surface with energy traps through incomplete reactions and due to the radiative recombination of electrons and holes [27]. It is possible that polycyclic aromatic compounds also have an influence on the behaviour of these hydrogels [28].

It can be noticed that the emission properties of the hydrogels based on cellulose nanocrystals are influenced by external stimuli, among them the pH, this is due to changes in the surface properties of these products as well as in the electronic transitions of these products [18]. The increase in pH causes a decrease in emission intensity but does not influence on the wavelength [28].

6 Cellulose Hydrogels and Mechanical Properties

6.1 Key Aspects of Measuring Mechanical Properties on Superabsorbent Hydrogels

Superabsorbent gels can have a swelling ratio in the order of 100-1000 for many gels, which is inversely proportional to the crosslinking density [22]. As the crosslinking density increases, the gels become soft and floppy and can be a challenge to work with as they tend to be slippery and difficult to grasp. However, gels that are highly cross-linked tend to be stiff but they are also generally quite brittle and tend to decrease its sensor capabilities [29]. This brittleness occurs due to the free-radical polymerization and the cross-linking in these structures is heterogeneous with many chain loops. If this gel is deformed, chain segments could deform more than others, leading to zones with high stress leading to possible failure at low deformations [30].

Most polymers are relatively poor in terms of mechanical properties in order of KPa [31] compressive strength and if the polymer is designed for biomedical applications such as bone healing – the bone is in the order of 170 MPa innovations with the polymer are necessary [32].

Another important aspect of measuring mechanical properties of hydrogels is that due to the relatively low pore sizes (1-100 nm range), it is one of the easiest ways to obtain the values of the behaviour of the hydrogel network (such as: mesh size, volume swelling coefficient, molecular weight between two adjacent crosslinking points) [31].

Measurement of mechanical properties needs to be evaluated carefully since they are time-dependent due to the viscoelasticity of the polymer network and time-dependent deformation fluid flow [31]. To exemplify the importance of this topic, hydrogels for tissue engineering tends to lack mechanical properties [33]; however, as cells proliferate and elongate on the hydrogel implant, they improve the mechanical properties as the time passes via reorganisation of fibres and production of extracellular matrix [34, 35].

Water evaporation can also affect the measurements of the hydrogel if these are performed in the swollen state [36]. Furthermore, further compression of the hydrogel could change the structure and the process is irreversible as it will be discussed.

6.2 Universal Test-frame

Universal Test-frame is the most common tool for mechanical characterization of materials which can perform several techniques such as tensile, compression, adhesive strength, torsion, among others. For tensile tests (Fig. 5a), the technique consists in applying a tensile force to the extremities of the material which are held between two grips. However, hydrogel samples tend to be hydrated which makes it difficult to grip so cardboards are used or performed with glue [31]. With this technique, a chart of stress-strain is obtained and values of Young's modulus, yield strength, maximum elongation at break are derived from the data tested. This test has some issues as it destroys the sample and only once it is possible to study each specimen, in terms of studying the hydrogel mechanical properties over time this test is not advised.

Bacterial cellulose possesses higher water holding capacity and superior tensile strength compared to plant cellulose [37]. In addition, bacterial cellulose has mechanical properties such as tear resistance, which is superior to many synthetic materials [38]. For these reasons, researchers developed a tube-shaped cellulose and assessed its potential as a substitute for blood vessels [39, 40] which demonstrated values of tensile strength around mN which is comparable to those of normal blood vessels, it could withstand the blood pressure of a rat [41]. Results showed that after four weeks, the tube was covered with oriented endothelial cells which enhances the stability under shear stress that occurs when blood flows through these vessels.

More recently, researchers have been investigating cellulose nanocrystals which are produced from chemical treatments from pulp cellulose fibres. These nanocrystals possess many desirable properties, such as high tensile strength (7500 MPa) and high stiffness (Young's modulus up to 140 GPa) with an abundance of surface hydroxyl groups [42, 43]. The addition of these nanocrystals into synthetic hydrogels can improve mechanical properties and exhibit controllable swelling ratio [44, 45].

In addition to tensile tests, Universal test-frame can also measure compression tests (Fig. 5b). In compression tests, values are obtained by the pressure applied to the surface of the hydrogel and distance which is compressed. Compression tests, however, has several limitations when testing hydrogels, such as expanding under compression and pressure might not be applied evenly. These limitations result in high standard deviation values if the sensor

of the machine is not very precise. To overcome these problems, samples are normally measured in a dry state, or in a confined compression chamber (Fig. 5c), after it reaches the equilibrium swelling ratios to improve its precision [46].

Superabsorbent hydrogels have many applications in agriculture due to the need of reducing water consumption and optimize water resources. The ability to store large quantities of water from these hydrogels makes it excellent in this field; moreover, these hydrogels store water even under significant compression [37]. The compressibility of a structure is also an indicator of the rigidity of a foam and cellulose hydrogels not only can improve the swelling capabilities of a synthetic polymer but also its stiffness [47].

