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Physical properties and antibacterial activity of chitosan/acemannan mixed systems

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ABSTRACT

The aim of the present study was to investigate the mechanical and thermal properties of mixed chitosan–acemannan (CS–AC) mixed gels and the antibacterial activity of dilute mixed solutions of both polysaccharides. Physical hydrogels of chitosan comprising varying amounts of non-gelling acemannan were prepared by controlled neutralization of chitosan using ammonia. As the overall acemannan concentration in the mixed hydrogel increased while fixing that of CS, the mechanical strength decreased. These results indicate that AC perturbs the formation of elastic junctions and overall connectivity as it occurs in the isolated CS network. Heterotypic associations between CS and AC leading to the formation of more compact microdomains may be at play in reducing the density of the gel network consolidated by CS, possibly due to shorter gel junctions. Micro-DSC studies at pH 12.0 seem consistent with the suggestion that molecular heterotypic associations between CS and AC may be at play in determining the overall physical properties of the mixed gel systems. In dilute solution, CS showed antimicrobial activity against *Staphylococcus aureus* but not against *Escherichia coli*; AC did not exert antimicrobial activity against any of the two bacterial species. In blended solutions of both polysaccharides, as the amount of AC increased, the antimicrobial activity of the system against *S. aureus* ceased. In conclusion, this study demonstrates that it is feasible to incorporate acemannan in chitosan–acemannan gels and that although the mechanical strength decreases due to the presence of AC, the gel network persists even at high amount of AC. This study anticipates that the CS–AC mixed gels may offer promise for the future development of biomaterials such as scaffolds to be used in wound therapy.

1. Introduction

The development of new biomaterials and improvement of existing ones to promote skin regeneration have been major areas of research in the field of polymers (Seal, Otero, & Panitch, 2001). Skin trauma caused by heat, chemicals, electricity, ultraviolet or radioactivity can result in several degrees of skin damage. In the more severe cases, these can lead to partial or complete damage of both dermal and subdermal tissues. Unfortunately, the body cannot heal itself dermal injuries properly. Recent advances in wound care, such as skin grafting have increased the survival rate of burned patients. Among the main challenges in the development of new materials and improvement of existing ones for wound healing and skin care, are to develop new biomaterial matrixes and approaches that serve to support, reinforce and in some cases organize the regenerating tissue. Approaches for full-thickness skin replacement often try to mimic the porous structure and mechanical characteristics of the extracellular matrix (ECM). Materials of this type have been developed to induce the three-dimensional regeneration of skin tissue by culturing allogeneic human keratinocytes and fibroblasts (Falanga et al., 1998).

Performance of the varied required biomaterial functions usually demands a porous scaffold microstructure that often is generated by freeze-drying of a solution or a hydrogel of a

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biopolymer (e.g. type I bovine collagen). Emerging biomaterials for skin care applications may in future benefit from responsive and functional hydrogels based on natural polymers able to convene different specific physicochemical and bioactive properties. In connection with this, in this work, we have addressed the development of hybrid hydrogels comprising chitosan (CS) and acemannan (AC) polysaccharides. Both are biodegradable, non-toxic biopolymers. Over the past decade or so, they have attracted increasing attention in the biomaterials, pharmaceutical and cosmetic fields and both are components of approved commercial products, particularly wound dressings and skin-care products (Kaya, Yapici, Savas, & Güngörmüş, 2011; Muzzarelli, Conti, Ferrara, & Biagini, 1988; Reynolds & Dweck, 1999; Tao, Xiao-Kan, Xu-Ting, & Da-Yang, 2012).

CS is an aminopolysaccharide that occurs in fungi and is obtained at commercial scale from the chemical N-deacetylation of chitin, the ubiquitous polysaccharide that occurs in the exoskeleton of crustacean, insects and the cell wall of fungi. CS defines a family of linear heteropolysaccharides that are comprised by (1 → 4)-linked 2-amino-2-deoxy-β-D-glucose (D-units) and 2-acetamido-2-deoxy-β-D-glucose (A units). Units of type A are usually present in lower proportions than D ones, and their content, given by the molar ratio of A groups to the total (A + D), is regarded as the degree of acetylation, which is expressed either as a percentage (DA%) or as a fraction (F_A). AC has been identified as the main polysaccharide in *Aloe* spp. gel. Chemically is described as the backbone of β-(1 → 4)-D-mannosyl residues acetylated at the C-2 and C-3 positions that exhibit a mannose monomer: acetyl ratio of approximately 1:1 and contain some side chains mainly of galactose attached to C-6 (Domard, 1996; Fan, Hu, & Shen, 2009; Femenia, Simal, & Rossello, 1999).

