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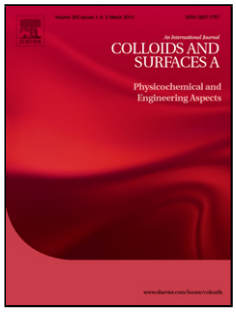

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pH-Responsive Chitosan Nanofilms Crosslinked with Genipin

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ABSTRACT

Crosslinked chitosan-based nanofilms (thickness approximately 40 nm) were prepared using a naturally occurring and nontoxic reagent, genipin, and the surface properties were studied as a function of crosslinking degree and pH, by combining three techniques: dynamic contact angle of sessile drops, quartz crystal microbalance with dissipation (QCM-D) and atomic force microscopy (AFM). The results showed a pH-response of these crosslinked chitosan nanofilms, which swelled at pH 3 and shrank at pH 6. This transition was facilitated by chitosan crosslinking, although the wettability, studied by contact angle measurements, decreased with crosslinking. QCM-D and AFM were used to study the swelling capacity and elastic properties of crosslinked chitosan nanofilms, demonstrating that the response of the nanofilms was fully reversible between pH 3 and 6, but irreversible changes occurred at pH 9. QCM-D showed variations in frequency (Δf) generated by the processes of swelling/shrinkage and variations in dissipation (ΔD), related to the changes in the structure of chitosan nanofilms under different pH values. Under various pH conditions, the AFM results also showed swelling at pH 3 and shrinking at pH 6, observing an increase in the elastic modulus from ≈500 MPa at pH 3 to ≈700 MPa at pH 6. These results allowed us to understand the pH-sensitive properties of genipin-crosslinked chitosan nanofilms, which can be very useful in smart biomaterial-based textiles.

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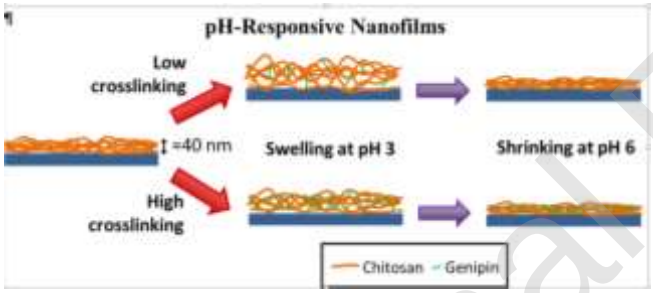
Graphical Abstract



ABSTRACT

Crosslinked chitosan-based nanofilms (thickness approximately 40 nm) were prepared using a naturally occurring and nontoxic reagent, genipin, and the surface properties were studied as a function of crosslinking degree and pH, by combining three techniques: dynamic contact angle of sessile drops, quartz crystal microbalance with dissipation (QCM-D) and atomic force microscopy (AFM). The results showed a pH-response of these crosslinked chitosan nanofilms, which swelled at pH 3 and shrank at pH 6. This transition was facilitated by chitosan crosslinking, although the wettability, studied by contact angle measurements, decreased with crosslinking. QCM-D and AFM were used to study the swelling capacity and elastic properties of crosslinked chitosan nanofilms, demonstrating that the response of the nanofilms was fully reversible between pH 3 and 6, but irreversible changes occurred at pH 9. QCM-D showed variations in frequency (Δf) generated by the processes of swelling/shrinkage and variations in dissipation (ΔD), related to the

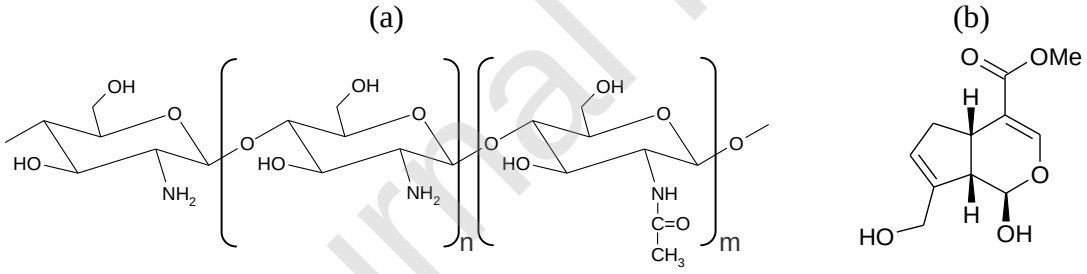
changes in the structure of chitosan nanofilms under different pH values. Under various pH conditions, the AFM results also showed swelling at pH 3 and shrinking at pH 6, observing an increase in the elastic modulus from ≈500 MPa at pH 3 to ≈700 MPa at pH 6. These results allowed us to understand the pH-sensitive properties of genipin-crosslinked chitosan nanofilms, which can be very useful in smart biomaterial-based textiles.

INTRODUCTION

Currently, the combination of conventional textile materials with smart functional finishing technologies offers a wide range of high value-added products. The development of smart textiles for the nonconventional sectors such as biomedical, sportswear and hazard protection includes the deposition of microcapsules, hydrogel thin films and surface modification with plasma.[1-4]

An innovative strategy for the functional finishing of textile materials is based on surface coating with a thin layer of stimulus-sensitive hydrogels.[5-8] In the past decade, stimuli-responsive hydrogels have been extensively investigated because of their responsiveness to environmental stimuli (i.e. pH, temperature, light, electric or magnetic field) as well as their wide range of applications in biomedicine.[9-11] The importance of these smart textile systems lies in their response being tailored to respond to a specific external stimulus, tuning the swelling or shrinkage of the polymer coating and the subsequent delivery of an active component. These stimuli-responsive properties open wide applications in many different fields and demonstrate the importance of designing new smart textiles, using a wide variety of approaches.

The use of polyelectrolytes, natural-based polymers such as dextran derivatives, chondroitin sulfate, hyaluronan and chitosan, has been considered an attractive option in the formation of responsive coatings.[12-15] In this context, chitosan (Scheme 1a) has been the focus of a great attention due to its interesting properties, such us biocompatibility, biodegradability, nontoxicity, low price, and adsorption properties.[16-19]



Scheme 1. Chitosan (a) and genipin (b) molecules.

