Carbohydrate Polymers 108 (2014) 91–98

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Genipin-crosslinked chitosan/poly-L-lysine gels promote fibroblast adhesion and proliferation

Mina Mekhail[a,1], Kaushar Jahan[b,2], Maryam Tabrizian[a,b,*]

[a] Department of Biomedical Engineering, McGill University, Montreal, Quebec, Canada
[b] Faculty of Dentistry, McGill University, Montreal, Quebec, Canada

ARTICLE INFO

*Article history:*
Received 6 February 2014
Received in revised form 12 March 2014
Accepted 13 March 2014
Available online 21 March 2014

*Keywords:*
Chitosan
Poly-L-lysine
Genipin crosslinking
Regenerative medicine
2D gels

ABSTRACT

Chitosan blends have been widely investigated to create biomaterials with desirable physicochemical and biological properties for tissue engineering applications. A recurring difficulty, however, has been to maintain their stability in an aqueous environment. The rationale behind this study was to demonstrate that genipin crosslinking can improve and maintain the stability of chitosan/poly-L-lysine (PLL) blends. Four gel formulations were prepared by varying the weight ratios of chitosan and PLL. Electron microscopy revealed that genipin crosslinking provided a more homogenous gel surface compared to uncrosslinked gels. Moreover, it was discovered that 3 h was sufficient to stabilize the gels. In vitro studies using fibroblasts demonstrated that genipin-crosslinked gels enhanced fibroblasts' attachment as compared to uncrosslinked gels. Moreover, cell viability was significantly improved by 1.6 times on 60:40 gels, and 6.5 times on 50:50 gels after crosslinking. Finally, proliferation was enhanced up to 5 times on 60:40 gels.



## 1. Introduction

In the past two decades, there has been increased interest in developing new biomaterial compositions for various regenerative medicine applications (Stevens & George, 2005). Among these, naturally derived biomaterials continue to play a major role in advancing the field of soft tissue engineering due to their biocompatibility, resemblance to natural extracellular matrices and wide availability (Malafaya, Silva, & Reis, 2007). Chitosan, a cationic polysaccharide has been used extensively in regenerating various tissues, such as: cartilage (da Silva et al., 2010; Feijen et al., 2009; Marra, Tan, Chu, & Payne, 2009; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012), bone (Hasirci, Yilgor, Tuzlakoglu, Reis, & Hasirci, 2009; Kim, Venkatesan, Qian, Ryu, & Kumar, 2011; Misra & Thein-Han, 2009), skin (Muzzarelli, 2009a; Sethuraman, Dhandayuthapani, & Krishnan, 2010; Yao et al., 2009; Yu, Chen, Huang, Liu, & Gu, 2011), peripheral nerves (Gu et al., 2010), the spinal cord (Cho, Shi, & Borgens, 2010; Shoichet, Kim, & Tator, 2011) and vascular tissue (Fan et al., 2009). Early optimization of chitosan involved the manipulation of its molecular weight and its degree of deacetylation (Matthew & Madihally, 1999). This provided some promising results; however, there was still a need to enhance cell compatibility, improve mechanical properties and control biodegradability of chitosan constructs. This prompted the investigation of blending chitosan with natural polymers such as collagen and alginate (Gao et al., 2003; Zhang, Li, Ramay, Hauch, & Xiao, 2005) or synthetic polymers such as poly(vinyl alcohol) and poly(caprolactone) (Qi, Yu, Zhu, Zhou, & Wu, 2013; Zhu & Chan-Park, 2005) to harness the desirable physiochemical and biological properties of these polymers. This was evident from a recent study which demonstrated that a chitosan/polypyrrole–alginate blend enhanced cell attachment, distribution and overall biocompatibility after blending (Sajesh, Jayakumar, Nair, & Chennazhi, 2013). In another report, blending chitosan and gelatin with the addition of silicon oxide enhanced osteoblasts' attachment, proliferation and differentiation (Kavya, Jayakumar, Nair, & Chennazhi, 2013).

