Novel hydrogels based on yeast chitin-glucan complex: Characterization and safety assessment

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ABSTRACT

Chitin-glucan complex (CGC) was used for the first time for the preparation of hydrogels. Alkali solvent systems, NaOH and KOH solutions, either at 1 or 5 mol/L, were used for CGC dissolution using a freeze-thaw procedure (freezing at -20°C and thawing at room temperature; four cycles). The CGC solutions thus obtained were subjected to dialysis that induced the spontaneous gelation of the biopolymer, yielding translucent hydrogels with a yellowish coloration. Although all CGC hydrogels exhibited porous microstructures, high water content (above 97%) and good mechanical properties, their morphology, viscoelastic properties and texture were influenced by the type of solvent system used for CGC dissolution, as well as by their ionic strength. The K-based hydrogels presented a less compact network with larger pores and exhibited lower elastic properties. The Na-based hydrogels, on the other hand, exhibited a denser structure with smaller pores and a stiffer gel structure. These results show that it is possible to prepare CGC hydrogels with differing characteristics that can be suitable for different applications. Furthermore, all hydrogels were non-cytotoxic towards L929 fibroblasts and HaCaT keratinocytes. This study demonstrates CGC can be used to prepare biocompatible hydrogels with properties render them promising biomaterials.

1. Introduction

Hydrogels are three-dimensional macromolecular networks capable of adsorbing high amounts of water, saline and physiological media in their structure and swell [1–3]. These soft and pliable structures can be based on synthetic polymers (e.g. acrylic acid, acrylamide, poly(vinyl alcohol)) or on biopolymers (e.g. gelatin, starch, alginate) [2–4]. The latter have attracted considerable interest due to their biocompatibility, biodegradability and tissue-mimicking consistency, and are used in applications that range from agriculture and water treatment, to hygiene products and biomedicine [1,5]. Hydrogels can be divided into two types, namely, physical or chemical, depending on the type of procedure used to cross-link their polymer chains. Physical hydrogels are obtained by the establishment of physical interactions (e.g. chain entanglements, van der Waals forces, hydrogen bonds or ionic interactions), which are driven by exposing a polymer solution to specific temperatures, pH values and/or ionic strength. On the other hand, in chemical hydrogels, chemical cross-linkers (e.g. epichlorohydrin, succinic

anhydride) are used to induce the formation of a hydrophilic network stabilized through covalent linkages [4,6].

Several natural polysaccharides have been used to obtain hydrogels, including starch, alginate, hyaluronic acid, cellulose and chitin. These biopolymers hold great potential due to the high number of hydrophilic functional groups (e.g. hydroxyl groups) in their molecules. Most hydrogels are usually prepared using a procedure that involves polymer dissolution in a suitable solvent system, followed by gelation [1,4]. This constitutes a problem for biopolymers such as cellulose and chitin, whose solubility is limited to toxic and/or corrosive organic solvents (e.g. dimethylacetamide/lithium chloride, tetrabutylammonium chloride/dimethyl sulfoxide). Ionic liquids [6], deep eutectic solvents [7] and alkali or alkali/urea aqueous solutions [4,8,9] have been proposed as alternative solvent systems for the dissolution of cellulose and chitin, and the production of hydrogels.

Chitin-glucan complex (CGC) is a water insoluble, highly hydrophilic biopolymer composed of *N*-acetylglucosamine and glucose monomers [10]. It is one of the cell-wall components of several yeasts and fungi that confer stability and rigidity to the cell [11,12]. CGC is a biocompatible and biodegradable biopolymer that combines antioxidant, antibacterial and anti-inflammatory properties that render it a valuable biomaterial for several applications, including the treatment of obesity and diabetes [13], cosmetic ingredient

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[14], as food additive (GRAS notice No. 412, 2012; <http://www.fda.gov>), wine clarification [15] and wound healing [16].

Similarly to cellulose and chitin, CGC is insoluble in water and aqueous solutions, and also in most organic solvents. Therefore, the development of CGC into commercial products has been hindered by its intractability. In this study, aqueous alkali systems based on NaOH or KOH were used for the first time for CGC dissolution by the freeze/thaw method, and the resulting solutions were subjected to dialysis to induce the biopolymer's gelation. The CGC hydrogels thus obtained were characterized in terms of morphology, rheology, mechanical properties and biocompatibility.