Chitosan is obtained from chitin, which is the second most abundant polysaccharide found in nature. It has interesting biological properties such as antibacterial, haemostatic and antitumoral activities, in addition to promoting wound healing.[18-20] Chitosan is soluble in acidic media (pK_a ≈6.5) because of the protonation of amino groups, rendering the molecule positively charged. Viscous solutions of chitosan can be used to produce pH-responsive gels in various forms, e.g., beads, membranes, coatings, fibres and sponges.[18] Crosslinking of gels is a suitable strategy for modifying the properties of chitosan coatings or films, which can have a profound influence on different properties, such as stiffness, wettability and stability.[21-27] Several reagents have been

used in the crosslinking of chitosan: glutaraldehyde, formaldehyde, epoxy derivatives, etc.[28-31] However, none of these are free from problems caused by physiological toxicity.[32-36]

Genipin (Scheme 1b), is a natural crosslinking agent present in *Genipa americana* and it is also derived from components of *Gardenia jasminoides Ellis* fruits. It was used by some American indigenous tribes for tattooing, since it spontaneously reacts with amino groups at room temperature and neutral pH, producing an intense dark blue colour in presence of oxygen from air.[37-40] It is well known that chemical reactions of genipin and amino groups lead to intermolecular covalent bonds that crosslink polymers,[37,38] allowing the formation of chitosan-based hydrogels[40] or films.[41] Interestingly, genipin can also polymerize itself to form oligomers such as dimers, trimers or tetramers, forming long spacing chains.[37-39] Therefore, genipin constitutes a crosslinker with a relatively long spacing chain. In addition, it is also known that genipin has a much lower toxicity than other crosslinking agents, such as glutaraldehyde,[36,42] encouraging us to investigate the application of genipin in pH-responsive crosslinked chitosan films on fabrics.

In previous works, we deposited chitosan/genipin films on textile fabrics, and such composite materials were studied for the delivery of cosmetically active components (carnitine or dihydroxyacetone),[43-44] demonstrating the utility of chitosan/genipin coated textiles in cosmeto-textile applications. In addition, another previous work showed that these films increased the ability of fabrics to absorb water vapour,[45] thus improving comfort of textile materials. In the present work, the main objective was to study the properties of crosslinked chitosan films at the nanometre scale, as well as understand the relationship between macroscopic and nanometric properties. To our knowledge, this is the first study of chitosan nanofilms formed by direct deposition from a chitosan/crosslinker mixture. Here, we add a low concentration of genipin to a chitosan solution and deposit layers on a flat substrate. We take advantage of the slow kinetics of the chitosan-genipin reaction to crosslinking after deposition. The main objective of studying crosslinked chitosan nanofilms is their possible application in textile finishing, to impart smart pH-responsive properties to textiles. This paper describes a simple and interesting approach that can be applied for coating textile fabrics.

The swelling of chitosan/genipin nanofilms at various pH values and crosslinking degrees was investigated. For this purpose, three different techniques were used: dynamic contact angle, quartz crystal microbalance with dissipation (QCM-D) and atomic force microscopy (AFM). Contact angle determination is one of the most established methods for investigating the wettability properties of films. Quartz crystal microbalance (QCM) yields information about swelling/shrinking processes and is useful for sorption studies at the solid/liquid interface.[46] This technique can allow real-time measurements of the changes in the mass and viscoelastic properties of chitosan nanofilms induced by pH changes. Atomic force microscopy (AFM) enables the morphological characterization of chitosan nanofilms, providing information about thickness, roughness and viscoelastic properties.

Characterization of the pH-response of textiles coated with crosslinked chitosan nanofilms is difficult because of the extremely high surface roughness of textile materials. To overcome this issue, flat surfaces of silicon wafers were used instead as substrates, and chitosan/genipin nanofilms were prepared and adhered onto those silicon surfaces with covalent anchoring.

MATERIALS AND METHODS

Materials

Chitosan (CHT, medium molecular weight) was purchased from Sigma-Aldrich, with 85 % deacetylation. Genipin was supplied by Challenge Bioproducts Co (Taiwan), who obtained from *Gardenia jasminoides* fruits. [3-(2,3-epoxypropoxy)-propyl]-trimethoxysilane (abbreviated as EPTMS) was purchased from Merck. Sodium chloride (NaCl) with a purity of 99.5% was from Sigma-Aldrich. Glycine with a purity of 99.9%, stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) with a purity of 98% and ninhydrin were purchased from Sigma-Aldrich. Citric acid monohydrate, purity > 99.5%, was purchased from Fluka. Ethylene glycol monomethyl ether, SDS, purity ≥ 99.5% and 2-propanol, purity ≥ 99.5% were also supplied from Sigma-Aldrich. Acetone was from VWR with a purity of 100 %. Silicon wafers with (100) surface orientation were used. Quartz crystal sensors coated with silicon were obtained from Q-Sense (Sweden). All chemical products were used as received. All solutions were prepared using Milli-Q water (resistivity > 18 MΩcm), purified with a Milli-RO 10 Plus pre-treatment unit and a Q-PAK unit. The outgoing water was filtered through a 0.2 μm filter.

Preparation of crosslinked chitosan nanofilms on substrates

Initially, substrates were cleaned in a 2 vol% Hellmanex® solution for 1 h. Then, substrates were rinsed with Milli-Q® water and ethanol (99.5%) and dried with a nitrogen stream. Prior to coating with chitosan or chitosan/genipin solutions, substrates were functionalized with an EPTMS solution (10 vol% in acetone) for 15 h at room temperature. This was performed by dipping the silicon wafers into EPTMS solutions with anhydrous solvents. Then, substrates were rinsed with acetone to remove excess silane, dried under a nitrogen flow and heated in an oven at 100 °C for 1 h, anchoring the silanated layer, by covalent bonds, on the surface of silicon wafers.[47]

Chitosan solutions were prepared at 1 wt% in aqueous acetic acid 1 vol%. Solutions were stirred overnight and then filtered (Fisherbrand) to ensure the total removal of insoluble impurities. Genipin was dissolved in phosphate buffer solution ($Na_2HPO_4 \cdot 2H_2O$ / KH_2PO_4) at pH 7.4, with [$Na_2HPO_4 \cdot 2H_2O$] = 11.876 g/L and [KH_2PO_4] = 9.078 g/L, to prepare solutions at 0.05 wt%. Chitosan and genipin solutions were mixed in a 1:1 weight ratio (reaching a final concentration of 0.5 wt% chitosan and 0.025 wt% genipin) for 15 min and this solution was applied to the substrates to prepare the crosslinked nanofilms on silanated substrates in a closed environment with measurement of relative humidity (RH = 80%), to avoid the evaporation of solvents that could influence silane-chitosan and/or chitosan-genipin couple reactions. The concentrations of chitosan and genipin (0.5 and 0.025 wt%, respectively) were selected based on previous works,[40,41] to achieve partial crosslinking in 1 hour and to reach almost complete crosslinking in 6 hours.