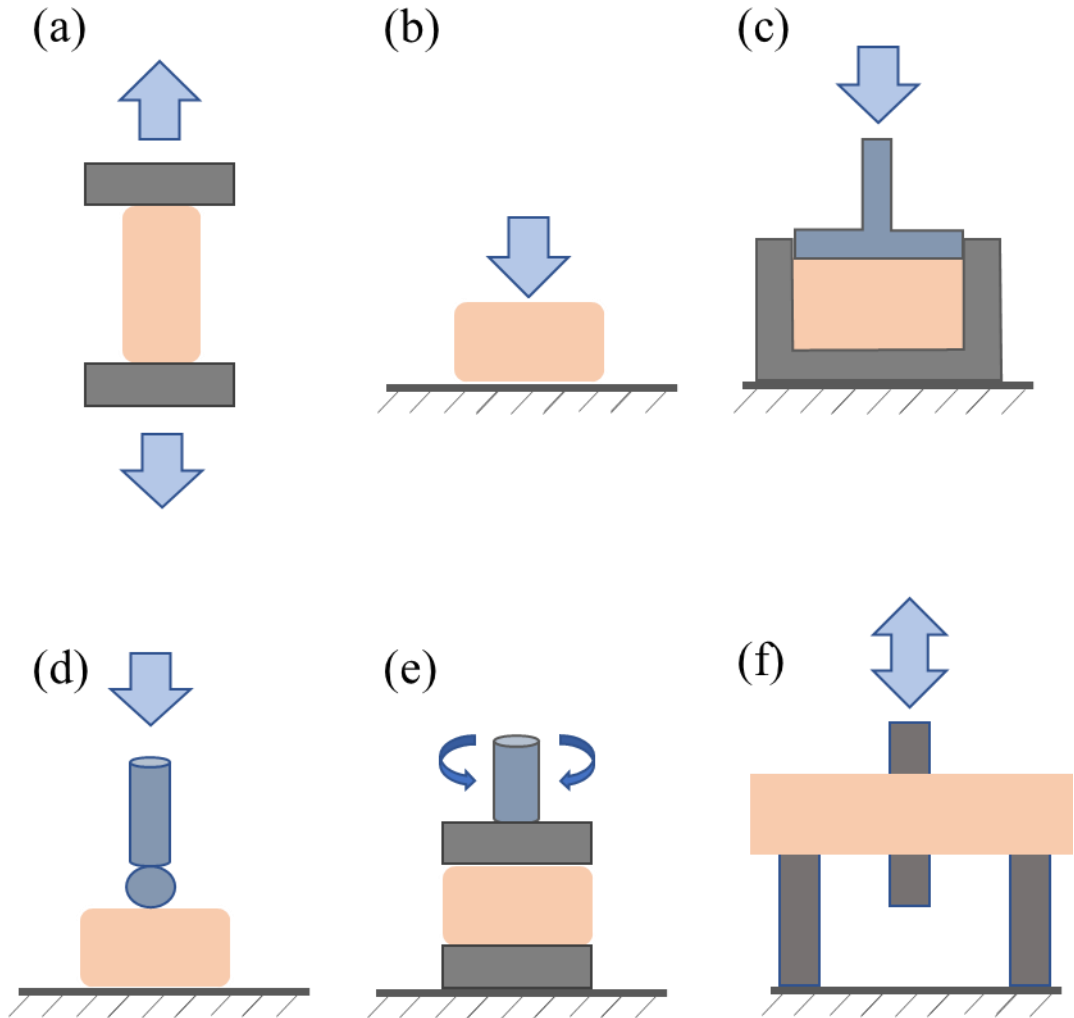


Fig. 5 Different types of tests that can be performed for mechanical properties. (a) Tensile; (b) Compression; (c) Confined compression; (d) Indentation; (e) Rheology; (f) Dynamic mechanical analysis.

6.3 Instrumented Indentation

Instrumented indentation is a versatile technique that aims to measure elastic and plastic properties in micrometric and nano-scales. It is an expansion of the capabilities of the traditional hardness test and consists of penetrating a diamond tip into the material, controlling and recording the load and the depth of penetration on nanometre scale with large amount of data (Fig. 5d), which are plotted in a force-displacement diagram, forming a load-unloading curve. It is used to measure mechanical properties of materials with modified surfaces, thin films, among others [48].

As this technique involves some knowledge of mathematical models, some brief introduction to the theoretical methods will be given.

Fig. 6 exhibits the surface of a sample after being penetrated by an indenter to the depth h of the surface due to the application of a force P . At this depth, there is elastic and plastic deformation forming an impression of the formed tip used for any depth of contact h_c . After the tip is removed the part of the material that has suffered elastic deformation is recovered.