The primary structure of these polysaccharides shares in common a central chain of different sugars linked by β(1 → 4)-glycosidic linkages and the presence of either O-acetyl (in AC), or N-acetylated amino (in CS) substituent groups. CS is produced at commercial scale by chemical N-deacetylation of chitin (Montembault, Viton, & Domard, 2005). In acidic media chitosan is a polycation (pK_o, ~6.0, Rinaudo, Milas, & Le Dung, 1993). AC is neutral in solution. AC and CS are known to exhibit a number of distinctive biological activities relevant in the development of innovative biomaterials. CS is biodegradable by enzymes that occur in various tissues of mammalians such as lysozyme and chitinase enzymes (Kean & Thanou, 2010), and it is also known to be mucoadhesive (Sogias, Williams, & Khutorianskiy, 2008). Besides, CS activates the immune system by stimulation of macrophage function (Djeraba, 2000; Peluso et al., 1994) and promotes wound healing (Chatelet, Damour, & Domard, 2001). Once placed on the wound, films of CS with a high DA, have been found to adhere to and accelerate the growth of fibroblast and to favor the proliferation of keratinocytes and thereby epidermal regeneration. It has also been found to accelerate the growth of fibroblast and collagen (Howling et al., 2001). In addition, CS has been found to accelerate blood coagulation, mediated not only by platelet (Okamoto et al., 2003). Also, CS is well known to inhibit the growth of a wide range of bacteria and fungi (Liu, Guan, Yang, Li, & Yao, 2001; Rabea et al., 2003; Strand, Vandik, Varum, & Ostgaard, 2001). Although the precise mechanisms of antimicrobial action of chitosan are yet to be fully elucidated, there is a consensus that the interaction of positively charged chitosan in solution with the negatively charged cell membranes, leading to the leakage of proteinaceous and other intracellular constituents, is a determinant. The range of bioactive properties convened by CS along with its amenability to being transformed into a wide range of different materials such as hydrogels, films, scaffolds, fibers, micro- and nanoparticles, makes it a unique building block for the design innovative biomaterials. In turn, a number of biological activities *in vitro* and *in vivo* have also been attributed to AC. Among these, modulation of the immune function, particularly the activation of macrophages and the production of cytokines, nitric oxide and increase the expression of some adhesion molecules and receptors have been reported (Pugh, Ross, ElSohly, & Pasco, 2001; Zhang & Tizard, 1996). Other documented bioactivities claimed for AC include enhancement of antibody production ('t Hart, Van den Berg, Kuis, Van Dijk, & Labadie, 1989); induction of anti-inflammatory activity and of the proliferation of fibroblasts (Jensen, Seeley, & Gillin, 1998; Kahlon et al., 1991; Peng et al., 1991). These bioactive effects have been suggested to have important implications in the physiologic mechanisms of host defense against invading bacteria, virus, and in the wound healing effects of aloe gel. *Aloe vera* gel has been used in the fabrication of biomaterials for skin-care (e.g. mucositis, dermatitis) and wound healing dressings for oral care (Poor, Hall, & Poor, 2002).

Chitosan physical hydrogels obtained from alkali chitin have been shown to be pH sensitive and to exhibit a lower critical solution temperature (LCST) (Goycoolea et al., 2007; Ueno, Mori, & Fujinaga, 2001). Below the LCST, CS hydrogels are swollen, hydrated and hydrophilic, and above it, the gels are, dehydrated and hydrophobic. The gelation of CS by different mechanisms has been studied by a number of different biophysical techniques, including oscillatory dynamic rheology (Argüelles Monal, Goycoolea, Peniche-Covas, & Higuera-Ciapara, 1998), dynamic light scattering (de Morais, Pereira, & Fonseca, 2012), among other methods.

This study aimed to investigate the behavior of a mixed gel of CS added with AC as a first step toward the future design of novel biomaterials that could benefit from the biological activities of both polysaccharides that may lead to achieve a synergic effect, particularly for wound healing and skin care applications. We show that incorporating varying amounts of AC in a gel matrix of CS weakens the mechanical properties of a CS gel network and have also studied the thermal properties as well as on the antibacterial activity of obtained gels against two model strains of Gram(+) and Gram(−) bacteria, *Staphylococcus aureus* and *Escherichia coli*, respectively.

2. Experimental

2.1. Materials

All the reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA), except ammonium hydroxide that was from Acros Organics (Geel, Belgium). Deionized water was used throughout.

2.2. Purification of polysaccharides

Chitosan sample was originally obtained by heterogeneous deacetylation of chitin isolated from blue shrimp head waste (*Lithopenaeus stylyrostris*) in a pilot plant facility of the Laboratory of Biopolymers of Centro de Investigación en Alimentación y Desarrollo, A.C, Hermosillo Sonora. For purification, CS was fully dissolved at 0.5% (w/v) in a 5% excess of stoichiometric amount of aqueous acetic acid. After complete dissolution it was filtered successively through membranes (Millipore) of 3, 0.8, 0.45 and 0.22 μm pore size. Then, the solution was precipitated with dilute ammonia up to a constant pH of 9 and centrifuged. The precipitate was rinsed with water until a neutral pH was achieved then it was centrifuged and freeze-dried.

Acemannan, the water soluble polysaccharide fraction from the inner gel of *Aloe vera* leave, was extracted from freshly cut aloe leaves sourced from commercial plants purchased from a local retailer. Briefly, the leaves were cleaned, allowed to drain off the yellow sap from the rind, washed extensively with water and diced into ~2–3 cm pieces. They were ground and centrifuged so as to separate the inner transparent gel from the insoluble plant matter.

The clear supernatant was by mixed with 3 volumes of 95% (v/v) ethanol. The resulting precipitate was collected by centrifugation and re-dissolved in water until complete dissolution. It was filtered successively through membranes (Millipore) of 3, 0.8, 0.45 and 0.2 μm pore size, dialyzed against four water changes per day during 48 h and freeze-dried.