2. Materials and methods

2.1. Materials

Yeast biomass was obtained by cultivation of the yeast *Komagataella pastoris* (DSM 70877) using glycerol as the sole carbon source, as described by Farinha et al. [10]. CGC represented 20 wt% of the cell dry mass. It was extracted from *K. pastoris* biomass by the hot alkaline procedure described by Araújo et al. [17].

2.2. Preparation of CGC hydrogels

Two aqueous alkali solvent systems, namely, NaOH and KOH, were tested at different concentrations (1 mol/L and 5 mol/L). CGC powder (0.5 g) was dispersed in the alkali solvent systems (25 g) and the suspensions were kept at -20°C for 48 h. During this period, four freeze-thaw cycles were performed in which the thawed suspensions were extensively stirred (at 500 rpm, for 1 h), at room temperature. The insoluble fractions of the suspensions were separated from the soluble ones by centrifugation ($20,000 \times g$, 30 min, 4°C). CGC hydrogels were prepared by dialysis of the soluble fractions with a 12,000 MWCO membrane (Nadir®, dialysis tubing, Carl Roth) against deionized water for 48 h, at 20°C , until neutral pH and constant conductivity values ($20 \mu\text{S}/\text{cm}$) were achieved. The hydrogels prepared with NaOH or KOH, 1 mol/L and 5 mol/L, were labelled as Na1 and Na5, or K1 and K5, respectively.

2.3. Chemical characterization of CGC hydrogels

For quantification of sodium and potassium in the hydrogels, freeze dried CGC hydrogel samples ($\sim 5 \text{ mg}$) were hydrolyzed with nitric acid (5 mL HNO_3 5%, v/v), at 120°C , for 2 h, under constant stirring. Hydrolyzed samples were filtered ($0.2 \mu\text{m}$ nylon, WHATMAN) and their content in sodium and potassium was determined by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (Horiba Jobin-Yvon, France, Ultima, equipped with a 40.68 MHz RF generator, Czerny-Turner monochromator with 1.00 m (sequential) and autosampler AS500). Nitric acid solution (HNO_3 5%, v/v) was used as blank, being subjected to the same hydrolysis procedure as the samples.

The water content of the hydrogels was determined according to Eq. (1) [18]:

$$\text{Water content (\%)} = \frac{(\text{wet mass} - \text{dry mass})}{\text{wet mass}} \times 100 \quad (1)$$

where the dry mass (g) was determined by freeze drying a pre-weighed amount of the gels (wet mass, g).

The chemical composition of the hydrogels was analyzed by high performance liquid chromatography (HPLC) after two acid hydrolysis methods, as described by Araújo et al. [17].

2.4. Rheological properties

The rheological behavior of the CGC hydrogels was measured and analyzed using a controlled stress rheometer (HAAKE MARSIII, Thermo scientific) equipped with a plate-plate serrated geometry (diameter 20 mm) with a gap of 1 mm. The hydrogels samples were equilibrated at $25 \pm 0.03^{\circ}\text{C}$ for 5 min, after which the viscoelastic properties were evaluated by carrying out frequency sweeps at a constant tension within the linear viscoelastic region, for a frequency range from 0.01 to 1 Hz.

2.5. Texture profile analysis

The texture properties of the CGC hydrogels were determined with a texture analyzer TA-XT plus (Stable Micro Systems Ltd., Surrey, UK) equipped with a 50 N load cell. A double compression cycle was carried out up to 50% strain of the samples original height at a speed rate of 1 mm/s using an aluminum plunger with 60 mm diameter. A delay period of 5 s was allowed between the two compression cycles. The maximum tension of the first compression was defined as the hardness (Pa) and the ratio between the second and first compression distances until maximum forces was used to measure the springiness. Cohesiveness was defined as the ratio between the positive force area during the second cycle and that of the first compression cycle. All the measurements were performed in duplicate at room temperature ($20 \pm 0.2^{\circ}\text{C}$).