In Fig. 6, h_c is the contact depth between the tip and h_s the sample is the displacement of the contact perimeter surface. The depth h is related in equation 1.1

$$h = h_c + h_s \tag{1.1}$$

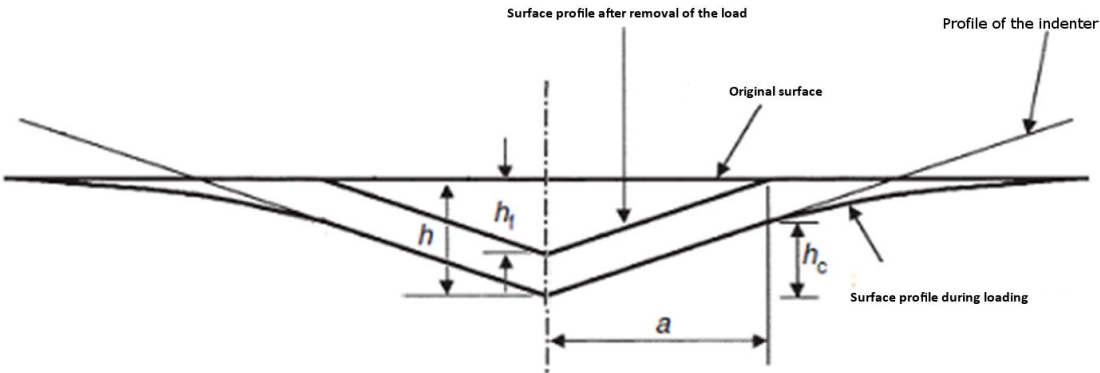


Fig. 6 Schematic of a section in two moments of a penetration. Original artwork.

After removal of the tip and recovery of the elastic deformation, a final residual impression remains. As E_R corresponds to elastic recovery, the relationship between these magnitudes are related in equation 1.2

$$h_{max} = h_f + h_s = h_c + h_s \quad (1.2)$$

The deformation of the diamond tip, however small, should be taken into account. Where it is necessary to define the equation 1.3

$$\frac{1}{E_R} = \frac{1-\nu^2}{E^*} + \frac{1-\nu_i^2}{E_i^*} \quad (1.3)$$

E is the modulus of elasticity, ν the Poisson ratio of the sample, ν_i, E_i, E_r , correspond to the Poisson's ratio, elastic modulus of the tip and reduced modulus of the indenter set and sample respectively.

The Graph of Fig. 7 exhibit a loading-unloading cycle (load applied as a function of the stress). The contact stiffness S can be determined using the maximum loading point of the unloading curve, that is, it corresponds to the slope of the first moments of the elastic recovery as related to equation 1.4

$$S = \frac{dP}{dh} \quad (1.4)$$

That is also related to the reduced module [49], by equation 1.5

$$S = \beta \frac{2}{\sqrt{\pi}} E_r \sqrt{A} \quad (1.5)$$

Where A is the contact area designed for maximum load and β is a dimensionless constant that corrects deviations in stiffness caused by the lack of axial symmetry of pyramidal indenters. This evaluation, made during the contact and not after the tip removal.

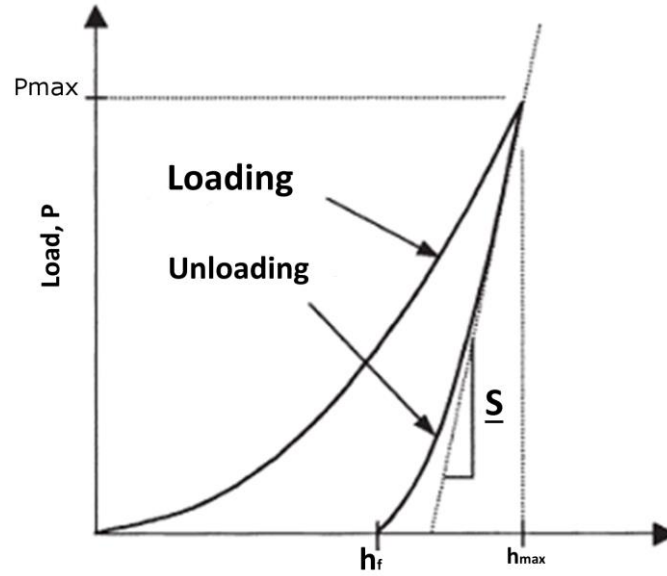


Fig. 7 Schematic representation of a load curve P by a displacement h for a complete load-discharge cycle [50]. Original artwork.

Assuming that the tip does not deform significantly, A is a function of the depth of contact between indenter and sample, which is obtained during tip calibration by the equation 1.6

$$A = F(h_c) \quad (1.6)$$

Where h_c is the depth of contact related to equation 1.7

$$h_c = h_{max} - h_s \quad (1.7)$$

the deflection of the surface h_s in the perimeter of contact depends on the geometry of the penetrator and is related via equation 1.8

$$h_s = \varepsilon \frac{P_{max}}{S} \quad (1.8)$$

The ε is a constant with a value of 1.0 for flat tips, 0.75 for paraboloids of revolution and 0.72 for conics; P_{max} is the maximum applied load.

