New crosslinkers for electrospun chitosan fibre mats. I.
Chemical analysis

Marjorie S. Austero¹, Amalie E. Donius¹, Ulrike G. K. Wegst¹,²
and Caroline L. Schauer¹,*

¹Department of Materials Science and Engineering, Drexel University,
3141 Chestnut Street, Philadelphia, PA 19104 USA
²Thayer School of Engineering, Dartmouth College, 14 Engineering Drive,
Hanover, NH 03755 USA

Chitosan (CS), the deacetylated form of chitin, the second most abundant, natural polysaccharide, is attractive for applications in the biomedical field because of its biocompatibility and resorption rates, which are higher than chitin. Crosslinking improves chemical and mechanical stability of CS. Here, we report the successful utilization of a new set of crosslinkers for electrospun CS. Genipin, hexamethylene-1,6-diaminocarboxysulphonate (HDACS) and epichlorohydrin (ECH) have not been previously explored for crosslinking of electrospun CS. In this first part of a two-part publication, we report the morphology, determined by field emission scanning electron microscopy (FESEM), and chemical interactions, determined by Fourier transform infrared microscopy, respectively. FESEM revealed that CS could successfully be electrospun from trifluoroacetic acid with genipin, HDACS and ECH added to the solution. Diameters were 267±199 nm, 644±359 nm and 896±435 nm for CS–genipin, CS–HDACS and CS–ECH, respectively. Short- (15 min) and long-term (72 h) dissolution tests (T₆₀₀) were performed in acidic, neutral and basic pHs (3, 7 and 12). Post-spinning activation by heat and base to enhance crosslinking of CS–HDACS and CS–ECH decreased the fibre diameters and improved the stability. In the second part of this publication, we report the mechanical properties of the fibres.

Keywords: electrospinning; chitosan; biopolymer; genipin; diisocyanate; epichlorohydrin

1. INTRODUCTION

Biopolymers, such as chitin and chitosan, are excellent candidates for a wide variety of applications, especially in the biomedical field, because they are renewable [1–3], biodegradable and biocompatible [4,5]. By forming fibres and fibre mats, the material’s surface-to-volume ratio can be markedly increased. The resulting increase in chemical functionality makes biopolymer fibres and mats highly attractive for food-processing and biomedical applications, specifically in the areas of active food packaging and filtration, tissue engineering and wound healing [6]. Electrospinning is a well-established technique with which fibres and mats can be manufactured in a simple and flexible process. Polymer solutions are placed in a syringe which is connected to a conductive collector and a high voltage source. Fibres and mats are spun from the solutions by applying the appropriate accelerating voltage to overcome the solution’s surface tension. Their properties such as fibre diameter, mat porosity and morphology can be carefully tailored for a given application through the electrospinning parameters such as the applied electric field strength, solution flow rate and tip-to-collector distance [7–9].

Chitin (N-acetyl-D-glucosamine), the second most abundant, naturally occurring polysaccharide, is the structural polymer of exoskeletons of crustaceans (such as crabs and shrimps) as well as squid pens [3,5,10,11]. When compared with chitin, chitosan (CS), the deacetylated form of chitin, is widely used in the food-processing and biomedical fields owing to its increased solubility in aqueous acid solutions.

CS has successfully been electrospun with a number of copolymers such as polyethylene oxide (PEO) [12] and polyvinyl alcohol (PVA) [13] using a variety of solvent systems; it has also been spun solo [14–18]. Neutralized CS fibre mats are non-toxic and biocompatible, and therefore have great potential for its use as filtration membranes and tissue engineering scaffolds. Because in these applications, membranes and scaffolds not only have to be stable in a wet environment, but also resistant to a combination of chemical and mechanical stresses, they need to be stabilized.

Crosslinkers are agents that stabilize polymers through the coupling and bonding of functional groups in the chains, thereby preventing dissolution. In the
form of beads, films and membranes, the crosslinking of CS has been reported with crosslinkers such as glutaraldehyde (GA) [18–21], genipin [22–24], diisocyanates [25,26] and epoxides [27]. Recently, Schiffman and Schauer [19] reported the successful one-step crosslinking with GA of CS fibres electrospun from trifluoroacetic acid (TFA), and their improved chemical stability and mechanical properties.

GA (figure 1e) is a homobifunctional crosslinker, which reacts with CS via either a Schiff base reaction, leading to imine functionality, and/or through Michel-type adducts with terminal aldehydes, leading to the formation of carbonyl groups (figure 1i). GA’s disadvantage is that it is cytotoxic in its unreacted form, a fact that has lead to reservations towards GA-crosslinked CS for biomedical applications and encouraged our search for alternative, more biocompatible crosslinkers.