Fig. 1 schematically shows the reaction between crosslinked chitosan and the silane film, where silane epoxy groups easily react with chitosan amino groups, forming imine bonds.[47,48]

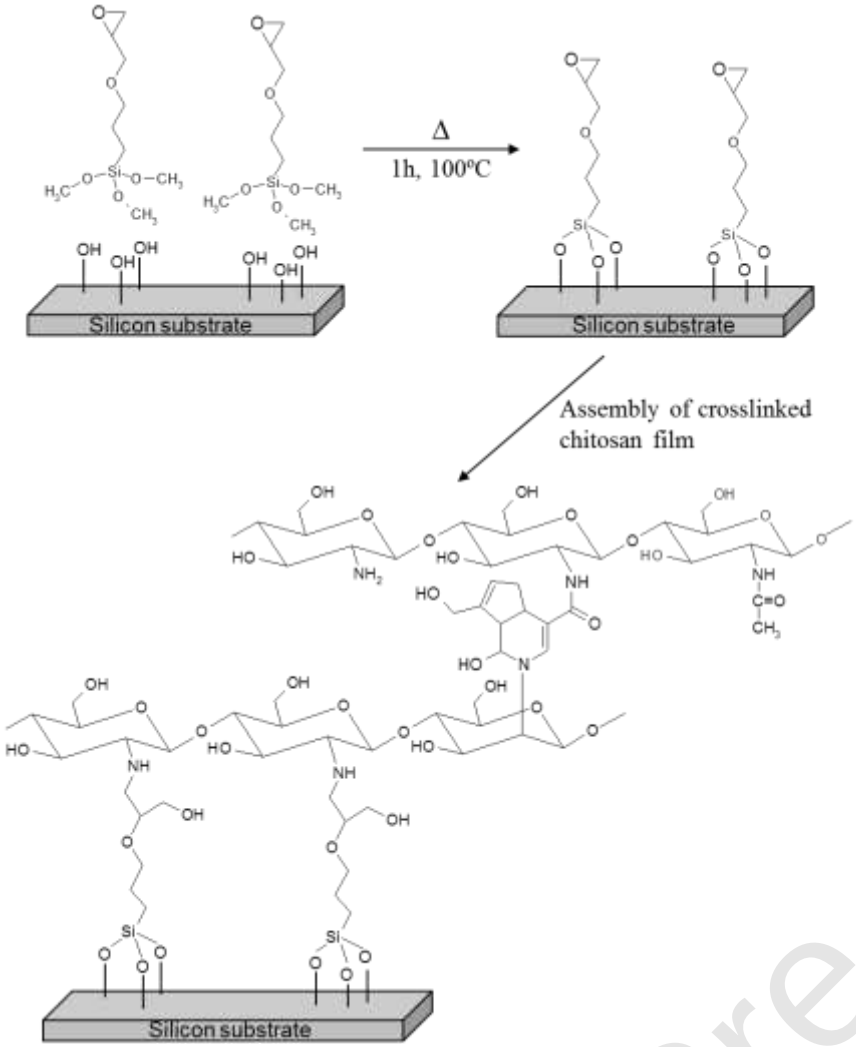


Fig. 1. Proposed scheme for the assembly of crosslinked chitosan nanofilms onto silicon substrate. The chitosan nanofilm was chemically anchored on silicon substrates through silanation with EPTMS.

Chitosan nanofilms were prepared on silicon wafers or QCM-D crystals by immersion of substrates in chitosan-genipin solution or chitosan solution to obtain crosslinked or uncrosslinked chitosan nanofilms, respectively. The substrates were immersed in the chitosan-genipin solution for 1 or 6 h, to study the influence of chitosan crosslinking time in the pH-response of nanofilms.

Chitosan crosslinking reaction with genipin was carried out at room temperature in presence of air, where the carboxymethyl group of genipin forms secondary amides with chitosan amino groups, as described in literature.[37-39] As expected, a gradual increase in the viscosity of the chitosan/genipin aqueous mixtures was observed, as well as the appearance of dark blue color, confirming the formation of chitosan-genipin-chitosan covalent bridges. The increase in viscosity and formation of elastic hydrogels, which occurred at long reaction times, made difficult the preparation of thin nanofilms and therefore the maximum contact time of 6 h was selected.

Uncrosslinked chitosan nanofilms were also prepared, for comparison, by immersing the substrates in the chitosan solution for 6 h, in the absence of genipin. These substrates were rinsed with Milli-Q® water to remove loosely adsorbed chitosan from the substrates and finally dried at room temperature for 24 h.

The pH-response of the chitosan nanofilms, prepared on silanated substrates, was studied using 10 mM sodium chloride (NaCl) solutions at three different pH values: 3, 6 and 9. pH was controlled by the addition of either hydrochloric acid or sodium hydroxide.

Evaluation of the crosslinking of chitosan with genipin

Chitosan-genipin aqueous mixtures were prepared with the same chitosan concentration of films (0.5 wt%) and various genipin concentrations (0.025, 0.005 and 0.0025 wt%) and the visual aspect of these mixtures was followed by visual observation for 24h.

Estimation of the degree of crosslinking

The degree of chitosan crosslinking was evaluated by quantifying the amount of chitosan amino groups (-NH_2) that remain accessible after crosslinking, using the ninhydrin method for the quantification of proteins described by B. Starcher[49] and adapted to chitosan by Y. Yuan[50]. Briefly, a solution with 1.05 g citric acid monohydrate, 10 g NaOH (1M), 0.04g $SnCl_2 \cdot H_2O$ and 15 g milliQ water was prepared. Then, a second solution with 1 g ninhydrin and 25 g ethylene glycol monomethyl ether was added and the mixture was kept in a dark bottle. For the assay, 7.5 mg of either crosslinked chitosan film or pure chitosan powder was added to 5 g of the ninhydrin mixture. Then, the vial was sealed and heated at 100 ºC in an oil bath for 20 min. The solution was cooled to room temperature, centrifuged at 2500 rpm for 5 min to remove precipitate and 1 mL of supernatant was extracted and diluted in 5 mL isopropanol 50%. Finally, the optical absorbance at 570 nm was measured by UV-Vis spectrophotometry (PerkinElmer Lambda 365). The reduction in amino groups was quantified as follows (Eq. 1):

$$\boldsymbol{Amino\ reduction} = \frac{[\boldsymbol{NH_2}]_{\boldsymbol{uncrosslinked}} - [\boldsymbol{NH_2}]_{\boldsymbol{crosslinked}}}{[\boldsymbol{NH_2}]_{\boldsymbol{uncrosslinked}}} \qquad \text{Eq. 1.}$$

where $[\text{-}NH_2]_{uncrosslinked}$ is the concentration of free amino groups in the uncrosslinked pure chitosan powder and $[\text{-}NH_2]_{crosslinked}$ is the concentration of free amino groups in the chitosan-genipin crosslinked film. Two replicates were measured for each sample and the average was calculated.

The concentration of free amino groups was determined from the calibration curve of glycine concentration as a function of absorbance (Supporting information, Figure S2) to determine the glycine concentration equivalent, for both uncrosslinked and crosslinked samples. Glycine concentrations from 0 to 5 mg/mL were selected for the calibration curve.