By extracting the values of P_{max} , h_{max} and S from a load-unloading curve, the elastic modulus E of the material is calculated from the combination of expressions (1.3) and (1.8). The hardness H is given by equation 1.9, load P_{max} :

$$H = \frac{P_{max}}{S} \quad (1.9)$$

Instrumented indentation for superabsorbent hydrogels has several advantages over other conventional mechanical characterisation techniques [33, 51] such as quick, online and real-time measurements of materials. Several important characteristics of the hydrogel can be obtained using instrumented indentation. However, the instrumented indentation was not commonly used for hydrogels due to the limitations of commercial instruments which were originally developed for stiff, engineering materials [52]. In addition, the jelly characteristic of these materials leads to a significant effect on adhesion between the indenter tip and the sample resulting in large errors for any known mathematical correction model [53, 54].

The adhesion of a hydrogel surface attracts the indenter tip and result in a negative load at the beginning of the experiment measured by the indentation machine. It is important to define the initial point of contact of the indenter tip because such point is important for calculating the contact area and the elastic modulus [55], error in the initial point of contact can lead to inaccurate values by the equipment.

Recent advances [53, 56] shows promising results and correct values for measuring the mechanical properties of hydrogels in the range of nanoscale. Basically, the method uses a model proposed by Johnson-Kendall-Roberts (JKR) that consider the adhesion of the gel surface [57]. This model in the recent years only worked accurately with spherical shapes for nanoindentation of hydrogels samples but the recent work of Jin C. et al., (2016), recently brought the attention of a possible utilization of the JKR model to Berkovich and flat indenters [54].

However, Wei J. et al., (2016) used a multi-indentation to determine the initial indentation depth for the Oliver-Pharr mathematical method correction, the researchers used multiple indents with determined preloads to find the values of preload and maximum apparent indentation depths so they could calculate the initial true initial indentation depth [58].

6.4 Rheology

Rheology is an important technique of characterization of polymers, which mainly aims to evaluate the flow of materials in liquid as well as semi-solid states. The obtained data is fundamental to the behaviour of hydrogels (Fig. 5e) in different conditions or external stimuli [18].

Knowledge about the viscoelasticity of a hydrogel or gel has great importance for the understanding of the interactions of the polymers used in its production as it is also fundamental to the evaluation of the viability according to its application. Cellulose-based hydrogels may present as a rigid cross-linked gel, with a rubbery consistency, which is optimal for hydrogel application to bone implant or drug delivery systems [59].

The association of cellulose nanofibers with gelatin hydrogels promotes a significant increase of up to 150% in the system's modulus storage, which implies that the cellulose nanofibers provide a viscous consistency to the hydrogels while maintaining the mechanical resistance of the product, a conformability that favors its use, especially as a membrane [24].

Liu et al., (2017) produced dual stimuli responsive cellulose hydrogel and observed that the elasticity of these hydrogels are influenced by the concentration in the product formulation. A positive relation with this mechanical property is observed; however, this positive relation is only detected until a certain cellulose concentration, after this limit, there is no significant influence [60].

The increase in the mechanical properties of hydrogels is a feature that makes cellulose a biopolymer of choice when the objective is a compound that exhibits viscosity associated with a high elasticity. This characteristic is often associated with the molecular weight of the cellulose [60].

6.4.1 Role of Water in Mechanical Properties of Cellulose Hydrogels Investigated with Rheology

Water is one of the most important components of polysaccharide-based hydrogels (90% of their weight when in their swollen state) and it plays an important role in the mechanical properties of polysaccharide hydrogels. The water inside a hydrogel can bound or semi-bound with the polymer structure, this is important because it forms a hydrogen bond and it is a key role in mechanical properties [61]. Although, these polysaccharides hydrogels can be squeezed through the needle of a syringe, also called “injectable hydrogels”; researchers [62, 63] have analysed that the squeeze of a needle could change the mechanical properties of this polymer [64, 65].

As such, researchers have been trying to understand the role of water in the mechanical properties of polysaccharide hydrogels; Pasqui et al., (2012) found that in rheology, values of storage modulus (G') and elastic modulus (G'') reduces after these hydrogels are squeezed through a syringe or stressed prior the test but more cycles of stress or passing through a syringe does not vary the values.

The stress performed by the rheometer machine with a raw hydrogel also changes the values of the modulus in polysaccharide hydrogels and, if more tests are made after this first attempt, it changes the values but becomes continuous with more tests. This is related to a recovery effect once the material is subjected to further cycles of stresses [65]. However, the structure or degree of crosslinking of the hydrogel does not alter after being stressed but the entangled polymer chains unroll when a stress occurs and align towards the direction of the stress, leading to a swelling by more than 92% of the native cellulose hydrogel due to the free unprotonated COO^- which increases the electrostatic repulsions between the polymer chains.