Genipin (figure 1b) was first isolated from extracts of the *Genipa americana* plant; it is also found in low concentrations (<0.1%) in *Gardenia jasminoides* Ellis fruits. At present, genipin is isolated after hydrolysis of geniposide, from the gardenia plant, but in higher concentrations (3.06–4.12%). The hydrolysis process uses β-glucosidase from *Penicillium nigricans* [28]. Because genipin is a naturally occurring crosslinker with 5000–10 000 times less cytotoxicity than GA [29], it has widely been studied as an alternative crosslinker for CS-based biomaterials. Genipin spontaneously crosslinks CS gels, microspheres, films or cast membranes in 0.5–5.0% acetic acid (AA). Most of the time, however, it is crosslinked in blends with synthetic polymers such as PVA [30], poly (vinylpyrrolidone) (PVP) [31] and PEO [32] and with the biopolymers gelatin [33,34] and silk fibroin [35]. The mechanism of crosslinking is the spontaneous reaction of genipin with the NH₂ group of CS (figure 1a,b,f) [24] or a protein with a reactive amino-group; it forms dark blue or green pigments [22–24].

---

**Figure 1.** A schematic of the select functional groups of: (a) chitosan, reacting with: (b) genipin, (c) HDACS, (d) ECH and (e) GA resulting in the formation of crosslink products: (f) CS–genipin, (g) CS–HDACS, (h1,h2) CS–ECH and (i) CS–GA, respectively.
Another known CS crosslinker is hexamethylene-1,6-diaminocarboxysulphonate (HDACS; figure 1c). HDACS, a water-soluble and stable blocked-diisocyanate, forms urea linkages (figure 1g) after crosslinking with the NH₂ group of CS at basic pH or at elevated temperatures [25,26]. Like genipin and HDACS, the epoxide, epichlorohydrin (ECH) (figure 1d), can crosslink at the NH₂ group, but has also been demonstrated to crosslink with primary OH groups in CS films [36], beads [21,27] and wet-spun fibres [37–39] (figure 1b/1h/2). The mechanism of CS–ECH crosslinking is temperature-dependent [36].

To the best of our knowledge, to date only GA (figure 1e) has been successfully used to crosslink electrospun CS fibres [18,19] (figure 1f). Explored in this study is the use of a novel set of crosslinkers, genipin, HDACS and ECH, for the processing of crosslinked electrospun CS fibres and mats. Their effect on fibre and mat morphology and the chemical stability of as-spun and crosslinked fibre mats are reported here. Their effect on the mechanical performance of the mats is described in part 2 of this publication series [40].

2. MATERIAL AND METHODS

2.1. Reagents

Medium molecular weight CS (75% DD, MW = 190–310 kDa), TFA (99% ReagentPlus), GA (50 wt% in water), ECH, AA (>99.7% ACS Reagent) and sodium hydroxide (NaOH) were used as received from Sigma Aldrich, MO, USA. Genipin was purchased from Wako Pure Chemicals Industry, Ltd., (Japan). HDACS was prepared using the protocol reported by Welsh et al. [25]. All aqueous solutions were prepared with doubly distilled water.

2.2. Preparation of chitosan solutions

CS solutions were prepared with 2.7 wt% CS in 99 per cent TFA, mixing the solutions overnight at room temperature on an Arma-Rotator A-1 (Bethesda, MD, USA). Solutions of 2.7 wt% CS in 1 per cent TFA/water were also prepared for conductivity measurements.

2.3. Crosslinking

The electrospinning and crosslinking procedures were a modification of the work of Schiffman and Schauer [19]. The respective crosslinker was added to the CS/TFA solution and mixed for 2 min immediately before electrospinning. The amount of crosslinkers added was as follows: genipin (0.1 wt%); 5:1 (wt%) CS : HDACS [25,41]; 10:1 (vol%) CS : ECH [21,27,42]. For comparison, as-spun mats were likewise prepared by adding 1 ml of GA to a 2.7% (w/v) CS/TFA solution [19].

The pH and conductivity of the solutions prior to spinning were determined using universal range (pH 0–14) pH indicator sticks (J.T. Baker, Deventer, The Netherlands) and an Oakton CON 5110 conductivity meter (Vernon Hills, IL, USA), respectively. All tests were performed at room temperature (25 ± 4°C) and with three trials for each solution composition.