2.3. Characterization of chitosan and acemannan

The degree of acetylation (DA) of CS was determined by ^{1}H-NMR spectroscopy spectra recorded on a Bruker 250 spectrometer (250 MHz) at 25 °C. Ten milligrams of chitosan were solubilized in 1 g of D_2O containing 0.21% by weight of HCl. The DA was then evaluated as proposed by Hirai, Odani, and Nakajima (1991). The molecular weight distribution parameters were determined by gel permeation chromatography (GPC) coupled online with differential refractometer (DRI, Waters 410) and a multi-angle laser light scattering (MALLS) detector operating at λ = 632.8 nm (Wyatt Dawn DSP). GPC–HPLC analysis was performed in a multidetection instrumental system comprising: An IsoChrom LC pump (Spectra Physics) connected to Protein Pack glass 200 SW and TSK gel 6000 PW columns. A 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH = 4.5) was used as eluent. The flow rate was adjusted to 0.5 mL/min. The polymer solutions were prepared by dissolving 1 mg of polymer in 1 mL of buffer solution, then filtered on a 0.45 μm pore size membrane (Millipore) before injection of 100 μl. The parameter *dn/dc* of CS was determined with a differential interferometer operated at λ = 632.8 nm (NFT Scan Ref). The water content of freeze-dried chitosan samples was evaluated on a DuPont Instrument 2950 thermogravimetric analyzer (TGA) operated at a ramp of temperature of 2 °C/min under a flow of helium and accounted for in all calculations.

Contents of mannose, glucose and galactose was analyzed by anionic interchange of chromatography of high resolution (HPAEC-PAD) according with a modified method described for cell wall of grapefruit. The analysis were performed by use of a BioLC system, equipped with a CarboPac PA20 (3 mm × 150 mm) analytical column and an ED50 electrochemical detector and controlled via Chromeleon 6.60 Sp2 Build 1472 software (Dionex Corp., Sunnyvale, CA). Monosaccharides were expressed as percentage. The water content of freeze-dried acemannan sample, weight-average molecular weight (M_w) and the parameter *dn/dc* were evaluated as described above for chitosan. But, the solvent was sodium nitrate 0.1 M and an Aquagel-OH column (8 μm i.d., PL) was utilized.

2.4. Preparation of gels

The general protocol of CS gels preparation was that previously developed (Montembault et al., 2005). This protocol was slightly modified to allow the inclusion of AC. Briefly, CS solution (4% w/w) was made by dispersing in water and hydrochloric acid was added so as to achieve the stoichiometric protonation of the $-NH_2$ sites. After complete dissolution of CS, AC solution was added so as to achieve AC final concentrations varying from 0.5 to 7.0% (w/w). The same total weight of 1,2-propanediol was further added. The blend was stirred magnetically (~100 rpm) for 30 min. Gels were obtained by leaving the solutions quiescently to evaporate at 50 °C until gelation occurred, to evaporate in Petri dishes in a stove at 50 °C during 24 h. Once formed, the gels were neutralized by adding 30 mL of 28–30% (w/w) ammonium hydroxide directly to the Petri dish to fully regenerate the neutral amine form of CS and to avoid any re-dissolution. They were then washed exhaustively with water to eliminate both 1,2-propanediol and the excess of ammonium hydroxide, as monitored by the pH of the washing water. The gels were stored in water at 4 °C until used.

Table 1
Physicochemical characteristics of chitosan and acemannan samples.

Sample	Degree of acetylation (DA %)[a]	dn/dc[b]	M_w[c]	M_n[c]	I_p[c]
Chitosan	23.7	0.18	280,000	168,471	1.66
Acemannan	18	0.16	408,300	179,314	2.28

[a] Degree of acetylation (DA) as determined by ^{1}H NMR spectroscopy.
[b] Differential refractive index determined by interferometry.
[c] Molecular weight distribution parameters determined by GPC–HPLC with multidetection (MALLS-DRI): weight-average molecular weight (M_w); number-average molecular weight (M_n) and polydispersity index ($I_p = M_w/M_n$).

2.5. Rheological determinations

The rheological properties of the gels were investigated by low-amplitude oscillatory tests using a strain-controlled rheometer fitted with a parallel plate tool (25 mm diameter) (Rheometrics Mod. A34980, Piscattaway, NJ, USA). The gap was registered precisely from a micrometer and the normal force transducer was used to measure the first contact between the hydrogel surface and the upper plate and it varied in the range 2.0 ± 0.2 mm. The strain was fixed in such a way to avoid nonlinear viscoelastic deformations (0.2–1.0%) as verified by strain sweeps on all the gels tested. Drying of the samples was prevented by a glass ring of ϕ = 60 mm placed around the measuring geometry, and the annulus was filled with the appropriate buffer solution. Temperature control was exerted by a water circulating bath.

2.6. Microcalorimetric determinations

The thermal transitions of gels of CS 4% and CS–AS (4:1.5%) at pH 7.0 and 12.0 were investigated by micro-DSC in a Setaram equipment (μDSC-IIIa, Caliure, France). To this end, a piece of each gel was loaded into the calorimetric pan and the buffer solution was added. An accurately weighed aliquot of the same buffer was loaded in the reference pan.