However, to stabilize chitosan blends and enhance their mechanical, biodegradability and cellular adhesion properties, crosslinking using chemical or physical methods (e.g. UV, ionic crosslinking) (Airoldi & Monteiro, 1999; Gorgieva & Kokol, 2012; Song, Jin, & Hourston, 2004; Sun, Li, Li, Wei, & Tian,

* Corresponding author at: Department of Biomedical Engineering, 3775 University Street – Duff Medical Building, Room 313, McGill University, Montreal, Quebec H3A 2B4, Canada. Tel.: +1 514 398 8129; fax: +1 514 398 7461.
*E-mail addresses:* mina.mekhail@mail.mcgill.ca (M. Mekhail), kaushar.jahan@mail.mcgill.ca (K. Jahan), maryam.tabrizian@mcgill.ca (M. Tabrizian).
[1] Present address: Department of Biomedical Engineering, 740 Penfield – Genome Building, Room 4300 McGill University, Montreal, Quebec H3A 1A4, Canada.
[2] Present address: Faculty of Dentistry, 740 Penfield – Genome Building, Room 4300 McGill University, Montreal, Quebec H3A 1A4, Canada.

http://dx.doi.org/10.1016/j.carbpol.2014.03.021
0144-8617/

2011; Tsai, Chen, Liu, & Lai, 2011) is commonly employed. For instance, chemical crosslinkers such as glutaraldehyde, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and genipin have been used to stabilize chitosan (Butler, Ng, & Pudney, 2003; Dal Pozzo et al., 2000; Kawase et al., 1997; Sano et al., 1999). Genipin, a natural molecule, has been emerging as a favorable crosslinking agent due to its low cytotoxicity compared to widely used glutaraldehyde (Sung, Huang, Huang, & Tsai, 1999), and its ability to self-polymerize, which could be exploited to produce biomaterial constructs with a wide range of mechanical properties, porosities and degrees of swelling (Butler et al., 2003; Mi, Shyu, & Peng, 2005; Shyu, Mi, & Sung, 2000). Genipin is derived from geniposide, found in the fruit of *Gardenia jasminoides Ellis*, and crosslinks primary amine groups (Butler et al., 2003). It has been extensively investigated in crosslinking both 2D gels and 3D scaffolds fabricated using amine-containing polymers such as chitosan, collagen and gelatin (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002; Mekhail et al., 2010; Moura, Figueiredo, & Gil, 2007; Muzzarelli, 2009b; Silva et al., 2008; Song et al., 2004).

One of the promising approaches for enhancing the cellular response of chitosan is to blend it with the highly cationic polymer Poly-L-lysine (PLL) in order to improve cell attachment and proliferation. PLL is a homopolymer of the essential amino acid L-lysine and has been used as a standard tissue culture coating to promote cellular adhesion through electrostatic interactions. Films formed by blending PLL and chitosan have been previously fabricated to enhance cellular attachment to chitosan (Cheng et al., 2004; Zheng, Wei et al., 2009; Zheng, Zhang et al., 2009). However, no crosslinking was employed to stabilize the chitosan/PLL blends and none of these investigations reports on how the repulsive forces of two positively charged polymers might lead to leaching out of PLL.

The aim of this study was to assess the chitosan/PLL blends stability and investigate whether or not PLL leaches out from of chitosan/PLL in an aqueous environment if no crosslinking was applied. The presence of highly cationic PLL in solution disrupts cellular membranes and has adverse affects on cellular viability. Therefore, genipin crosslinking was applied to the chitosan/PLL blends to prepare a 2 D gel that prevents PLL dissolution, thereby to provide a more adequate 2D culture environment for cell growth and proliferation.