2.5. Electrospinning of crosslinked chitosan solutions

The CS/crosslinker solution was loaded into a syringe (Becton Dickinson & Co., Franklin Lakes, NJ, USA) to which a 21-gauge Precision Glide needle (Becton Dickinson & Co.) was attached. The syringe was then placed on an advancement pump (Harvard Apparatus, Plymouth Meeting, PA, USA), set at 1.0 ml h⁻¹ flow rate and a distance of 10 cm from the collecting plate, which was a 90 × 90 mm copper plate wrapped with aluminium foil. A high voltage supply (Gamma High Voltage Research Inc., Ormond Beach, FL, USA) was connected to the needle (positive electrode) and the plate. A voltage of 15 kV was applied while advancing the solution at a set flow rate. The set-up was run at 23–25°C and 20–35% RH. All mats were stored at the same conditions for a maximum of one week prior to conducting further tests.

2.6. Heat and base activation

As-spun and crosslinked samples underwent either heat or base activation. The respective temperatures and thermal and base activation times are shown in table 1. Base activation was carried out in a 110 × 80 × 50 mm gas vapour chamber (VWR Scientific Products, Bridgeport, NJ, USA) containing 10 ml 1 M NaOH, which was allowed to vapourize at 23°C. The CS–GA and CS–genipin samples were exposed neither to heat nor to base activation.

2.7. Field emission scanning electron microscopy

Fibre and mat morphologies of the electrospun samples were observed in Zeiss Supra 50VP field emission scanning electron microscopy (FESEM; Carl Zeiss NTS, LLC, North America) after sputter coating the samples with approximately 5 nm-thick platinum/palladium for 30 s and 40 mA using Denton Vacuum Desk II (Denton Vacuum, LLC, Moorestown, NJ, USA). Mean fibre diameters (n = 50) were measured using ImageJ (v. 1.41o, National Institute of Health, USA).

2.8. Fourier transform infrared microscopy

Infrared spectra for CS fibres post-crosslinking were taken using Fourier transform infrared microscopy (FTIR; Varian Excalibur FTS-3000, Varian, Inc., Palo Alto, CA, USA). All spectra were taken using the attenuated total reflectance module in the spectral range of 4000–500 cm⁻¹ by accumulation of 64 scans at 4 cm⁻¹ resolution.

J. R. Soc. Interface
3. RESULTS AND DISCUSSION

3.1. Solution properties, electrospinning and fibre morphology

3.1.1. Solution colour, mat colour and pre-activation fibre morphology

All CS solutions prepared in this study were electrospinnable. Owing to the corrosiveness of the TFA solutions, viscosities were taken using the Ubbelohde glass viscometer. Viscosities did not drastically change the spinning process (3 h) (figure 2m); however, it is important to note that, as expected, the relative viscosity of CS/TFA increased after the addition of genipin and ECH. The addition of HDACS, however, decreased the solution viscosity and also led to the formation of a cloudy white dispersion that phase separates through time, requiring the constant replacement and mixing of a freshly prepared solution into the syringe every hour. Owing to this, CS–HDACS solutions were filtered prior to measurement of viscosity to prevent clogging the capillary tube.

Mat characteristics varied depending on the crosslinker added and/or the activation conditions used. Table 1 shows the observed solution and fibre mat colours. It is important to note that initially, all CS/TFA solutions were clear, light yellow in colour.

Genipin-crosslinked fibre mats were light pink in colour with both unbranched round and flat fibres. This is in contrast to observed dark blue or green colour that is observed when genipin is added to CS/AA in films or beads [24]; the addition of genipin to CS/TFA changed the colour to a darker yellow. As electrospinning progressed, the solution turned brownish red. Yellow and brownish-red intermediate pigments had been observed previously when genipin was added to methylamine [24]. Researchers have noticed that further exposure of the pigments to O2 leads to the formation of a blue pigment [22,23]. We also noticed that the CS–genipin mats turn blue when placed in a NaOH–ethanol solution.

Genipin’s degree of crosslinking with CS is pH-dependent [24]. At a higher pH, a higher degree of self-polymerization is likely to occur than at lower pH. This self-polymerization occurs before genipin reacts with CS, leading to a lower degree of crosslinking. Genipin-crosslinked CS networks at a lower pH consist of primary CS chains and short crosslink bridges of genipin, while higher pH leads to networks with long crosslink bridges of genipin [24]. Because the spinning solution is at pH ~ 2 (figure 2o), the brownish red colour of the solution is probably owing to short crosslink bridges of genipin when crosslinking to the NH2 group on CS.