2.7. Antimicrobial activity of gels

The antibacterial activity of CS, AC and CS–AC blended systems in solution against *S.aureus* and *E. coli* was studied by the method developed by Jorgensen, Turnidge, and Washington (1999), as modified by Velazquez et al. (2007). To this end, solutions of AC (7.021% in water), QS (0.112% in dilute HCl), and mixtures of AC (2.77%)–QS (0.0839%) and AC (1.75%)–QS (1.28%) were assayed. The mixtures were diluted with Müeller Hilton broth, adjusting the total polymer concentrations to 200, 100 and 50 μg/mL. Hydrocloric acid (90 mM), Müeller Hilton culture medium and gentamicin mixed with 15 μL of bacterial inoculum (*S. aureus* or *E. coli*) were the control groups. The microplates were incubated to 35 °C by 48 h. The optical density of each well was read at λ = 630 nm in an automatic microtiter plate reader (Benchmark, Bio-Rad, Hercules, CA, USA) at different incubations times (0, 6, 12, 24 and 48 h). All treatments were assayed in triplicate. The whole biological assays were made in duplicate to ensure the reproducibility of the results.

3. Results and discussion

3.1. Characteristics of the polysaccharides

The general characteristics of the purified CS and AC polymer samples are given in Table 1, namely, the degree of acetylation (DA%), differential refractive index (*dn/dc*), weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (I_p) of both biomaterials. In turn, Table 2

Table 2
Sugar composition of acemannan sample as determined by HPLC-PAD.

	Sugar residue		
	Mannose	Glucose	Galactose
Contents (%)	83.2	14.1	2.6

shows the sugar composition of AC determined by HPAEC-PAD. The contents of mannose, glucose, and galactose determined in AC were in good agreement with previous studies (Gowda, Neelisiddaiah, & Anjaneyalu, 1979; Ni, Turner, Yates, & Tizard, 2004; Wozniewski & Gerhard, 1990).

3.2. Gelation experiments

In the formation of CS polymer gel networks, the balance between hydrophilic and hydrophobic interactions is well known to be at play on the process of gel formation and in the final mechanical and thermal properties of the hydrogel materials (Goycoolea et al., 2007). CS is a weak polybase that dissolves in dilute aqueous acidic solutions through the protonation of its free amino groups and the formation of the corresponding salt. The role of the added 1,2 propanediol during the gelation process was to reduce the dielectric constant of the medium. As a result, since electrostatic force is inversely proportional to dielectric constant, the intensity of attraction of dipoles (induced or not) will increase and likely are at play in setting-up the gel network. Hydrophobic interactions between acetyl groups-rich sites (enveloped by water molecules) present on both CS and AC would result in the release of water molecules to the solvent, with the consequent overall increase in entropy, thus yielding a virtual crosslinking point. On the other hand, the two hydroxyl groups in 1,2 propanediol may interact via hydrogen bonding with acetyl groups present. Regardless of the precise mechanism governing the association junctions in the gel network, these are bound to be greatly reinforced by the evaporation of water, alcohol and hydrochloride acid (Montembault et al., 2005). In addition, a key parameter in the gelation process is to make sure that the initial polymer concentration is well above the critical concentration for chain entanglement. As the solvents are almost fully removed during the evaporation step, this condition is fully met. At the last step, the hydrogel is neutralized and washed thoroughly with water to eliminate hydrochloride acid and 1,2-propanediol. Thus, the final gel was comprised only by the polysaccharides and water (95% w/w) (Boucard, Viton, & Domard, 2005).

3.3. Rheological measurements

The effect of the addition of the different concentrations of AC (0.5–7.0% w/w) to CS on the viscoelastic properties of the obtained hydrogels was evaluated by small amplitude oscillatory rheological determinations. Fig. 1 shows the dependence of the storage, G', and loss, G'', shear moduli and of the complex viscosity, η^*, with frequency, ω, of control CS gel (no added AC) as well as for a mixed CS–AC (4.0 and 1.5% w/w, respectively) gel at pH 7.7 and 40 °C. These mechanical spectra represent the rest of the series of mixed gels. The mechanical spectra of both hydrogels, the one of CS alone one and that of the mixed gel (CS–AC), show all the hallmarks of the typical behavior characteristic of a permanent gel network with $G' > G''$, almost negligible dependence of both moduli and a linear negative dependence of η^* with ω. A closer inspection of both spectra also reveals that the addition of AC in the mixed hydrogel resulted in a marked weakening of the strength of the gel network, where the G' values decreased by almost half as much with respect to the CS control gel.