6.5 Dynamic Mechanical Analysis (DMA)

The dynamical mechanical analysis is an important characterization tool which provides important data about the mechanical and viscoelastic properties of hydrogels, a sinusoidal stress is applied and the strain of the material is measured (Fig. 5f). This tool can still simulate pre-determined conditions, such as the physiological conditions of the human body and able to obtain behavioural data and responses of hydrogels on these conditions [66].

The choice of the polymer for hydrogel formulation should take into account several factors, including the objective of using the hydrogel. Since the properties of the hydrogels are directly related to the polymers used in the production, among them the mechanical characteristics which can be influenced, and thus manipulated, according to the concentration of the polymers used [67].

It is possible through DMA; perform the characterization of mechanical properties of hydrogels on different external conditions, such as simulating adverse situations and evaluating the response of these polymers to these stimuli. Cellulose-based hydrogels exhibit excellent mechanical properties, even under conditions of high humidity, while maintaining elasticity with a high tensile modulus [68]. The addition of cellulose nanocrystals to the

hydrogels formulation promoted an increase in the stiffness, this property of the cellulose biopolymer is also reported in several other characterization techniques, this is probably due to the cellulose inducing an effect on reinforcement, and more importantly, via DMA. Through the DMA, it was possible to observe that cellulose concentration in a hydrogel containing hyaluronic acid and cellulose nanocrystals promotes a direct positive effect on the stiffness, and can promote up to 135% increase over the modulus storage, however, this positive effect is limited to a critical quantity to the cellulose concentration [66].

Basu et al., (2017) demonstrated that hydrogels based on bacterial cellulose have a significant increase in its mechanical stability, this is probably due to the strong hydrogen bonds interactions between the cellulose chains [59]. This is also observed with cellulose biopolymers as shown by Lavoratti et al., (2016) where it was used an unsaturated polyester resin (UPR) and cellulose nanofibers (CNFs) obtained from dry cellulose waste of softwood (*Pinus sp.*) and hardwood (*Eucalyptus sp.*), a significant increase occurs in viscoelasticity and the activation energy, which corresponds to the amount of energy required to initiate the mobility of the polymer chains. The study suggests that a better fibre/matrix interface due to cellulose biopolymer promotes a better interaction between the hydrogel [69].

7 Thermal Methods of Analysis

The state of water in hydrogels gives valuable information about their properties. Melting, crystallization and glass transition temperatures of water in hydrogels reflect the state of the water-polymer interaction and can further improve the understanding of the hydrogel mechanism.

7.1 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) independently measures the rate of heat flow to a substance and its reference material at the same temperature. Heat flow is monitored and recorded as a function of temperature from which data is derived. Through the application of heat to the system, the properties of the substances are measured by thermal analytical methods.

DSC technique is mainly used to characterize the behavior of different polymers, and their associations in relation to structure and consistency when subjected to temperature

variations, either as heating or cooling the sample [5]. DSC can also be used to assess thermal stability with respect to degradation at body temperature and provide a thermal characterization of the materials used to produce hydrogels.

The characterization of hydrogels using DSC can evidence the response to the change in the physical state of the polymer against different temperature conditions. This behaviour plays an important role in the indication of the hydrogels usage, which makes it preferable to use as a basis or combination with the formulation, for polymers that promote a certain thermal stability [68].

Cellulose biopolymers incorporated into Polyvinyl alcohol fibres have the feature of promoting thermal stability. This ability is due to the property of acting as structural reinforcement the polymer chains of the hydrogels, reflecting an increase of the thermal and mechanical properties of the system [68].

By using heating and cooling cycles in methylcellulose hydrogels, it can be seen from the DSC that gelation rate tends to rise, probably due to the cooperation of the hydrophobic interaction among methyl groups with the intermolecular hydrogen bonds among of hydroxyl groups [70].

Patchan et al., (2013) tested different microcrystalline cellulose derived from various sources and noticed on DSC that cellulose increases the thermal degradation to 250°C, due to the high melting point of the cellulose. The low-temperature peak position increase with cellulose concentration, possibly because of the high level of linked water in hydrogels with increasing concentration [71].

Barros et al., (2015) investigated a polymer composed of chitosan and (hydroxypropyl)methyl cellulose and reports that the cellulose presents thermal resistance and stability to the external stimuli like temperature and pH, without significant alterations in the thermal behaviour of these products after variations of pH and temperature [72].

Despite the stability promoted by cellulose against some external stimuli, hydrogels based on this biopolymer can suffer a decrease in the thermal and mechanical characteristics after their degradation. As indicated by the work of Demitri et al. (2016), where this decrease

was due to the hydrolysis of the glycosidic bonds of the polymers, causing a partial degradation of the hydrogel [15].

7.2 Thermal Mechanical Analysis (TMA)

The thermomechanical analysis or TMA consists in a technique of characterization of polymers that seeks to determine the thermal stability of these products, obtaining important data regarding the behaviour and durability of the polymers in front of different temperatures conditions [59].