Liu et al. [46] reported that to fully crosslink CS, 0.025 wt% genipin is sufficient. In this study however,

<table>
<thead>
<tr>
<th>sample</th>
<th>activation</th>
<th>solution colour</th>
<th>mat colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS/TFA</td>
<td>none</td>
<td>clear, light yellow</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>60°C, 24 h</td>
<td>clear, light yellow</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>120°C, 2 h</td>
<td>clear, light yellow</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>NaOH, 24 h</td>
<td>clear, light yellow</td>
<td>white</td>
</tr>
<tr>
<td>CS–GA</td>
<td>none</td>
<td>light to dark yellow</td>
<td>yellow</td>
</tr>
<tr>
<td>CS–genipin</td>
<td>none</td>
<td>yellow to brownish red</td>
<td>light pink</td>
</tr>
<tr>
<td>CS–HDACS</td>
<td>none</td>
<td>light yellow, turbid</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>120°C, 2 h</td>
<td>light yellow, turbid</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>NaOH, 24 h</td>
<td>light yellow, turbid</td>
<td>yellow</td>
</tr>
<tr>
<td>CS–ECH</td>
<td>none</td>
<td>light yellow</td>
<td>white to glossy opaque</td>
</tr>
<tr>
<td></td>
<td>60°C, 24 h</td>
<td>light yellow</td>
<td>opaque</td>
</tr>
<tr>
<td></td>
<td>NaOH, 24 h</td>
<td>light yellow</td>
<td>opaque</td>
</tr>
</tbody>
</table>

2.9. Solubility test

Stability of the fibre mats was tested in acidic, neutral and basic conditions. A modified procedure of the solubility tests described by Samuel et al. [43], Gotoh et al. [44] and Park et al. [45] was performed. Samples (10 × 10 mm) were submerged in separate solutions, each containing 20 ml 1 M AA (pH 3), H2O (pH 7) and 1 M NaOH (pH 13) in a 100 × 15 mm round glass Petri dish (Becton Dickinson & Co.). Moreover, the dissolution of mats was monitored by measuring the transmittance of each solution at 600 nm (T600) using a spectrometer (USB2000 Miniature Fibre Optic Spectrometer, Ocean Optics, Inc., Dunedin, FL, USA). Aliquots (1.5 ml) were taken, transferred to cuvettes and measured at an ambient temperature after 15 min and 72 h. Three trials were conducted for each measurement. The same volume reagent (1.5 ml) was replaced into the solution after every sampling. Each mat condition was also visually observed and noted. For reference values, 1 M AA, H2O and 1 M NaOH were used, and all the compositions were normalized with respect to the corresponding reference solution (i.e. 1 M AA) rather than the reference-crosslinker (i.e. 1 M AA + genipin) solutions.

2.10. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was carried out with a TGA Q50 Thermogravimetric Analyzer (TA Instruments, New Castle, DE, USA). The samples were placed in a humidity chamber at 65 per cent RH for 1 h before testing. Samples of 3 mg from each composition and treatment were individually loaded into a platinum pan and examined to determine the water loss difference between the fibre mats. Runs were carried out under N2 gas and with a ramp rate of 10°C min–1 up to 150°C. The per cent weight loss (% wt. loss) was normalized per milligram (mg) of the mat.

Table 1. Thermal and base activation conditions, and the solution and mat colour of CS samples.
0.10 wt% was chosen for two reasons. First, excess of the crosslinker was added to saturate all the crosslinking sites of CS. Second, at concentrations less than 0.10 wt%, the mats were soluble in acidic and neutral solutions, indicating that the mats were not fully crosslinked. The 0.10 wt% used had shown improvements in stability, as will be briefly described later in this paper.

HDACS crosslinking produced white fibre mats with both round and flat non-uniform diameters. Addition of HDACS made the CS/TFA solution turbid without an observed colour change. Amounts added for both HDACS [25,26] and ECH [21,27,42] were based on previous studies on CS films or hydrogels. Keeping in mind that the CS used is 75 per cent DD, the crosslinker amounts used here were modified to estimate a 1:1 molar ratio of amine : crosslinker group. ECH-crosslinked fibre mats were initially, after 10 min of electrospinning, transparent and glossy, but turned white after 5 h. Fibres were round, highly branched and tree-root-like.

3.1.2. Pre-activation fibre diameter

Figure 2 displays the fibre surface morphologies of all the spun mats. Electrospinning the CS/TFA solution yields a mean fibre diameter of 133 \pm 53 nm. When adding the crosslinker genipin, HDACS and ECH, the mean fibre diameters increased. The CS–genipin, CS–HDACS and CS–ECH mats consisted of fibres with mean fibre diameters of 267 \pm 199 nm, 644 \pm 359 nm and 896 \pm 435 nm, respectively. For comparison, CS–GA crosslinked mats were also spun. The yellow-coloured CS–GA mats have a mean fibre diameter of 112 \pm 33 nm, which is lower than that of the other three tested crosslinkers. This value is lower but within the standard deviation of the earlier reported value of 128 \pm 40 nm [18,19].