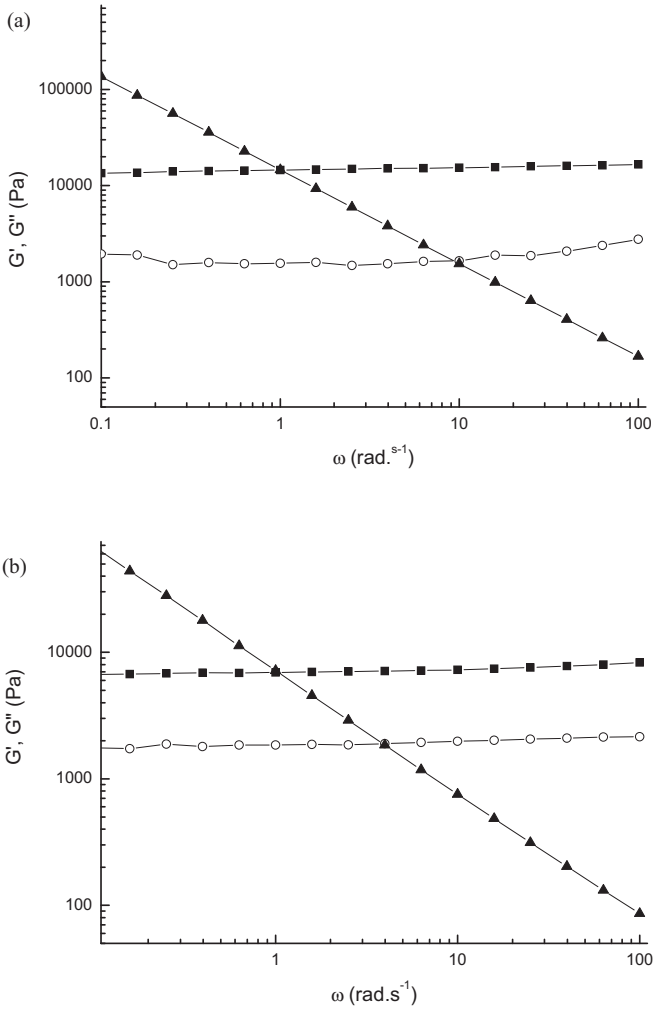

Fig. 1. Frequency (ω) dependence of the storage, G' (■), and loss, G''(○), moduli and of the complex viscosity, η^* (▲), of: (a) chitosan (4% w/w) and (b) chitosan (4% w/w)–acemanann (1.5% w/w) mixed gels (strain, $\gamma = 0.2\%$; 40 °C and pH 7.7).

Fig. 2 shows the variation of G' values, of the CS–AC gels as a function of the composition. Notice that the inclusion of AC in the mixed systems, even at the lowest concentration (1.0% w/w) to a fixed concentration of CS (4.0% w/w), results in a monotonic decrease

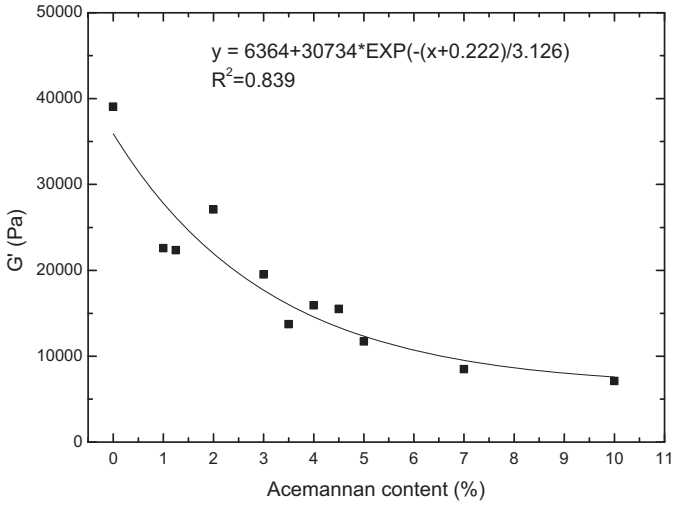

Fig. 2. Variation in storage modulus G' (■) with acemannan contents (23 °C pH 7.0; $\omega = 10$ rad s^{-1}, strain 0.2% CS c. 4% w/w).

of G' values with acemannan concentration. The data were fit to a non-linear exponential decay function thus affording a high correlation coefficient ($R^2 = 0.839$). This experiment, thus, confirms that the presence of AC invariably leads to a weakening of the mechanical strength of the mixed CS–AC gels. This same overall response has previously been observed in mixed CS films 2% added with *Aloe vera* extracts at 30, 40 and 50% (Khoshgozaran-Abras, Hossein, Hamidy, & Bagheripoor-Fallah, 2012) and in gelatin–aloe films (Chen, Wang, & Weng, 2010). Considering the fact that the concentration of CS in the full series of mixed CS–AC co-gels was kept fixed at 4.0% (w/w) the results are consistent with the notion that the presence of AC somehow interferes with the overall density of crosslinks of junctions that govern the connectivity of the CS gel network (Treolar, 1975). Soluble purified AC did not show evidence of gelling by itself (Fig. S1 in Supporting information), hence, it would be expected that if only CS is responsible for the formation of the gel network in the CS–AC co-gels, then a semi-interpenetrated network (semi-IPN) could be formed, in which disordered AC occurs as filler in the sol fraction of the CS network. In such case, the overall mechanical rigidity of the network would only depend on the net concentration of CS. It is therefore unlikely that a semi-interpenetrated (semi-IPN) network model could account for the observed decrease in G' with the increasing amount of AC in the system. In true semi-IPN comprising identical amounts of CS and varying concentrations of AC, constant G' values would have been anticipated regardless of the concentration of AC (Ferry, 1980).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.07.064.