## 2. Materials and methods

### 2.1. Materials

High Molecular Weight Chitosan (Degree of Deacetylation > 90%; 3000 cp viscosity) was purchased from MP Biomedicals LLC (Cat. No.150597); PLL (MW 20–30 kDa) from Sigma–Aldrich (Cat. No. P2636); Genipin from Wako Pure Chemical Industries Ltd. (Cat. No. 078-03021); CellCrown™ inserts from Scaffdex (Cat. No. C00001S); Live/dead staining kit from EMD chemicals (Cat. No. QIA76); Anti-Ki67 antibody from Abcam (Cat. No. ab15580); DMEM (Cat. No. 30-2002), and 3T3 fibroblasts (Cat. No. CRL-1658) from ATCC; finally, Calf Bovine Serum (Cat. No. 16030074) and PenStrep (Cat. No. 15140-122) from Invitrogen.

### 2.2. Chitosan/PLL 2D gel fabrication

Four solutions of 1 M acetic acid (total volume 40 ml) were prepared. Four different chitosan: PLL weight ratios were investigated; 100% chitosan (40 mg), 80:20 (32 mg of chitosan and 8 mg of PLL), 60:40 and 50:50. Chitosan was first dissolved in acetic acid solutions followed by dissolving PLL. Solutions were poured into glass Petri dishes (Ø = 9 cm) and left to dry in a fume hood for 48 h. The 2D gels were neutralized by adding 1 M NaOH (30 ml/Petri dish) for 24 h followed by thorough washing in anhydrous ethanol until the pH of gels was 7–7.4. PLL is insoluble in anhydrous ethanol and therefore it was used instead of water to wash the gels. Samples were stored in anhydrous ethanol until crosslinking.

### 2.3. Stability of uncrosslinked chitosan/PLL gels in an aqueous environment

Uncrosslinked chitosan/PLL gels were placed in PBS for 2 h. Samples were then removed and 25 mg of genipin was added to the solutions to see if any PLL leached out into PBS. Genipin changes color upon reaction with primary amine groups, and if any PLL was in the storage solution then a color change would take place.

### 2.4. Genipin-crosslinking

Four solutions of anhydrous ethanol containing 10% PBS (total volume of 20 ml) were prepared and 0.005 M genipin (25 mg) was dissolved in each solution. The genipin concentration used was always in excess, and the degree of crosslinking was controlled by increasing the time of crosslinking. The addition of 10% PBS was necessary to accelerate the crosslinking reaction (Mekhail et al., 2010). Chitosan/PLL gels were immersed in genipin solutions at 37 °C incubator for 3, 6, 24 and 48 h.

### 2.5. Physicochemical characterization

#### 2.5.1. Fourier Transform Infrared (FTIR) Spectroscopy

Infrared measurements were performed using a Perkin Elmer FTIR spectrometer with an ATR attachment (Pike Technologies). The spectra were collected in absorption mode, using 64 scans, and a resolution of 4 $cm^{-1}$.

#### 2.5.2. Scanning Electron Microscopy (SEM)

All gels were dried using critical point drying. Samples were initially dehydrated to ethanol followed by amyl acetate prior to critical point drying. A Leica EM CPD030 critical point dryer was then used to dry the samples. Post-drying, gels were coated with Gold/Palladium and observed using a Hitachi S-4700 FE-SEM at 2 KeV and a current of 10 μA.

#### 2.5.3. Percent swelling

Gels were dried overnight in a 60 °C oven and dry weights were recorded ($W_{dry}$). Gels were then placed in PBS overnight at 37 °C followed by thorough washing in distilled water. The surface water was removed by tapping the gels once on a Kim wipe followed by measurement of the wet weight ($W_{wet}$). Eq. (1) was used to calculate the percent swelling.

$$\frac{W_{wet} - W_{dry}}{W_{dry}} \times 100 \quad (1)$$

#### 2.5.4. Degree of crosslinking

Gels were dried overnight in a 60 °C oven and the dry weights were recorded ($W_{dry}$). Each gel was placed in a vial containing 2 ml distilled water and 1 ml 2% ninhydrin solution. Vials were placed in an 80 °C water bath for 15 min. The vials were then left to cool down for 10 min at room temperature. The purple supernatant produced was diluted 10 times. A volume of 100 μl from each vial was placed in a 96 well-plate and the absorbance was read at 570 nm. A standard curve was prepared using L-lysine at the concentration range 0.13–0.008125 mg/ml. Ninhydrin assay solution (0.5 ml) was added to 1 ml of each concentration. The absorbance measurements from each gel composition were converted to concentrations using