The increase in fibre diameters upon addition of the crosslinker to the CS solution is probably owing to several factors. In the case of CS–genipin, the fibre increase was very minimal, indicated by minimal change in...
viscosity (figure 2m) or conductivity (figure 2p) of the solution. However, it is suggested that fibre diameter increase is probably owing to the slow formation of short crosslinks within the polymer chains at low pH (figure 2o), preventing thinning of the strand. In ECH, since the crosslinker does not ionize in solution (figure 2o,p) pH and conductivity changes were not substantial. However, the marked increase in diameters might be owing to initial crosslinker modification of the CS backbone (i.e. a bifunctional crosslinking binding to only one CS functional group instead of two; figure 1) and/or formation of stronger polymer chain with crosslinker bonds (i.e. increased formation of covalent bonds that increase polymer chain rigidity), supported by the increased relative viscosities of the solution prior to spinning (figure 2n), all of which prevent thinning of the strand during fibre formation. CS–HDACS exhibited an opposite behaviour to ECH, but it is important to remember that unlike the solutions used for viscometry tests, the spinning solution was constantly replenished, used unfiltred and ionized in solution, leading to increase in conductivity (figure 2p).

Moreover, the strong acid and solvent TFA (pH 1–2, figure 2o) used in this study protonates the amine groups (pK\textsubscript{a} 6.3), leading to a highly charged backbone (i.e. the uncharged amines become charged), which increases chain repulsion and/or the swelling capability of the fibres [47]. Owing to the differences in the chemistries of the added crosslinkers, the presence and swelling effect of residual TFA in the mat itself maybe expected to vary from one crosslinker type to another. This is supported by TGA investigations (figure 3a) in which smaller fibre diameters correlates to higher weight loss owing to surface adsorbed water.

### 3.1.3. Post-activation fibre morphology and diameter

Full crosslinking of CS with HDACS [25,26] or ECH [21,27,36–39] requires either heat (120°C and 60°C, respectively) or base activation. Figure 2 shows scanning electron microscope (SEM) micrographs of the post-activation mats.

For CS–HDACS-120°C, mean fibre diameters were 285 ± 139 nm, a 55 per cent decrease from the as spun CS–HDACS. Like heat activation, exposure of CS–HDACS-base to 1 M NaOH for 24 h showed a decrease in mean fibre diameter to 339 ± 179 nm, a 47 per cent decrease. In both activation conditions, no changes in mat colour and shape were observed. A decrease in mean fibre diameters was also observed for CS–ECH-60°C (870 ± 490 nm), a 3 per cent decrease, and CS–ECH-base (394 ± 263 nm), a 56 per cent decrease. In both cases, the mats changed from glossy/white to glossy/rough/white in texture.

The decrease in fibre diameters may be attributed to the loss of water from the fibre mats brought about by the increased temperature and/or the full crosslinking at higher pH. This is also supported by the per cent weight loss (figure 3a) as determined by TGA, which indicate that mats with lower fibre diameters had exhibited higher water loss, which was also expected because of the surface-adsorbed water. It must be noted that all the spun mats have been conditioned at 65 per cent RH prior to running TGA to ensure that the effects are only owing to the crosslinker and/or activation conditions. The minimal decrease in fibre diameter for CS–ECH-60°C might be owing to the incomplete evaporation of water as a result of lower activation temperature or because ECH crosslinking is more pH- [38] than temperature-mediated.

The comparison of the mean fibre diameters of the as-spun with the pre- and post-activated crosslinked CS mats (figure 2) revealed that in the case of the as-spun mat (CS), both the heat and the base treatments (CS–60°C, CS–120°C and CS–base) resulted in an increase in mean fibre diameters. Interestingly, the opposite, a decrease in fibre diameter, was observed in the case of the pre-activation samples (CS–HDACS and CS–ECH) after crosslinker activation with heat or base (CS–HDACS-120°C, CS–HDACS-base, CS–ECH-60°C and CS–ECH-base) owing to chemical crosslinking.

### 3.2. Fourier transform infrared microscopy

FTIR spectra (figure 3) of as-spun and crosslinked fibre mats were taken to determine their respective chemical interactions, especially any expected covalent crosslinking. Characteristic CS peaks (figure 3) are from amide I (1673 cm\textsuperscript{-1}), amide II (1532 cm\textsuperscript{-1}), C–N stretch (1431 cm\textsuperscript{-1}), bridge ether oxygen (1202 cm\textsuperscript{-1}) and alcohol C–O (1085 cm\textsuperscript{-1}) [18,19].