Cellulose type I and II nanofiber supports high temperatures before starting its degradation process, which occurs from 150 °C, initially occurring a mild degradation up to 250 °C, above this, it is observed the complete degradation of the cellulose due to the breaking down of its molecular structure. These thermal properties can be altered according to the variation of the biopolymer, where the cellulose nanocrystals have a higher thermal resistance, being degraded at temperatures of approximately 350 °C [73].

The use of cellulose nanowhiskers in the poly(lactide) PLA via graft method using n-octadecyl-isocyanate provides an increase in the resistance and thermal stability which is greatly influenced by the concentration used. However, the concentrations that increased the thermal characteristics of the product were the same ones that allowed a reinforcement action in the hydrogel network [74].

A comparison of the thermal stability between the natural fibre cellulose biopolymers with nanocrystals formed from mercerized fibres reveals that the latter has a higher stability against temperature changes, this is due to the stronger hydrogen bonds, another factor that contributes to this is the high purity and crystallinity of the cellulose nanocrystals [74].

A data that can be obtained through the TMA is the coefficient of thermal expansion (CTE), this is an important thermophysical property for polymers, mainly thermosetting resins, where the lower the CTE the greater the dimensional stability of the polymer or hydrogel. Due to better dispersion and morphology of cellulose fibres, hydrogels formulated with these polymers have a lower CTE and also influences other thermophysical aspects such as a better thermal stability of the product [69].

8 Biological Techniques

Cellulose hydrogels have many applications in biomedicine. However, it is important to analyse its biocompatibility including cytotoxicity and how cells proliferate and differentiate into these structures for further studies *in vivo*. This section introduces some basic concepts of biological techniques and the behaviour of cellulose hydrogels.

8.1 Cell Culture and Adhesion

The ability of a hydrogel to maintain cell adhesion and proliferation on its surface as well as control its behaviour are essential requirements for the successful use of these hydrogels in the field of tissue engineering [66].

The addition of cellulose nanocrystals in hyaluronic acid hydrogels has shown good interaction with cells. The cultures of adipose cells in these materials have shown proliferation and elongation after 24 hours of culture. The study suggests that presence of cellulose nanocrystals corresponds with the density, morphology and cytoskeleton organization of the studied cells [66].

Hossain et al., (2014) studied PLA fibres coated with blends of cellulose nanowhiskers compared to uncoated PLA fibres. The results have shown that hydrogels based on cellulose nanowhiskers present an excellent environment for cell adhesion and proliferation, which occurs intensely and rapidly. In addition, after 24 hours of cell culture, it was possible to observe the formation of a confluent structure with several cell layers, which suggests a cellulose stimulus for cell attachment as observed in Fig. 8 [75].

When performing cell culture derived from bone marrow in a PEGDA700 hydrogel incorporated with CMCNa porous implant. The interaction observed is that these hydrogels act to stimulate cell adhesion and proliferation, with intense cell growth being observed at 14 days. In addition, during this period, an increase in cell differentiation and activity of osteoblasts occurs on samples with cellulose. This, confirms the ability of the cellulose-based implant to withstand osteoblasts differentiation [15].

Rauci et al., (2014) studied hydroxyl ethyl cellulose (HEC) incorporated with CMCNa using a chemical treatment that induces $-COOH$ functional groups and investigated this hydrogel using human mesenchymal stem cells line (hMSC) . The results show that the

cellulose hydrogel exhibits its potential to be used as implants or biological membranes, which have excellent biocompatibility for the most diverse tissues that make up living organisms. In addition, low cytotoxicity was detected which allowed the adhesion of these cells on hydrogels with rapid and intense cellular development on its surface compared to standard samples. [76].