For these measurements, crosslinked chitosan films were prepared on simple glass substrates without anchoring. The procedure for film formation consisted of casting the mixture of chitosan and genipin on a glass plate and drying in air at room temperature for approximately 24h.

Dynamic contact angle

The wettability of crosslinked and uncrosslinked chitosan nanofilms was assessed by means of contact angle measurements for 10 min. The contact angle was measured using a Gardco PG-X goniometer, which allows image capture of droplets in real time. The measurements were carried out at room temperature using a volume droplet of 2 μL. Contact angle measurements were performed on silicon wafers with an area of 1.3 x 3 cm^2 and three replicas were taken for each

sample. The evolution of contact angle (θ, the angle between the baseline of the drop and the tangent at the drop boundary) was monitored using the image-processing software supplied by Gardco.

Quartz crystal microbalance with dissipation monitoring (QCM-D)

The QCM-D technique allowed the simultaneous monitoring of the changes in frequency (Δf) and dissipation energy (ΔD) during the processes of swelling or shrinkage of crosslinked chitosan nanofilms at different pH values (3, 6 and 9). This technique is well known and has been described in detail elsewhere.[51] Briefly, QCM is a highly sensitive sensor of mass variation (resolution in the ng·cm^{-2} range) that also tracks energy dissipation properties of the adsorbed mass. It is one of a few techniques that can be used to directly observe the adsorption process in situ.[52-54] The mass change results from the variation of the normalized resonant frequency (Δf/n) of an oscillating quartz crystal when adsorption occurs on the surface of the piezoelectric crystal. The variation in oscillation frequency (Δf) is proportional to the mass of the adsorbed layer (Δm) by the Sauerbrey equation (Eq. 2). This equation implies a linear relationship between Δf and Δm, for thin and rigid nanofilms. However, some deviations from the Sauerbrey model may be expected for viscoelastic nanofilms. In that case, the adsorbed mass (Δm) is measured as dissipation energy (ΔD).

$$\Delta m = -C\,\Delta f / n \qquad \text{Eq. 2}$$

where n is the overtone number (n = 1, 3, 5, 7, 9, 11) and C is the mass sensitivity constant, which depends on the QCM-D crystal (for quartz crystal sensor C = 17.7 ng·cm^{-2} Hz^{-1}).

The pH-responses of chitosan nanofilms at crosslinking times of 1 h and 6 h were monitored in situ using an E4 device (Q-Sense, Sweden), by the preparation of these nanofilms on QCM-D crystals. The sensor crystals (Q-Sense) are AT-cut quartz with gold plated polished electrodes. The quartz crystals were excited at their fundamental frequency ($f_0 \approx 5$ MHz) as well as at the overtones (n) 3rd, 5th, 7th, 9th and 11th, corresponding to 15, 25, 35, 45 and 55 MHz, respectively.

All measurements were performed in a temperature-controlled flow chamber, at 25 ºC and 0.3 mL/min, at three different pH values (3, 6 and 9) in the presence of 10 mM NaCl. Initially, a baseline was established in 10 mM NaCl at pH 6, prior to each measurement.

Atomic force microscopy (AFM)

For a better understanding of the pH-response of crosslinked chitosan nanofilms, AFM was employed to assess topography and measure the surface elastic modulus. AFM measurements were performed with a Multimode Nanoscope III device (Bruker). Samples were scanned in PeakForce mode using silicon nitride cantilevers of triangular shape (ScanAsyst-Fluid+ supplied by Bruker), with a nominal spring constant of 0.7 N/m and a resonant frequency of 150 kHz. All measurements were performed at room temperature with a fluid cell (Bruker) with 10 mM NaCl solutions at pH 3, 6 and 9. Scan size was 2 x 2 μm^2. Before any measurement, samples were maintained in 10 mM NaCl at pH 6, for 1 h.

RESULTS AND DISCUSSION

Nanofilms were prepared by immersing EPTMS-functionalized substrates in chitosan-genipin aqueous solutions, just after preparing the mixtures. It is known that chitosan crosslinking with genipin is a slow process,[40,55,56,57] and such crosslinking often takes hours or even days to be completed. In the present work, chitosan and genipin were mixed just before immersing the substrates (time zero), and a long time before gelation occurred[40]. Therefore, it was assumed that crosslinking was negligible and very close to zero when immersing.

Monitoring of chitosan crosslinking with genipin

The reaction between chitosan and genipin was studied by visual observations, replicating a procedure described before by S. Vílchez et al.[40] at 0.5 wt% chitosan concentration and with various amounts of genipin (0.025, 0.005 and 0.0025 wt%). The increase in viscosity was evaluated by turning the vials upside down. The results (images included as Supporting Information, Fig. S1) showed that crosslinking greatly increased viscosity, to a point where the solution did not flow if turned upside down, demonstrating the formation of hydrogels. Sample gelation with 0.025 wt% genipin had already occurred within 1 hour, while it required 24 h in the case of 0.0025 wt% genipin. These results are consistent with those previously reported,[37-38,57] and after gelation samples become dark blue, as expected.

Evaluation of the degree of crosslinking

The crosslinking degree was evaluated using the ninhydrin method that determines the reduction in the amount of accessible amino groups, as described in the experimental section. A total of 7.5 mg of chitosan-genipin films (containing 7.125 mg of chitosan and 0.375 mg of genipin) were prepared by solvent evaporation for 24 h, on glass substrates without anchoring with EPTMS. This procedure was selected as an alternative to the thinner films (≈40 nm thick) used for QCM and AFM determinations, due to the problems of manipulating undried nanofilms, peeling them off from the substrate and analysing tiny film amounts. Therefore, the applied method can be assumed to reveal the maximum possible crosslinking. As a blank control, another film with 7.5 mg of pure chitosan was also analysed. The decrease in average absorbance, measured by the ninhydrin method, was from 0.360 to 0.244, allowing us to obtain the glycine equivalents of pure chitosan and crosslinked chitosan of 1.42 and 1.00 mg/mL, respectively, based on 7.5 mg of chitosan. This reduction in glycine equivalents (corresponding to 1.89×10^{-5} and 1.33×10^{-5} mol/mL, respectively, of NH_2 amino groups) indicates that approximately 30% of the chitosan free amino groups were consumed during genipin-induced chemical reactions. This result confirms that the chitosan-genipin films are crosslinked, as expected because of the dark blue colour of these films.

Wettability of crosslinked and uncrosslinked chitosan nanofilms

The wettability of nanofilms is an important parameter regarding surface properties. The wettability of chitosan nanofilms was studied by means of contact angle determinations. Table 1 presents the values of initial contact angle (θ_0) for both uncrosslinked and crosslinked nanofilms, as a function of pH (3, 6 and 9) and as a function of crosslinking time (0, 1 and 6 h).