With the addition of crosslinkers, changes in CS IR peaks were observed. CS–genipin (figure 3) exhibited amide I peak broadening, which can be attributed to NH\textsubscript{2} group deformation [24]. The reaction mechanism of crosslinking of genipin with CS is chemically complex and pH-dependent [22–24]. Under acidic and neutral pH, genipin crosslinking involves the attack of the NH\textsubscript{2} of CS, resulting in a possible formation of bifunctional linkages to the crosslinker [24,48] (figure 1f).

HDACS crosslinks CS at the NH\textsubscript{2} group of the polymer, creating a urea linkage [25] (figure 1g). When fully crosslinked, this appears as a strong peak at around 1650 cm\textsuperscript{-1} [25]. In this study, a medium peak at around 1652 cm\textsuperscript{-1} (figure 3) was evident for the CS–HDACS fibres even before heat (figure 3) or base exposure (figure 3), indicating partial covalent crosslinking. The same peak increased in height after heat (figure 3) or base exposure (figure 3), indicating increased crosslinking.

ECH is another known CS crosslinker, which is favoured owing to its ability to couple the polymer at the OH group, leaving more chemical functionality to the polymer owing to the available NH\textsubscript{2} groups. However, crosslinking is not limited to the OH groups (figure 1h1), but also occurs at the NH\textsubscript{2} groups (figure 1h2) depending on the temperature [36]. At less than 40°C, ECH crosslinks CS at the NH\textsubscript{2} groups but at more than 40°C, both the OH and the NH\textsubscript{2} groups participate (figure 1h1,h2), forming a denser crosslinked network [36].

The FTIR spectrum (figure 3) of CS–ECH mats show amine deformation at 1638 cm\textsuperscript{-1} and an increase in C–N stretch peak (1431 cm\textsuperscript{-1}) suggesting crosslinking occurs at the NH\textsubscript{2} groups of CS. These peaks were also taller and broader after heat or base exposure indicating an increase in amine group deformation, which might be used in crosslinking. Moreover, the peak at 1085 cm\textsuperscript{-1} (figure 3) that corresponds to the C–O stretch was observed to increase and broaden. This is an indication that the OH groups are
crosslinking at elevated temperatures, even under basic conditions (figure 3).

3.3. Solubility test

Crosslinking improves the dissolution of CS films, beads and fibre mats [18,19] under a wide pH range. Figure 4 and table 2 summarizes the results of the dissolution test. As dissolution criteria, transmittances at 90 [43] and 50 [45] were taken as cutoffs (figure 4). If the transmittance \( T_{90} \) of the aliquot solutions from the immersed mats is greater than 50, the mats were considered partially dissolved. Further, if the \( T_{90} \) is greater than 90 and the mat is visually present in the

![Figure 3](http://rsif.royalsocietypublishing.org/)
solution, it is considered not dissolved; otherwise, it is fully dissolved (figure 4 and table 2).

Knowing that CS (figure 5d) does not dissolve under 1 M NaOH owing to the neutralization of the amine groups of CS, we are interested in knowing which crosslinker and/or activation conditions are able to keep the fibrous structures of the mats after immersion in solutions with the lowest pH at a longer time. Figure 5 displays the SEM micrographs of these mats. At neutral to acidic pH, CS is soluble owing to the protonation of amines (pKa 6.3), unless the polymer is crosslinked or the functional groups are modified. Hence, improved mats are those that are stable at lower pHs at a longer time. Here, like as-spun CS, CS-60°C (figure 5h) is stable only at 1 M NaOH. Heating of CS (CS-120°C, figure 5k) mats partially improved stability up to 15 min at pH 3, which is attributed to the modification of the amine groups by the formation of an amide between the ammonium salt and the trifluoroacetate at temperatures greater than 100°C [49]. CS-base (figure 5j) is only partially stable for 15 min at pH 3.

Improved stability of the mats was observed upon the addition of the crosslinkers. Addition of genipin rendered the mats partially insoluble at pH 3 even after 72 h (figure 5g), supporting the suggestion that genipin formed partial crosslinks during spinning. Interestingly and as expected, the addition of HDACS rendered the

Figure 4. Transmittance ($T_{600}$) of aliquot solutions from dissolution testing of CS-based mats in 1 M AA, H$_2$O and 1 M NaOH for 15 min (unfilled bars) and 72 h (blue bars). Solid horizontal bars indicate $T_{600} = 90$, while broken line mark $T_{600} = 50$. (Online version in colour.)