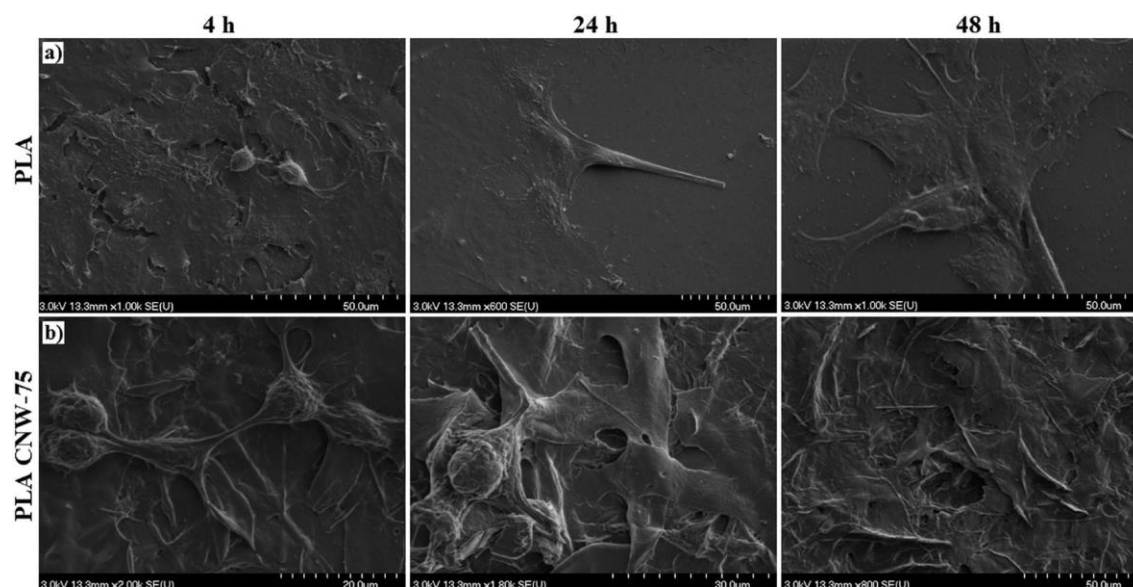


Fig. 8 NIH-3T3 mouse fibroblast cell morphology and spreading at varying time points (4, 24 and 48 h). (a) Polylactic acid; (b) Polylactic acid with cellulose nanowhiskers. Reprinted with permission from [75]. Copyright © 2017, ACS.

8.1.1 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

The MTT (3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide) assessment seeks to analyze the viability of cultured cells in hydrogels by adding information on the cell functionality effect. The test provides fundamental data for the biocompatibility and the application of polymers or hydrogels that are aimed to be used as biomaterials, such as implants that promote the acceleration of tissue healing [77].

Peng et al., (2016) investigated a novel quaternized cellulose (QC) and native cellulose in NaOH/urea aqueous solution. These hydrogels present excellent biological characteristics, besides allowing excellent cell adhesion and proliferation. The cellulose-based hydrogels have low cytotoxicity, where MTT assays exhibited that up to 80% of the cells

growth remained viable, mainly due to the improvement of the hydrogel cytocompatibility due to the cellulose network [77][78].

The cytotoxicity of the cellulose biopolymer in various cells has already been reported in literature [79], these properties favor the use of this biopolymer in the biomedical area, artificial blood vessels [80], implants for bone tissue [25], drug delivery system [81], and application in the regeneration of peripheral nerve damage [82].

Such improved biocompatibility is shown in Fig. 9 where work of Cheng et al., (2014) investigated a thermoresponsive polysaccharide hydrogel based on nanofibrous cellulose and elastin-like polypeptide (ELP). High fibroblast viability was obtained (Fig. 9a) indicating a non-cytotoxic hydrogel while cells were spread towards the surface of the hydrogel (Fig. 9b) and they were capable of proliferating even after 7 days of incubation (Fig. 9c-d).

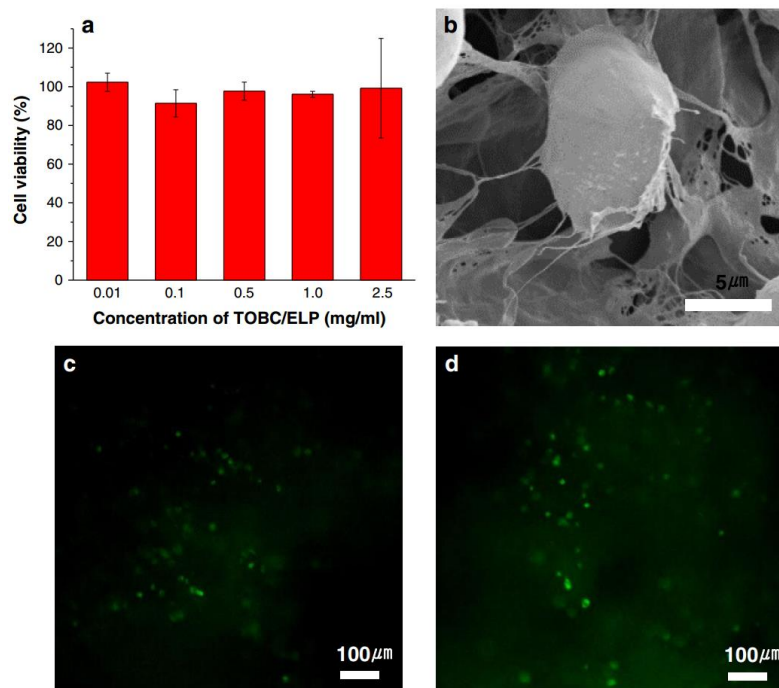


Fig. 9 a) MTT results of the TOBC/ELP solution. b) FESEM micrographs of cells encapsulated in a TOBC/ELP hydrogel. Fluorescence microscopic images of fibroblast cells encapsulated in a TOBC/ELP hydrogel after c) 1 day and d) 7 days of incubation (cells were live/ dead stained.). Reprinted with permission from [83]. Copyright © 2017, Springer.