Table 1. Initial contact angle (θ_0, º) for uncrosslinked (t=0) and chitosan nanofilms crosslinked for 1 or 6 h, at different pH values. t indicates the crosslinking duration.

	Uncrosslinked chitosan films (t=0)	**Crosslinked chitosan films**	
		1 hour	**6 hours**
pH=3	25 ± 3	37 ± 9	51 ± 8
pH=6	34 ± 9	33 ± 3	38 ± 2
pH=9	25 ± 3	41 ± 3	45 ± 2

Uncrosslinked chitosan nanofilms showed the lowest contact angle values, indicating a more hydrophilic surface, which could be attributed to a larger surface density of either $–NH_2$ deprotonated groups at high pH or protonated $–NH_3^+$ at pH values lower than pK_a (≈6.5).[18] Moreover, at all pH values, it was observed that contact angle slightly increased by extending the crosslinking time. This result demonstrates that crosslinking truly occurred and led to less hydrophilic surfaces, probably by formation of less polar amide bonds. However, since low contact angles imply higher hydrophilicity of nanofilms, the small contact angle at pH 9 of the uncrosslinked nanofilm (25 ± 3°) seems anomalous. One would expect this value to be higher, since chitosan is fully deprotonated and less hydrophilic. However, chitosan chains might have some changes in conformation, as it will be discussed in next sections.

pH–response of crosslinked chitosan nanofilms monitored by QCM-D

The influence of pH on nanofilm swelling (1 h and 6 h) was investigated by QCM-D. The experiments were started at pH 6 until a stable baseline was achieved. pH was varied following a 6-3-6-9-6 cycle, as indicated in Fig. 2. The variation in frequency and dissipation were monitored during the 30 min experiment. The variations in both frequency (Δf) and dissipation (ΔD) can be associated with structural changes in the chemically adsorbed nanofilms, since samples had been prepared before, outside QCM chambers, allowing the evaluation of swelling or shrinking of nanofilms. The pH response of the crosslinked nanofilms, monitored by QCM-D, is shown in Fig. 2.

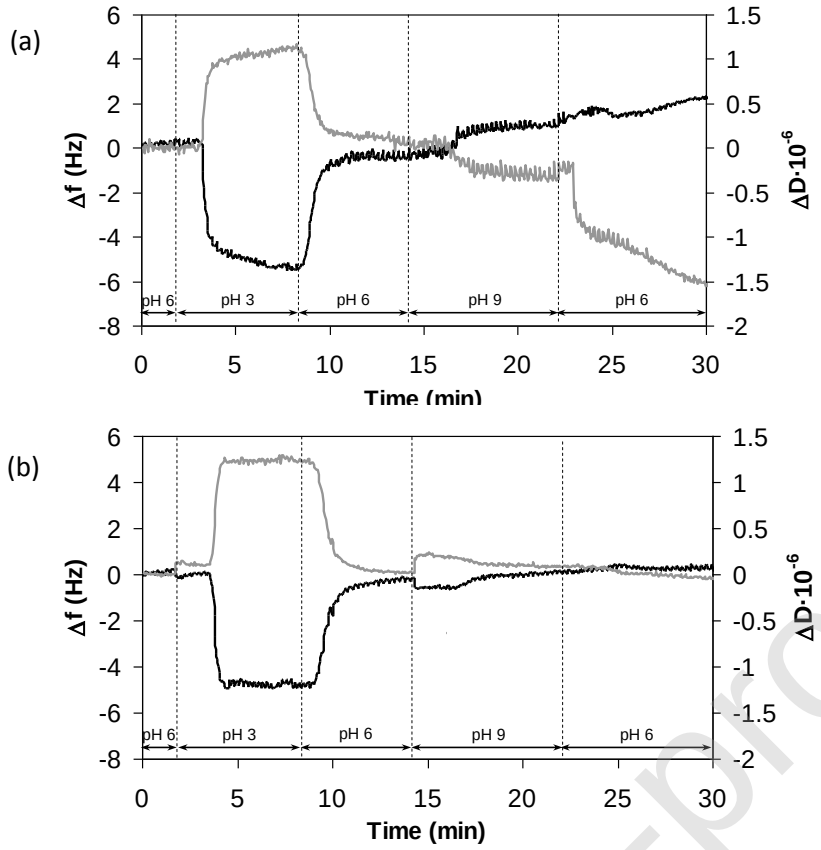


Fig. 2. Frequency, Δf, (black line) for the fifth overtone at 25 MHz, and energy dissipation, ΔD, (grey line) as a function of time: (a) 1 h Crosslinking time and (b) 6 h crosslinking time. The pH in the chamber was varied at time intervals, as indicated on the graph.

Fig. 2 shows that frequency and energy dissipation greatly change when pH decreases from 6 to 3, for two nanofilms with different crosslinking times (1 and 6 h). The frequency difference, Δf, varied from 0.2/-0.3 Hz at pH 6 (baseline) to around -5/-5.5 Hz at pH 3, and the dissipation difference, ΔD, shifted from $0.1 \cdot 10^{-6}$ (pH 6) to $1.1 \cdot 10^{-6}$ (pH 3) for the two nanofilms, with values almost independent of crosslinking time. Although Δf and ΔD were very similar for the two crosslinking conditions, some small differences were observed at pH 3. At t=1 h (Fig. 2a), the nanofilm showed a slower swelling. In contrast, the swelling behaviour of nanofilm at t=6 h (Fig. 2b) shows a rather flat frequency variation, indicating no further swelling for the highly crosslinked nanofilm. Certainly, the decrease in pH produces the protonation of chitosan amino groups, which causes swelling of the chitosan film, since pH 3 is much below the pK_a of chitosan (≈6.5).[18] Afterward, when the pH was increased again to pH 6, the frequency and dissipation returned to their initial values. Therefore, the two nanofilms swell reversibly between pH 3 and 6, expanding from pH 6 to 3 and shrinking from pH 3 to 6. This was attributed to the partial deprotonation of amino groups, producing a reduction in electrostatic repulsions and causing rearrangements of chitosan molecules, as also observed in other chitosan films.[52] Chitosan is a polycationic polymer with $pK_a \approx 6.5$, and consequently, it is highly charged at low pH but becomes neutral at pH values higher than 6.5. Therefore, both crosslinked and uncrosslinked chitosan become more hydrophobic and less hydrated at basic pH, collapsing to more condensed

conformations. In conclusion, chitosan films crosslinked with genipin are pH-responsive and undergo reversible swelling/shrinking between pH 3 and 6. However, uncrosslinked chitosan films do not behave reversibly, since they are water-soluble and dissolve at low pH.