The rheological evidence suggests that AC perturbs the formation of the same density of elastic junctions as in the CS alone network, and hence the CS–AC gels exhibit a lower storage modulus than CS gels. Whether CS and AC interact by heterotypic association or else, cannot be established conclusively at this stage. In synergistic blended polysaccharide systems comprising a gelling polymer (e.g. carrageenan or agarose) and a non-gelling one (e.g. glucomannan or galactomannan), it has been observed an increase in the strength of the gels as a direct consequence of the formation of heterotypic interactions that extend the agarose or carrageenan gel networks (Goycoolea, Richardson, Morris, & Gidley, 1995). It seems that in the case of CS–AC systems the consequence of the interactions are to shorten the density and possibly the length of the junctions that harness the gel network.

3.3.1. Micro-DSC analysis

The single and mixed gels were characterized by differential scanning microcalorimetry. Only at the extreme condition of pH 12 it was possible to identify a small step in the heat flow of the heating DSC thermogram (Fig. 3) that can be diagnostic of phase transition that it is only evident at high pH and not at the other assessed pHs (results not shown). The onset of this transition for CS alone (CS 4%) and mixed CS–AC (CS 4% and AC 1.5%) gels was centered at 20.36 and 21.48 °C, respectively. The heat capacities values of the temperature transitions for the gels of CS and CS–AC, were very close to each other, −0.05 and −0.041 J/g K, respectively. This result suggests that the overall segmental mobility of the chitosan gel network is not influenced by the presence of AC in the system. A phase transition at ~20 °C has been evidenced in previous studies carried out by our research group centered in chitin hydrogels obtained by phase separation using oscillatory rhelology and micro-DSC (Goycoolea et al., 2007). The existence of two co-existing network phases, namely a rigid network whose connectivity determines the mechanical strength (i.e. the storage modulus) and a second viscous phase that experiences a collapsing transition has been proposed in these systems. By contrast with the chitosan hydrogels harnessed from phase separation of alkali chitin and their subsequent

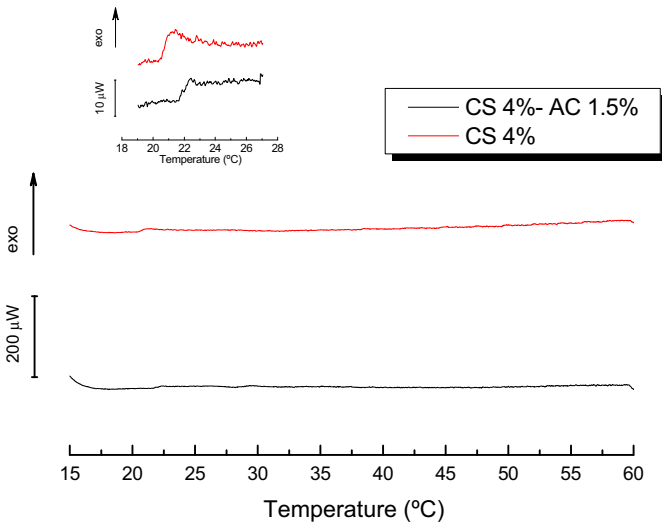

Fig. 3. Micro-DSC traces during heating (1 °C/min) of CS and CS–AC hydrogels (as shown in label) equilibrated at pH 12. Inset shows the same traces in the amplified region from 18 to 28 °C.

neutralization, the CS–AC hydrogels of the present study exhibited a phase transition only at pH 12.0 and not at lower pHs, as the former. This result may suggest that only when $-NH_2$ groups are fully neutralized and that the hydroxyl groups in the polysaccharides are ionized, it is possible to observe a coil to globule transition involving presumably fractions of CS and AC that remain in the sol state, in good keeping with what has been as suggested for chitin hydrogels (Goycoolea et al., 2007). The use of other techniques to probe critical gel formation, for example dynamic light scattering (DLS) may be highly informative in future studies addressing these type of systems.

3.4. Antimicrobial activity of CS–AC

New approaches and materials to fight pathogenic bacteria are urgently needed as recently acknowledged by the World Health Organization (Conrad, Miller, Cielenski, & De Pablo, 2000; WHO report: Pérez, 1998). In this respect, natural compounds have attracted much attention as bacteriostatic or bactericide agents. CS is known to exhibit antimicrobial activity. Also CS chemical derivatives have been used in the control of Gram(−) and Gram(+) bacteria (Jumaa, Furkert, & Müller, 2002; Li, Kennedy, Peng, Yie, & Xie, 2006; Oh, Kim, Chang, & Kim, 2001; Rabea et al., 2003). However, in the field of new biomaterials based on CS there are only very few studies and mostly focused on chemical derivatives. For instance, a study by Loke, Lau, Yong, Khor, and Sum (2000), addressed the antibacterial activity of films of chitin, carboxymethyl–chitin and acetate–chitosan coated with clorhexidin–gluconate as antiseptic. These films were tested against *Pseudomonas aeruginosa* and *S. aureus*. The concentrations in the *in vivo* assays on burns were of 0.5–2.5% of clorhexidin–gluconate, where the optimal concentration of bacteriostatic inhibition was 1.0%. Another study conducted by İkinci et al. (2002), addressed the use of gels and films of CS against the periodontal pathogen (*Porphyromonas gingivalis*). An antibacterial activity was detected at a concentration of 1.0%. In the case of AC, as far as we are concerned, there are not previous studies on its antimicrobial properties. However, it has been mentioned that in the gel of *Aloe vera*, the gelled crystalline fraction impedes the growth of certain bacteria such as *Streptococcus pyogenes*, *Streptococcus faecalis*, *Bacillus subtilis*, among other (Cheng-Pei, Be-Jen, & Yih-Ming, 2010; Reynolds & Dweck, 1999). It has also been suggested that AC has antiviral properties (Reynolds & Dweck, 1999).