2.6. Cryo-Scanning Electron Microscopy

The CGC hydrogels were characterized in terms of their structure and morphology by Cryo-Scanning Electron Microscopy (cryo-SEM). The hydrogels were analyzed with a Tabletop Microscope TM3030 (Hitachi in High Technologies, America) equipped with a sample holder with refrigeration. Samples were observed at low temperature (-4°C) using a magnification in the range of 150–500 $\times$.

2.7. Cytotoxicity tests

The cytocompatibility of the CGC hydrogels was evaluated according to the International Standard ISO 10993-5:2009(E) through assessment of metabolic activity (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) tetrazolium reduction assay) in two cell lines, human keratinocytes HaCaT and mouse L929 fibroblasts. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) streptomycin/penicillin, and maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2 . For the cell viability assay, 5×10^4 cells per well were seeded in 96 well plates and cultured at conditions above described. After 24 h, the medium was removed and 100 μL of fresh medium containing serial dilutions, up to 1000 $\mu\text{g}/\text{mL}$, of freeze dried CGC hydrogels dissolved in alkaline solvent (NaOH 1 mol/L, at neutral pH) were added. Upon 24 h of exposure, the supernatant was replaced by the MTT solution (0.5 mg/mL) and incubated (37°C , 5% (v/v) CO_2) for 2 h. During incubation, tetrazolium dye MTT is reduced by dehydrogenase enzymes to insoluble formazan. Following the incubation step, the medium was eliminated and replaced with dimethylsulfoxide to dissolve the formazan crystals accumulated inside living cells. For the quantification of formazan produced, the absorbance was measured at 590 nm using a Synergy™ HT Multi-mode microplate reader (BioTek Instruments Inc., Winooski, VT, USA), subtracting the background read at 630 nm. Cells grown in fresh medium without treatment and cells treated with Triton™ X-100 (1% v/v) were used as positive and negative controls, respectively. Results were expressed as percentage of metabolic activity relatively to the values obtained at 2 h and considering the metabolic activity of non-treated cells as 100%.

Fig. 1. Schematic illustration of CGC hydrogels preparation, comprising polymer suspension in the alkali systems, application of freeze/thaw cycles for polymer dissolution and dialysis for polymer gelation.

3. Results and discussion

3.1. Hydrogels formation

Four alkali solvent systems, NaOH and KOH solutions, either at 1 mol/L or 5 mol/L, were used for CGC dissolution using a freeze-thaw procedure (Fig. 1), proposed for dissolution of chitin and cellulose [1,4,8,9]. At temperatures below the freezing point, the hydrated alkali component disrupts the polymer chain matrix by breaking the inter- and intramolecular hydrogen bonds. The hydrogels were obtained by dialyzing the CGC solutions, against deionized water until complete removal of the salt ions.

As shown in Fig. 2, the CGC hydrogels presented a yellow coloration and were translucent. The yellow color might be due to the presence of protein whose content in the gels was between 4.4 and 8.8 wt%. The gels' shape was molded by the dialysis tubing, thus showing that the CGC hydrogels obtained by this methodology can be molded into other shapes by inducing gelation in appropriate molds.

As shown in Table 1, no sodium was detected in Na1 and Na5 samples, and only traces of potassium (0.14–0.17 wt%) were detected in K1 and K5 hydrogels. In the alkali solutions, CGC molecules do not undergo gelation due to the strong ionic forces that promoted their solvation. During the dialysis procedure, the salt ions diffuse from the solutions into the dialysis water. The lack of ions available to promote

CGC molecules solvation results in the reduction of the ionic forces and allows more interaction among the CGC molecules, thus inducing gel formation.

The chitin:glucan molar ratios in all hydrogels ranged between 22:78 and 27:73 mol% (Table 1), which is similar to the composition of the original CGC sample (29:71 mol%) and also to that reported in previous studies (25:75mol%) [10]. Although a slight reduction of the chitin fraction in the co-polymer hydrogels was noticed in comparison with the original sample, these results show that CGC was effectively dissolved in the alkali solvents used. All hydrogels presented a water content above 90% which is characteristic of this type of structures. Nevertheless, hydrogels prepared by dissolution of CGC in NaOH solutions had a slightly higher polymer content (2.07–2.28 wt%) than the ones prepared with KOH (1.42–1.70 wt%) (Table 1).