The QCM-D experiment was extended by increasing the pH to 9 (Fig. 2), well above the pK_a of chitosan, where all $–NH_2$ groups are virtually deprotonated. At this pH, the two chitosan nanofilms behaved differently. In the nanofilm crosslinked for 1 hour (Fig. 2a), the frequency difference, Δf, changed from -0.15 Hz to 1 Hz and the dissipation difference, ΔD, varied from $0.05 \cdot 10^{-6}$ to $-0.3 \cdot 10^{-6}$. In the case of the nanofilm crosslinked for 6 h, the variations were smaller (Fig. 2b), since the frequency changed from -0.15 Hz to 0.13 Hz and dissipation from $0.02 \cdot 10^{-6}$ to $0.08 \cdot 10^{-6}$. Thus, smaller variations in frequency and dissipation are observed in nanofilms prepared with longer crosslinking times. Most likely, a higher degree of crosslinking leads to more rigid nanofilms.

It should be mentioned that chitosan is insoluble in water at pH values above its pKa,[57] where it has a semicrystalline nature because of inter- and intramolecular hydrogen bonds. Under acidic conditions, its amino groups are protonated producing repulsion between positively charged chains, allowing hydration and solubilisation. However, this does not occur above pH 6.5. Therefore, it can be presumed that the chitosan nanofilms became dehydrated and collapsed at pH 9.

Finally, when the pH was returned to 6 from 9, the nanofilms crosslinked for only 1 h showed a large decrease in dissipation, which could be caused by the formation of insoluble clusters of less-hydrated chitosan chains, when the pH was increased far above the pK_a value. This was not observed in the nanofilm crosslinked for 6 h, indicating a more stable nanofilm at a higher degree of crosslinking.

The low values of frequency (Δf) and energy dissipation (ΔD) could indicate that nanofilms prepared at crosslinking times of 1 h and 6 h present a high crosslinking degree. The low variations in frequency and dissipation observed are in agreement with other results achieved in a different chitosan-based system,[59] obtaining similar values of Δf (≈ -5) but slightly higher ΔD values ($\approx 6 \cdot 10^{-6}$) at pH = 2.7. It was concluded that these chitosan films were highly crosslinked, so they could not show a high swelling.[59] In addition, other researchers found that when ΔD is very low and the ratio $\Delta D/(-\Delta f) << 4 \cdot 10^{-7}$ Hz^{-1} nanofilms can behave as rigid systems.[60,61] Focusing on our results of Δf and ΔD at pH 3, the values show $\Delta D/(-\Delta f) = 2.2 \cdot 10^{-7}$ Hz^{-1} that could demonstrate the rigidity and consequently, the mechanical stability of these nanofilms.

In conclusion, a clear response as a function of pH has been found, which is associated with swelling/shrinking behaviour. Nanofilms expanded at pH 3 and shrank back to the initial condensed state at pH 6. The nanofilms stayed in this condensed situation at pH 9 and again at pH 6. However, in the case of the nanofilms prepared with 1 hour of crosslinking time, an irreversible change was observed when placing the specimen at pH 9. Certainly, chitosan is completely deprotonated at pH 9 and consequently it behaves as a more hydrophobic material, as shown by the higher contact angle. Therefore, the chitosan nanofilm with a lower degree of crosslinking (t=1 h) suffered irreversible changes in conformation at pH 9, whereas the more crosslinked nanofilm (t=6 h) seemed to be much less influenced by basic pH. In conclusion, QCM results also indicated that 6 hours of crosslinking is required to obtain stable nanofilms. The nanostructure can be studied in more detail by AFM, as described below.

Topographical study of pH-responsive chitosan nanofilms by AFM

First, the thickness of chitosan nanofilms, crosslinked for 1 h, was determined by PeakForce QNM mode in an air-dried nanofilm. Holes were created in the nanocoating by repeatedly scanning a small area of a nanofilm by contact mode with a high setpoint. This method allows scraping off the soft nanofilm until reaching the underlying silicon harder substrate.[62,63]