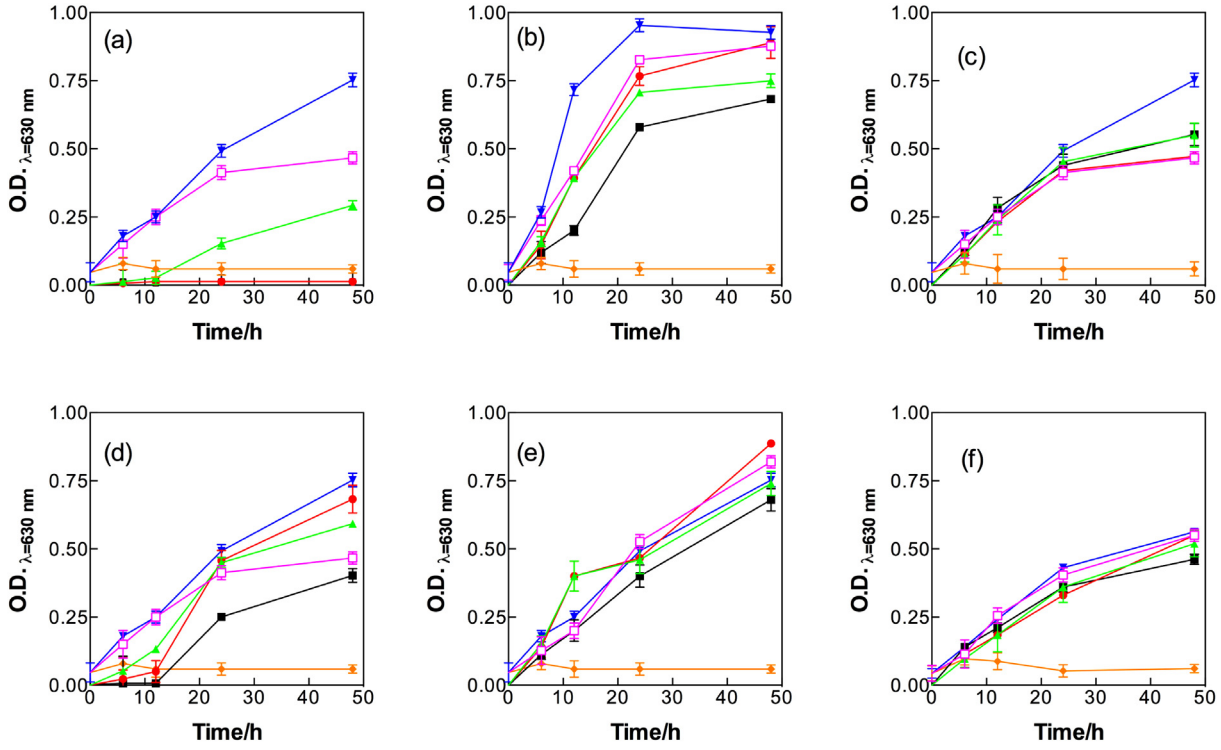

Fig. 4. Evolution of bacterial growth as determined by the optical density (O.D. λ = 630 nm) in Müeller Hilton culture medium (35 °C) after addition of chitosan to *S. aureus* 6538P (a) or *E. coli* 2028P (b); of acemannan (c), chitosan–acemannan (conc. ratio 1:2) (d), chitosan–acemannan (conc. ratio 1:1) (e) or chitosan–acemannan (conc. ratio 2:1) (f) to *S. aureus* 6538P. Key to the applied treatments in (a) and (b): -■- CS 200 μg/mL, -○- CS 100 μg/mL, -▲- CS 50 μg/mL, -□- control (Müeller Hilton culture medium), -◆- gentamicin, -▼- HCl 90 mM; in (c) the key to the applied treatments is identical as for (a) and (b) but acemannan was applied instead of chitosan; and in (d)–(f), chitosan and acemannan blends were applied at the above given concentration ratios and identical chitosan doses as in (a). The results shown are representative of at least two independent experiments and the values represent the average and standard error of triplicate determinations.

The present study addressed the antibacterial properties of mixed CS–AC solutions, with particular focus on possible synergistic effects. To this end, the effect of various treatments was evaluated on *Staphyloccocus aureus* 6538P and *E. coli* 2028P strains. Both are representative of saprophyte microorganisms (i.e. those that occur in a healthy host, but can also become pathogenic in the same host under given circumstances).