3.2. Morphological characterization

The morphological characteristics of the CGC hydrogels were assessed by cryo-SEM analysis (Figs. 3 and 4). Fig. 3 shows the K-based hydrogels microstructure, which consisted of a complex three-dimensional network made of CGC polymer chains, holding water inside. Both K-based hydrogels, K1 and K5, exhibited homogenous microstructures (Fig. 3a and c). Upon magnification (Fig. 3b and d), some differences of the pore sizes of K1 and K5 hydrogels can be noticed

Fig. 2. Photographs of: (A) the CGC solutions obtained by the freeze-thaw procedure in the alkali solvent systems: NaOH 1 mol/L (Na1 hydrogel) and 5 mol/L (Na5 hydrogel) and in KOH 1 mol/L (K1 hydrogel) and 5 mol/L (K5 hydrogel); (B) the corresponding CGC hydrogels obtained by dialysis of the alkali solutions against deionized water.

Table 1
Chemical characterization of CGC hydrogels (n.d. — not detected).

Sample	Polymer content (wt%)	Water content (wt%)	Na content (wt%)	K content (wt%)	Chitin:glucan ratio (mol%)
CGC	—	—	n.d.	n.d.	29:71
Na1 gel	2.07	97.93	n.d.	—	27:73
Na5 gel	2.28	97.72	n.d.	—	25:75
K1 gel	1.42	98.58	—	0.14	24:76
K5 gel	1.70	98.30	—	0.17	22:78

that suggest the influence of KOH concentration on the gels' network formation. Hydrogels prepared with KOH 1 mol/L presented a less compact network with larger pores (Fig. 3b) when compared to the hydrogels prepared with KOH 5 mol/L (Fig. 3d). A similar microstructure was reported by Nogueira et al. [18] for silk fibroin hydrogels prepared by dissolution in a ternary solvent of $\text{CaCl}_2:\text{CH}_3\text{CH}_2\text{OH}:\text{H}_2\text{O}$ followed by dialysis for calcium removal and hydrogel formation [19].

The microstructure of the Na-based hydrogels exhibited a more compact and denser network than the ones prepared with KOH (Fig. 4a and c). In addition, they presented heterogeneous and irregular structures. This fact may be explained by the difference in ionic radius of sodium and potassium atoms. During the hydrogelation process, the sodium atoms may have induced the formation of smaller pores within the hydrogel structure, while the bigger potassium atoms may have allowed the formation of larger pores in the polymeric structure. The NaOH concentration used for CGC dissolution and hydrogel preparation seems to have influenced the microstructure of the formed hydrogels. Na5 CGC hydrogels presented more regions of dense networks (Fig. 4c) than the Na1 CGC hydrogels (Fig. 4a). Upon magnification,

small pores with thick pore walls are detected that explain the tighter microstructure of the hydrogels (Fig. 4b and d). The wide area of dense regions of Na5 CGC hydrogels has limited a clear observation of the pore size (Fig. 4d). The porous structure CGC hydrogels present render them potentially suitable for use in applications such as drug delivery vectors and/or as matrices for cells in tissue engineering.

3.3. Rheological properties

The mechanical properties of hydrogels are determinant to envisage the functional purposes of these biomaterials. In order to assess those parameters, the viscoelastic properties of CGC hydrogels were studied. As shown in Fig. 5, for the two types of CGC hydrogels prepared with either NaOH or KOH as solvent systems, it can be noticed that the storage moduli (G') exhibited values one order of magnitude higher than the loss moduli (G'') in the whole range of frequencies. This fact is consistent with the gel-like nature of the samples. Similar profiles were obtained for hydrogels developed using hyaluronic acid (HA) cross-linked with divinyl sulfone [5]. Despite the identical behavior, CGC

Fig. 3. Cryo-SEM images of the K1 hydrogel prepared with KOH 1 mol/L (a, b) and of the K5 hydrogel prepared with 5 mol/L (c, d). Hydrogels' regions observed under magnification 500× (b, d) were expanded from images presented under magnification 150× (a, c).