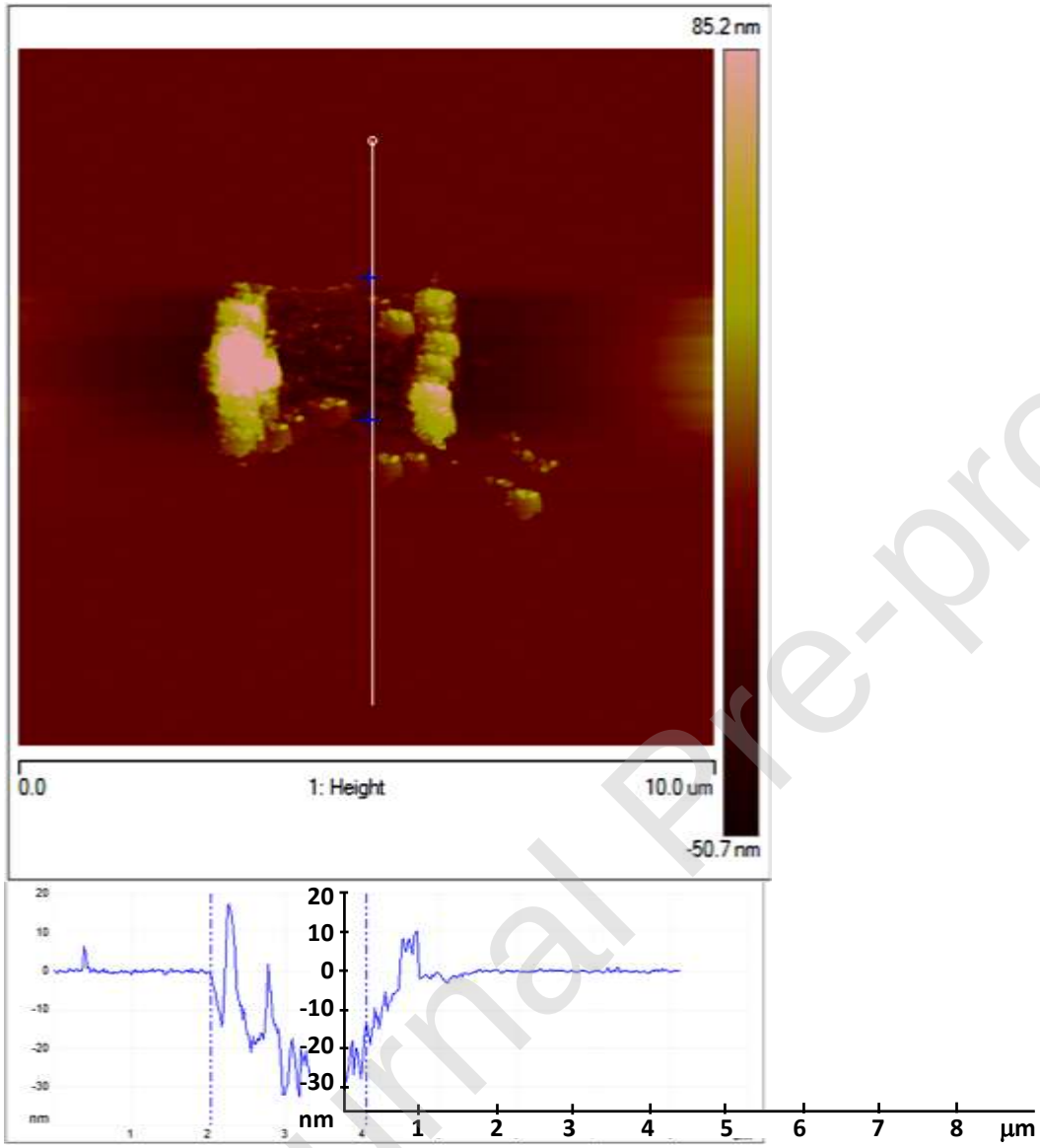
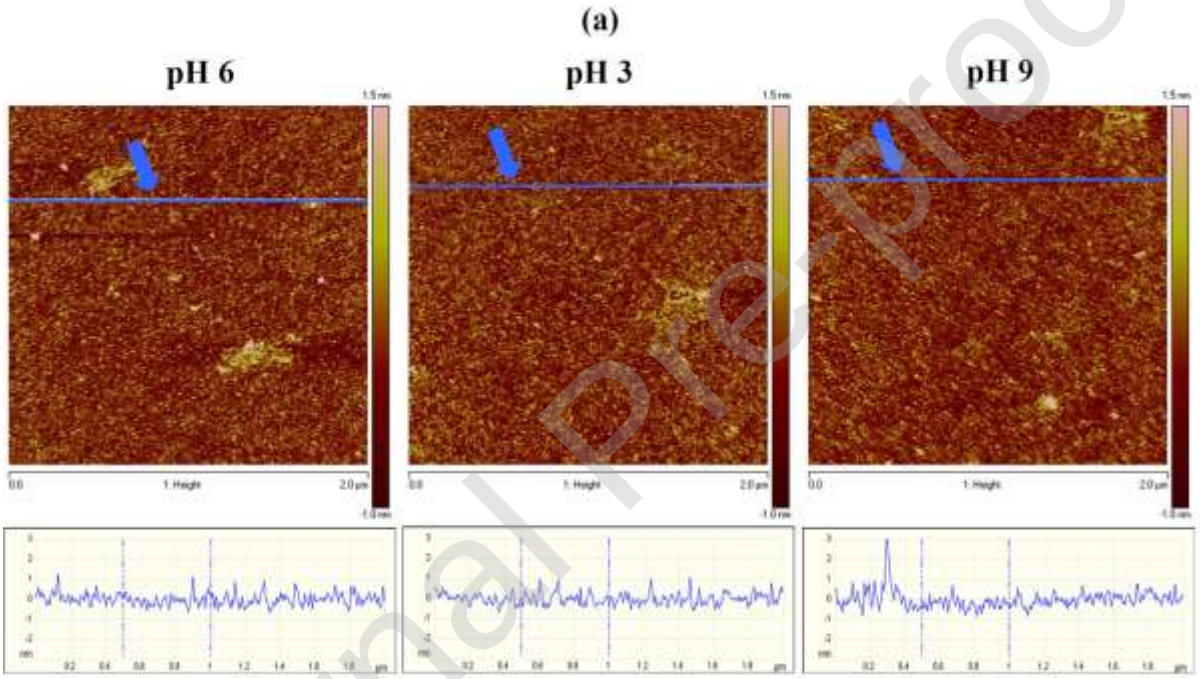


Fig. 3. Determination of the thickness of a dry chitosan nanofilm, crosskinked for 1 h, by applying PeakForce QNM mode in air. Cavities are indicated by dark colour, while protrusions by light colour tones. The vertical line indicates the selected direction for the scanning. The maximum depth was approximately ≈35 nm, as seen in the height profile.

An average film thickness of 40±14 nm was obtained from four measurements on the same sample, demonstrating that thin nanofilms were obtained. An example of these measurements is shown in Fig. 3. Interestingly, it was observed that the nanofilm surface was rather smooth, with a roughness much smaller than the film thickness (example shown in Fig. 3); thus, the nanofilm could be considered homogeneous.

The response of crosslinked chitosan nanofilms, in aqueous solutions at controlled pH, was studied for samples prepared with the same procedure for QCM-D measurements. First, nanofilms were characterized at pH 6, followed by pH 3 and finally pH 9. Between pH 3 and pH 9, samples were rinsed with a solution at pH 6. Fig. 4 shows the pH influence on the two types of chitosan nanofilms, obtained from crosslinking for either 1 or 6 h.

Rather flat surfaces were observed at the three pH values, with height profiles mainly within ±1 nm. However, the sample crosslinked for 6 h showed some roughness, resembling isolated islands, with diameters of approximately 0.2 μm and reaching ≈3 nm in height (Fig. 4b). These isolated clusters were observed at the three different pH values and attributed to possible ionic interactions of chitosan molecules to phosphate anions from the buffer solution containing genipin, occurring at longer times. Nevertheless, the maximum heights, approximately 3 nm, can be considered very small. The average thickness of a dry nanofilm was measured as ≈40 nm. It could be expected that wet films with absorbed water are probably much thicker, and therefore, protrusions of 3 nm high can be considered small. In conclusion, the nanofilms are rather flat with a smooth surface.