Fig. 4 shows the results of the effect of CS, AC isolated and CS–AC blended solutions on the growth of *S. aureus* 6538P or *E. coli* 2028P. The bacterial growth was monitored during 48 h post-treatment with varying concentrations of CS, AC and CS–AC. A clear dose–response behavior was evident, with most effective inhibitory concentrations observed for 200 and 100 μg doses of CS on the growth of *S. aureus* 6538P (Fig. 4a), that gave essentially the same inhibition as the positive control of gentamicin. When the antibacterial activity of CS was tested against the *E. coli* 2028P strain (Fig. 4b), almost no growth inhibition was observed at identical doses as those tested on *S. aureus* 6538P. This differential effect of CS on *S. aureus* 6538P and *E. coli* 2028P can be attributed to structural differences between the two bacteria. In *S. aureus* the external cell wall is comprised by peptidoglycan (N-acetyl-2-D-glucosamine, β-1,4-linked whith N-acetyl muramic acid). By contrast, *E. coli* in addition to peptidoglycan it contains an additional coating inside its cell wall comprised by lipopolysaccharide (ketodesoxy-octonate, heptoses, glucose, galactose, rhamnose, mannose and N-acetylglucosamine) (Forsythe, 2000). This difference in the composition of the bacterial cell wall seems to determine the different effect exerted by CS. In previous studies, it has been observed that CS of M_w 166,000 g/mol and degree of acetylation of 23.7% at concentrations of 0.25% showed no antimicrobial effect against *E. coli*, however, when tested against *S. aureus*, it inhibited the growth by 95%. At the same concentration, CS of M_w 48,500, inhibited the growth of *E. coli* by 30% and at M_w below 5000 g/mol the inhibition was 50% (Zheng & Zhu, 2003).

Micrographs of the membrane of *S. aureus* in these studies revealed that after the treatments with CS the membrane were weakened and broken whereas in *E. coli* the cell size appeared reduced. These effects indicated that CS may form a polymer layer at the cell surface, which can block the subsequent entry of nutrients into *S. aureus*, while CS oligomers may also enter into the cell (Zheng & Zhu, 2003). This explication agrees with the results of the present study. This is so, even when the M_w of the CS used in our study (280,000 g/mol) was somewhat greater than that of the previous studies.

In the case of the antibacterial activity of AC, it showed no effect when tested against *S. aureus* as shown in Fig. 4c. Interesting, although AC has a M_w of 408,300 g/mol it showed no inhibition of the bacterial growth and for the higher doses, it seemed to promote greater growth than the untreated controls. AC is comprised mainly by sugars (mannose, glucose and galactose) that instead of blocking the passage of nutrients into the cell, may serve themselves as a sources of nutrients. This suggestion is consistent with the results of previous studies (González, Domínguez-Espinosa, & Alcocer, 2008), who used *Aloe vera* gel as substrate for the growth of lactic acid bacteria (*L. plantarum* and *L. casei*) during the preparation of probiotic foods.

Fig. 4d–f show the antimicrobial activity of mixtures of AC (2.77%)–CS (0.0839%) and AC (1.75%)–CS (1.28%). It is noteworthy that the final mixture was diluted with culture medium so that final concentrations of test remain in a ratio CS–AC 1:2, CS–AC 1:1 and CS–AC 2:1, corresponding to Fig. 4d–f, respectively. It is evident that the onset of bacterial growth was delayed up to 12 h, while at 24 and 48 h the bacteria grew, though, at rates well below the controls (HCl 90 mM and Müeller Hilton culture medium) at all the

concentrations tested. Fig. 4e and f show the results corresponding to the CS:AC mixtures at 1:1 and 2:1 ratios, respectively. It was evident that the presence of AC antagonistically influences the antibacterial activity of CS. This is probably due to the interactions between the CS and AC as pointed out above. As a consequence of these interactions, the protonated amino groups of CS may lose capacity to interact with muramic acid groups of the cell wall of *S. aureus* and hence prevents the creation of an additional polymer membrane, while providing a nutrient input without any interference.

Although antibacterial studies could have also been performed in the gel state using a suitable experimental set-up (e.g. placing a piece of the hydrogels on an agar plate seeded with the bacteria and observing the inhibition halos), we consider that the information derived from the present study, in which the antibacterial activity was assessed in solution for the isolated and combined components, is highly valuable as it allows to understand the behavior of the two components in isolation or combined in a model system. Future studies could address the influence of hydrogels not only on bacterial growth but also perhaps on other bacterial traits such as biofilm formation.

4. Conclusions

Anhydroalcoholic process was used to set up mixed gel system of CS–AC. Rheological and calorimetric evidence showed that the inclusion of AC into the system weakens the mechanical strength of the CS gel network, even though the amount of CS remains constant. This is attributed to the establishment of heterotypic interactions between the two polysaccharides, possibly as a semi-interpenetrating gel network where AC chains disrupt the connectivity of the CS network. CS in dilute solution showed antimicrobial activity against *S. aureus* but not against *E. coli*, while AC on its own did not exert antimicrobial activity against the two bacteria. The mixture of both polysaccharides showed that as the amount of AC increased the antimicrobial activity of system against *S. aureus* decreased. It remains to be elucidated if the results observed for the antibacterial activity of blended dilute solutions are modified for mixed CS–AC hydrogels.

The results of our study may seem to reflect only "detrimental" effects of the incorporation of AC into CS hydrogels, namely in terms of mechanical strength and antibacterial properties. However, as we studied the effect of adding varying concentrations of AC, we can also conclude that the CS gels can tolerate the incorporation of AC while still preserving its mechanical strength and antibacterial activity. In the present study, it was not feasible to carry out further biological studies to evaluate the advantages that the presence of acemannan may confer to the mixed CS–AC hydrogels in terms of biological activity, namely enhancement of antibodies production and/or induction of anti-inflammatory activity. However, these aspects could be addressed in future studies on this system.

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