8.2 *In vivo* Characterization

For hydrogels designed for biomedical applications, *in vivo* biological characterization tests are fundamental, through this analysis it is possible to evaluate the behaviour of the hydrogel,

either as a controlled drug distribution system or as implants for tissue replacement or regeneration, on the conditions of living organisms [84].

Cellulose-based hydrogels are widely studied and employed in the biomedical area for various purposes such as wound dressing, which is one of the most reported, and its use has been researched for some time with the aim of treating wounds by burns and chronic injuries that are difficult to heal [84].

Portal et al., (2009) investigated the use of cellulose membranes for the application of healing chronic skin wounds, which are difficult to heal. The results suggest that cellulose membranes healed 75% of analysed wounds in 81 days, while conventional treatment took 315 days to heal 75% of the wounds. The study also evidences on cellulose membranes, a reduction in wound epithelization time of 74.5% when compared to conventional treatment. This indicates that cellulose on implants clearly benefits for the treatment of chronic injuries [85].

The elasticity and conformability present in implants made from cellulose lead these materials to obtain excellent adhesion, favouring their application in practically any part of the body. The use as a wound dressing for burn wounds in the face promotes a complete healing of wounds in 44 days, making it not necessary to use grafts as well as not being observed signs of extensive scars [86]. Cellulose membranes also have adequate properties on mechanical and biological for wound dressing. These membranes act as an accelerating factor in the healing of wounds, presenting ease of application and removal of the wounds while also being a painless product [87]. A cellulose membrane for wound healing being tested *in vivo* is shown in Fig. 10, and, due to the swelling characteristics of these hydrogels, it creates a moist environment hydrating the wound while also relieving the pain by delivering any drugs that can be introduced in its structure.

Cellulose membranes also work well in wounds that are difficult to heal, such as wounds in patients with diabetes, and an increase in wound healing rate has been reported as well as a reduction in the epithelization in wound healing time of these patients when compared to other treatment methods [88].

The use of a cellulose-based biosynthetic blood vessel as an implant in an ovine animal model revealed promising results, this cellulose implant could function even after 13 months, with the presence of endothelial cellularity in all segments of the implant [80].

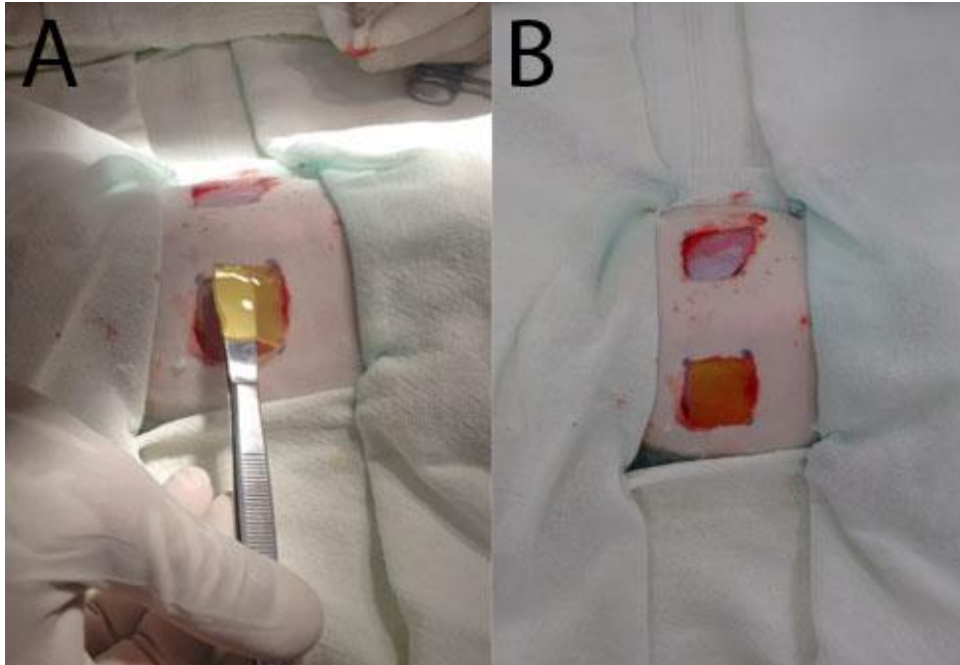


Fig. 10 (A) Placement of hydrogel membrane in the surgical wound; (B) Hydrogels dressing in the target of wound. Original artwork.

9 Conclusion

The cellulose biopolymer is a material that presents wide versatility of use in several areas, is a renewable resource and is present abundantly in nature which has properties that favour its use for various purposes.

The hydrogels based on or associated with, cellulose has a higher capacity to withstand mechanical forces maintaining a balance between stiffness and elasticity, besides increasing thermal resistance and to other external stimuli. Finally, this biopolymer has excellent biocompatibility and ability to maintain a favourable environment for cell growth and proliferation.

The characterization techniques allow evaluating the addition and permanence of all the positive properties of biopolymers as cellulose-hydrogels. These techniques are essential for

the research and production of increasingly improved and specialized hydrogels for a specific application.

10 References

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