J. R. Soc. Interface
mats fully insoluble after 72 h (figure 5a–c). This is comparable to CS–GA (figure 5f).

In the case of ECH addition, the improved mats were partially stable at neutral pH for a short time (figure 5l). Although chemical interactions indicating possible participation of hydroxyl groups at elevated temperatures for the CS–ECH-60°C (figures 3c and 5i) were observed, base activation resulted in a more stable mat (CS–ECH-base, figure 5e) that did not dissolve even after 72 h at pH 3.

Fibre morphologies of the surviving mats were similar to the pre-dissolution test samples. Moreover, the fibre diameters of the mats (figure 5) after the dissolution tests are still within the standard deviation of the initial mats.

Overall, the addition of crosslinker and/or post-activation steps resulted in mats with better dissolution, retained fibrous morphologies and mean fibre diameters in comparison to their corresponding controls (as-spun CS, CS-60°C, CS-120°C or CS-base; figure 5).

4. CONCLUSION

Novel sets of crosslinkers for electrospun CS fibre mats were investigated using the three crosslinkers genipin, HDACS and ECH. Both the type of crosslinker and post-spinning activation through exposure to temperatures of 60°C and 120°C or a base affected the fibre diameters and mat morphology. FTIR spectra revealed

Table 2. Transmittance ($T_{600}$) of aliquot solutions from dissolution testing of CS-based mats in 1 M AA, H$_2$O and 1 M NaOH for 15 min and 72 h with the visual observations of mat solubility. (Visual observations of mat solubility indicate N and Y for not visible and visible, respectively.)