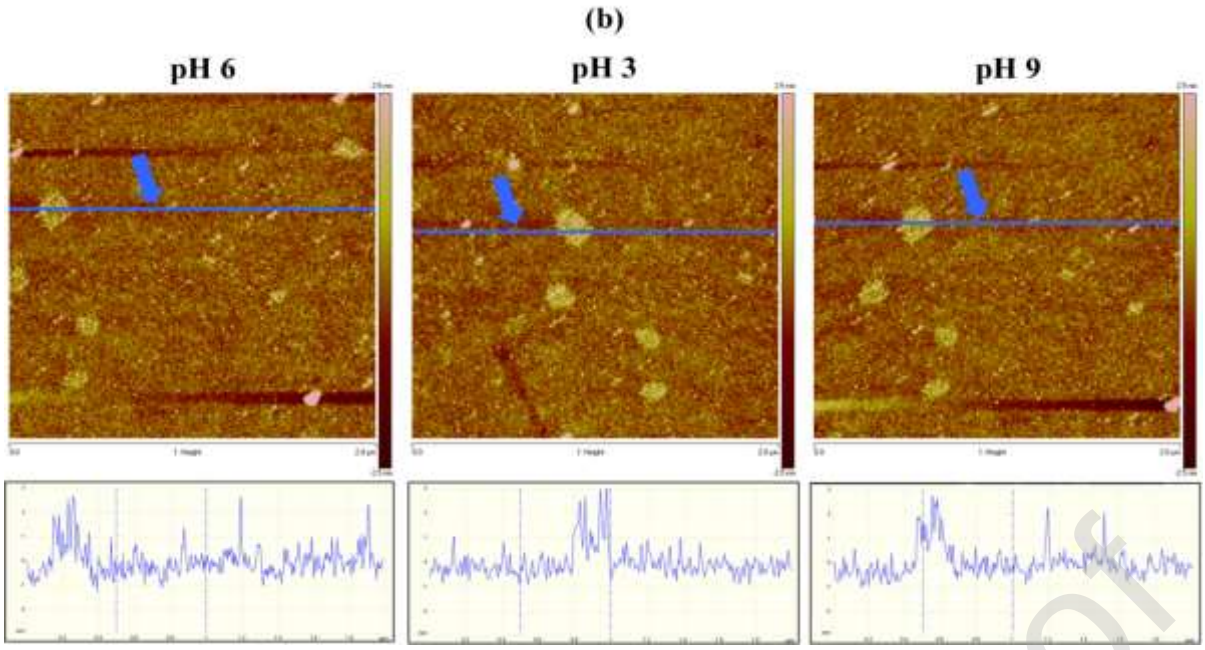


Fig. 4. AFM imaging and topography of nanofilms, for different pH values (3, 6 and 9). Island-like protrusions are indicated by lighter colour tones. Images show examples of chitosan nanofilms crosslinked for 1 h (a) and 6 h (b). Images cover 4 μm^2 areas, with 2 μm square size. Topographic profiles are shown below the AFM images, spanning distances of 2 μm (blue lines) with heights (Y-axis) ranging from -3 to +3 nm.

The height profiles (Fig. 4) did not show significant differences with modifications to pH, leading to the conclusion that roughness did not depend on swelling and/or shrinking. However, one cannot discard the possibility that variations in molecular conformation, as a function of pH, might influence nanofilm mechanical properties. Therefore, another method is required to evaluate the possible changes in chitosan conformation, and thus, nanofilm surface elasticity was studied (Fig. 5).

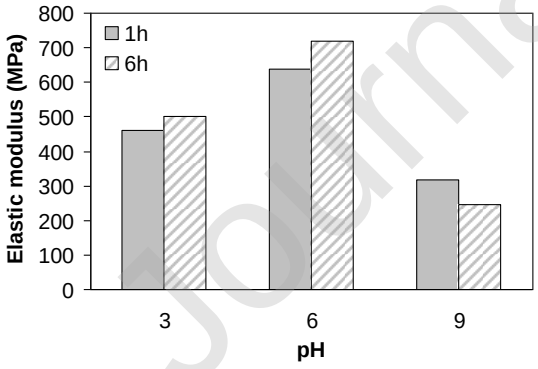


Fig. 5. Surface elastic modulus of chitosan nanofilms, as a function of pH (3, 6 and 9) at either 1 or 6 h crosslinking time.

In this context, determination of the surface elastic modulus of thin nanofilms by PeakForce AFM can allow the study of mechanical properties at the molecular level. Fig. 5 shows the results of the surface elastic modulus as a function of pH (3, 6 and 9). The modulus increases from pH 3 (swollen nanofilm) to pH 6 (shrank state), as expected for both crosslinking times. At pH 6,

higher values of elastic modulus are obtained because these films are more compressed, and most likely, they are less hydrated. These results were expected, since chitosan is highly charged at low pH and less charged at pH 6. Moreover, crosslinking for 6 hours seems to slightly increase the elastic modulus, which makes sense as crosslinks increase stiffness.

At pH 9, the QCM-D technique clearly identifies a collapse of the films (Fig. 2). However, the AFM results have not shown an increase in surface elastic modulus at pH 9 (Fig. 5), as it is commonly observed in the case of thick PNIPAm films.[64,65] The most likely explanation could be that when the quitosan-genipin layer undergoes a hydrophobic collapse, then this layer would expose its most hydrophilic groups towards the aqueous phase. This could maybe provide softer interfaces compared to a lower pH. It should be noted that the QNM PeakForce tapping method relies on very limited surface deformations (typically, around 1 nm thickness), and only the most external layer is deformed by the AFM tip. Consequently, a highly collapsed layer with exposed hydrophilic groups might provide lower surface elastic modulus.

In any case, it should be noted that the elastic modulus results confirm the reversible swelling/shrinking transition between pH 3 and 6 and that irreversible changes occur between pH 6 and 9. One possible application of these nanofilms would be textile fabric finishing for the design of smart fabrics. The chitosan/genipin functionalized textiles might have commercial interest because of sensorial perception dependence on pH, as well as water adsorption capability. As already shown in previous papers,[43-44] the deposition of chitosan/genipin films on textile materials is useful for the controlled delivery of cosmetic active components. Moreover, these films are able to absorb a significant amount of water vapour, as determined from water adsorption isotherms.[45]

CONCLUSIONS

Covalently crosslinked chitosan nanofilms have been anchored on a silicon substrate through silanation. These nanofilms were crosslinked with genipin, a low-toxicity natural reagent, obtaining ≈40 nm film thickness (with 1 hour of crosslinking), measured by AFM. These nanofilms are sensitive to pH, as demonstrated by the dynamic contact angle and quartz crystal microbalance with dissipation (QCM-D). The dynamic contact angle results showed that the nanofilms possessed a higher hydrophobicity after crosslinking, leading to higher contact angle values. This is attributed to the partial removal of $–NH_2$ groups and the formation of less polar amide bonds. A swelling/shrinking phenomenon was observed by QCM-D, induced by the protonation and deprotonation of chitosan amino groups, as a function of pH. This swelling/shrinking behaviour was reversible, shifting from a swollen nanofilm, at pH 3, to a shrunken state at pH 6. AFM of a dry nanofilm in air showed that the nanofilms were quite smooth and homogenous, since the surface roughness was only approximatey 3 nm high. The elastic modulus increased from pH 3 to pH 6, probably due to a higher degree of stiffness in less swelled nanofilms. Extended crosslinking time leads to tougher nanofilms, with a higher elastic modulus. These results have demonstrated that genipin crosslinking is a suitable and tunable method to control the swelling/shrinkage of pH-dependent chitosan nanofilms. Due to the low-toxicity nature of both chitosan and genipin, the prepared nanofilms have shown great potential in smart textile applications.

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Declaration of no competing interests

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