Fig. 4. Cryo-SEM images of the NaI hydrogels prepared with NaOH 1 mol/L (a, b), and of the Na5 hydrogels prepared 5 mol/L (c, d). Hydrogels' regions observed under magnification 500 $\times$ (b, d) were expanded from images presented under magnification 150 $\times$ (a, c).

hydrogels revealed to have improved mechanical properties compared to HA hydrogels. In particular, for the same frequency (0.1 Hz), Na-based CGC hydrogels presented storage moduli values of 389 Pa and 685 Pa (Na5 and Na1, respectively), while for the HA hydrogels G' lower value was reported (304.30 Pa). On the other hand, lower storage moduli (218 and 270 Pa) were obtained with K1 and K5 CGC hydrogels,

respectively. Lower values of G' indicate that K-based CGC hydrogels exhibited lower elastic properties when stress is applied.

As can be observed, CGC hydrogels prepared using NaOH (Fig. 5a) exhibited higher dynamic moduli than CGC hydrogels prepared with KOH (Fig. 5b), which is related to stronger mechanical properties. This result is in line with the morphology analysis, in which Na-based CGC

Fig. 5. Rheological properties of CGC hydrogels using (a) NaOH (Na1 and Na5 hydrogels) and (b) KOH (K1 and K5 hydrogels). Mechanical spectrum storage (G' , solid symbols) and loss moduli (G'' , open symbols).

Fig. 6. Texture profile analysis parameters for hydrogels prepared with NaOH 1 mol/L (Na1) and 5 mol/L (Na5), and with KOH 1 mol/L (K1) and 5 mol/L (K5): (A) hardness, (B) springiness and (C) cohesiveness.

hydrogels exhibited a denser structure with smaller pores with thicker pore walls (Figs. 3 and 4). As mentioned above, the characteristics of CGC hydrogels microstructure may be affected by the difference of ionic radius between sodium and potassium atoms.

Additionally, the mechanical spectra of CGC hydrogels shows that as the concentration of alkali solvents used increased, both dynamic moduli increased indicating a stronger structure (Fig. 5). In fact, higher values of both moduli, G' and G'' , were observed for the hydrogels prepared with 5 mol/L when compared to those for 1 mol/L, this fact being more evident for Na-based CGC hydrogels. The observed changes may be related with the higher ionic strength present during formation of the Na5 and K5 hydrogel structures. In fact, it was reported that the ionic strength has a significant impact on hydrogelation and modulation of hydrogels' properties [20]. For example, Martínez-Ruvalcaba and co-workers reported that for the same polymer content in chitosan-xanthan hydrogels (7%), the storage moduli increased around 6 times with the ionic strength of the different dispersion media used, namely distilled water and 0.05 mol/L potassium phosphate monobasic-sodium hydroxide buffer [21].

The differing properties displayed by the gels obtained under different ionic strength and cations render them potentially suitable for

distinct applications: the stiffer structures of the Na-based gels may be used, for example, for the development of tissue scaffolds, while the more elastic K-based gels would be more appropriate for example as wound dressings.

3.4. Texture profile analysis

Texture profile analysis (TPA) parameters were obtained by applying a 50% strain of the hydrogels' original height in a double compression cycle. The results obtained, including hardness, springiness and cohesiveness, are represented in Fig. 6. It may be observed that the hardness, which is related to the maximum force required to cause the selected deformation of the gel [22], was affected by the type of alkali and by the ionic strength of the solvent used to prepare the hydrogels (Fig. 6A). Thus, a considerably higher hardness was obtained for the Na5 hydrogels (7229 ± 781 Pa) when compared to Na1 hydrogels (1647 ± 411 Pa), showing that a higher ionic strength favored the formation of a stronger gel in the presence of NaOH. On the other hand, for the K-based CGC hydrogels, the hardness values were similar for both ionic strength values tested (3169 ± 768 and $3423 \pm$

Fig. 7. L929 fibroblasts and HaCaT keratinocytes viability when exposed to different concentrations of freeze dried CGC hydrogels dissolved in NaOH 1 mol/L, for 24 h. The dotted line at 70% represents the viability limits for non-toxic condition (ISO 10993-5, 2009).