<table>
<thead>
<tr>
<th>mats</th>
<th>15 min visual observation</th>
<th>$T_{600}$</th>
<th>comments</th>
<th>72 h visual observation</th>
<th>$T_{600}$</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M AA pH 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>N</td>
<td>95</td>
<td>fully dissolved</td>
<td>N</td>
<td>113</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS-60°C</td>
<td>N</td>
<td>84</td>
<td>fully dissolved</td>
<td>N</td>
<td>111</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS-120°C</td>
<td>Y</td>
<td>86</td>
<td>partially dissolved</td>
<td>N</td>
<td>101</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS-base</td>
<td>Y</td>
<td>83</td>
<td>partially dissolved</td>
<td>N</td>
<td>93</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS–genipin</td>
<td>Y</td>
<td>80</td>
<td>partially dissolved</td>
<td>Y</td>
<td>86</td>
<td>partially dissolved</td>
</tr>
<tr>
<td>CS–HDACS</td>
<td>Y</td>
<td>94</td>
<td>not dissolved</td>
<td>Y</td>
<td>105</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–HDACS-120°C</td>
<td>Y</td>
<td>93</td>
<td>not dissolved</td>
<td>Y</td>
<td>104</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–HDACS-base</td>
<td>Y</td>
<td>105</td>
<td>not dissolved</td>
<td>Y</td>
<td>114</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–ECH</td>
<td>N</td>
<td>86</td>
<td>fully dissolved</td>
<td>N</td>
<td>94</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS–ECH-60°C</td>
<td>Y</td>
<td>56</td>
<td>partially dissolved</td>
<td>N</td>
<td>88</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS–ECH-base</td>
<td>Y</td>
<td>88</td>
<td>partially dissolved</td>
<td>Y</td>
<td>105</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–GA</td>
<td>Y</td>
<td>87</td>
<td>partially dissolved</td>
<td>Y</td>
<td>103</td>
<td>not dissolved</td>
</tr>
<tr>
<td>1 M AA (reference)</td>
<td>—</td>
<td>98</td>
<td></td>
<td>—</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>H$_2$O pH 6-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>N</td>
<td>108</td>
<td>fully dissolved</td>
<td>N</td>
<td>79</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS-60°C</td>
<td>N</td>
<td>108</td>
<td>fully dissolved</td>
<td>N</td>
<td>91</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS-120°C</td>
<td>Y</td>
<td>100</td>
<td>not dissolved</td>
<td>Y</td>
<td>80</td>
<td>partially dissolved</td>
</tr>
<tr>
<td>CS-base</td>
<td>N</td>
<td>101</td>
<td>fully dissolved</td>
<td>Y</td>
<td>103</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS–genipin</td>
<td>Y</td>
<td>95</td>
<td>not dissolved</td>
<td>Y</td>
<td>86</td>
<td>partially dissolved</td>
</tr>
<tr>
<td>CS–HDACS</td>
<td>Y</td>
<td>93</td>
<td>not dissolved</td>
<td>Y</td>
<td>92</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–HDACS-120°C</td>
<td>Y</td>
<td>110</td>
<td>not dissolved</td>
<td>Y</td>
<td>91</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–HDACS-base</td>
<td>Y</td>
<td>95</td>
<td>not dissolved</td>
<td>Y</td>
<td>91</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–ECH</td>
<td>Y</td>
<td>69</td>
<td>partially dissolved</td>
<td>N</td>
<td>60</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS–ECH-60°C</td>
<td>Y</td>
<td>86</td>
<td>partially dissolved</td>
<td>Y</td>
<td>60</td>
<td>fully dissolved</td>
</tr>
<tr>
<td>CS–ECH-base</td>
<td>Y</td>
<td>99</td>
<td>not dissolved</td>
<td>Y</td>
<td>81</td>
<td>partially dissolved</td>
</tr>
<tr>
<td>CS–GA</td>
<td>Y</td>
<td>103</td>
<td>not dissolved</td>
<td>Y</td>
<td>90</td>
<td>not dissolved</td>
</tr>
<tr>
<td>H$_2$O (reference)</td>
<td>—</td>
<td>98</td>
<td></td>
<td>—</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>1 M NaOH pH 13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>Y</td>
<td>108</td>
<td>not dissolved</td>
<td>Y</td>
<td>123</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS-60°C</td>
<td>Y</td>
<td>108</td>
<td>not dissolved</td>
<td>Y</td>
<td>123</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS-120°C</td>
<td>Y</td>
<td>112</td>
<td>not dissolved</td>
<td>Y</td>
<td>120</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS-base</td>
<td>Y</td>
<td>112</td>
<td>not dissolved</td>
<td>Y</td>
<td>127</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–genipin</td>
<td>Y</td>
<td>113</td>
<td>not dissolved</td>
<td>Y</td>
<td>110</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–HDACS</td>
<td>Y</td>
<td>120</td>
<td>not dissolved</td>
<td>Y</td>
<td>133</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–HDACS-120°C</td>
<td>Y</td>
<td>121</td>
<td>not dissolved</td>
<td>Y</td>
<td>120</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–HDACS-base</td>
<td>Y</td>
<td>118</td>
<td>not dissolved</td>
<td>Y</td>
<td>114</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–ECH</td>
<td>Y</td>
<td>118</td>
<td>not dissolved</td>
<td>Y</td>
<td>133</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–ECH-60°C</td>
<td>Y</td>
<td>120</td>
<td>not dissolved</td>
<td>Y</td>
<td>111</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–ECH-base</td>
<td>Y</td>
<td>122</td>
<td>not dissolved</td>
<td>Y</td>
<td>116</td>
<td>not dissolved</td>
</tr>
<tr>
<td>CS–GA</td>
<td>Y</td>
<td>101</td>
<td>not dissolved</td>
<td>Y</td>
<td>121</td>
<td>not dissolved</td>
</tr>
<tr>
<td>1 M NaOH (reference)</td>
<td>—</td>
<td>99</td>
<td></td>
<td>—</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


12 Desai, K., Kit, K., Li, J. & Zivanovic, S. 2008 Morphological and surface properties of electrospun chitosan
cross-linking of chitosan and characterization of
two-step electrospun chitosan nanofibers.
Biomacromolecules 9, 2947–2953. (doi:10.1021
/bm7006983)

21 Chiono, V., Pulieri, E., Vozzi, G., Ciardelli, G., Aghiwalia,
L. & Giusti, P. 2008 Genipin-crosslinked chitosan/gelatin
blends for biomedical applications. J. Mater. Sci.
/10577130802126406)

22 Yu, C. M. 1987 The cross-linking of chitosan fibers.
1016/S1359-0497(02)00636-7)

2003 Chitosan cross-linking with genipin. J. Appl.
/app.28107)

24 Wei, Y. C., Hudson, S. M., Mayer, J. M. & Kaplan, D. L.
/pola.1992.08301013)

2004 In situ cross-linking of alternating polyelectrolyte
/la035798p)

26 Vondran, J. L., Sun, W. & Schauer, C. L. 2008 Crosslinked,
biodegradable drug-delivery vehicle: studies on the
cross-linked chitosan microspheres as a long-acting biode-
gradable matrix.

27 Sangsanoh, P. & Supaphol, P. 2006 Stability improvement
of chemical cross-linked chitosan beads.
Chemosphere 65, 53–60. (doi:10.1016
/j.chemosphere.2006.04.057)