450 Pa, respectively). Moreover, the hardness of both K-based hydrogels was lower than that of the Na5 hydrogel (Fig. 6A).

Springiness reflects how well the gel sample physically springs back after it has been deformed during the first compression and has been allowed to wait for a period between compressions [22]. It can be observed that similar values of springiness were obtained for all CGC hydrogels (Fig. 6B), which is indicative of an elastic behavior, as the fraction of the sample's original height is quite high, in a range of 0.85 to 0.93. Cohesiveness is related to how well the product withstands compression, so that, if a hard product maintains its internal structure without failures when subjected to compression, it would also present a high cohesiveness [22]. This fact was only substantially observed for Na5 hydrogels, presenting a value of 0.716 ± 0.043 (Fig. 6C), while the other hydrogels, where much less cohesive. The results suggest that the energy required for the second compression was significantly lower than that for the first compression, indicating that the gel matrix of the Na1 and K-hydrogels was disrupted during the first compression. Low strength of polymer chain molecular interactions may have led to this hydrogel behavior [2].

Concomitantly with the mechanical spectra (Fig. 5), the TPA results demonstrate that the ionic strength of the solvent used has more impact on Na-based hydrogels than on K-based hydrogels (Fig. 6). Additionally, the Na5 hydrogels presented improved mechanical properties since significantly higher hardness and cohesiveness values were observed.

Several authors reported the improvement of the texture properties of hydrogels by increasing the concentration of the polymer [23,24]. The same strategy could be used to improve mechanical properties of CGC hydrogels.

3.5. Biocompatibility of the CGC hydrogels

The evaluation of CGC hydrogels' biocompatibility was performed using L929 fibroblasts (International Standard ISO 10993-5:2009(E)). Fig. 7 shows the results of the cytotoxicity tests on the CGC hydrogels. The cell viability values on all tested hydrogels are greater than 90%, indicating nearly no cytotoxicity to the fibroblasts (Fig. 7A). The non-cytotoxicity of the solvent used to prepare the CGC samples (NaOH 1 mol/L) was confirmed by exposing the cells solely to this solvent (Fig. SM-1). Chang and collaborators [4] have reported similar biocompatibility results for chitin-based hydrogels towards 293T cells, most probably related to the biological nature of these macromolecules. Similarly, Abdel-Mohsen et al. [16] have demonstrated that chitin/chitosan-glucan complex (ChCsGC) revealed to be non-toxic for mouse fibroblasts cells (NIH-3T3) over the all concentrations tested (100–1000 µg/mL).

To further evaluate the biocompatibility of the CGC hydrogels for skin applications, HaCaT keratinocytes cells were used to assess the hydrogels for 24 h. Fig. 7B shows the viability of keratinocytes. Compared with the control group, the HaCaT cells exposed to the highest CGC hydrogels concentration (1000 µg/mL) exhibited reduced cellular viability to values around 70%. These results confirm that the CGC hydrogels could be used as an excellent matrix having biocompatibility, with potential applications in the field of skin wound and skin therapies. Biocompatibility of chitin-based hydrogels obtained by various methods has been reported in the literature through in vitro cytotoxicity tests [25–27].

4. Conclusions

This study reported for the first time the preparation of hydrogels based on the yeast biopolymer CGC. It was demonstrated that CGC gels form spontaneously during dialysis of CGC alkali solutions, obtained by the freeze-thaw procedure. The mechanical properties of the CGC hydrogels can be tailored according to the alkali solvent used for CGC dissolution, either NaOH or KOH, and also by changing the solutions ionic strength. Moreover, the best performing

hydrogels in terms of mechanical properties, namely, higher hardness and cohesiveness, were the ones obtained with NaOH 5 mol/L. Due to their biocompatibility, CGC hydrogels are promising materials for use in several biomedical areas. Their characteristics, namely, their porous structure and mechanical properties, supports their potential use as drug delivery vectors and/or as matrices for cells in tissue engineering.

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Author contributions

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