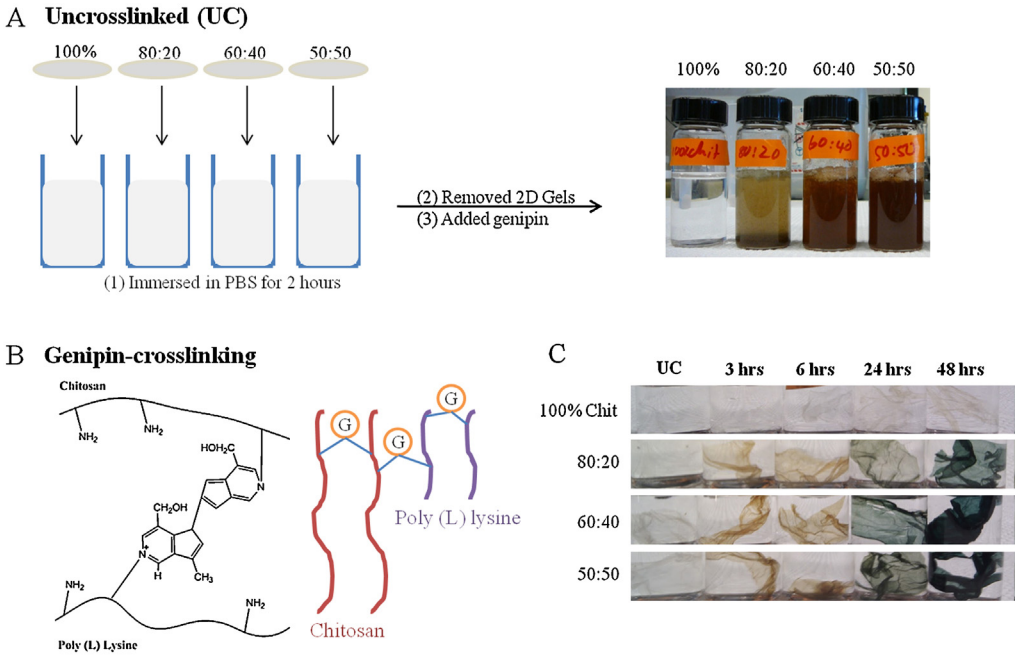


**Fig. 1.** (A) Schematic presentation of the methodology used to verify the stability of uncrosslinked (UC) chitosan/PLL gels in an aqueous environment and the results demonstrating PLL leaching out of chitosan/PLL gels. (B) A schematic representing genipin crosslinking between chitosan and PLL and an illustration of the intra-chain and inter-chain genipin crosslinking. (C) The change in color associated with genipin crosslinking.

the standard curve ($C_{free}$). The degree of crosslinking was calculated using Eq. (2).

$$1 - \left( \frac{C_{free} \times 10 \times 3}{W_{dry}} \right) \quad (2)$$

### 2.6. Cell seeding, staining and imaging

For all cell studies, only uncrosslinked and 3-h crosslinked 100, 80:20, 60:40 and 50:50 gels were used. Gels were washed thoroughly in culture media and Cell Crown™ inserts were used to fix the gels at the bottom of the wells prior to cell seeding.

#### 2.6.1. Immunolabeling and confocal imaging of 3T3 fibroblasts

DMEM supplemented with 10% Calf Bovine Serum (CBS) and 1% Pen-Strep was used to culture the fibroblasts. Fibroblasts were first expanded, and a concentration of $5 \times 10^4$ was seeded on the gels for 48 h. For confocal microscopy, the samples were washed twice in PBS and then fixed using paraformaldehyde for 30 min. Hoechst and Texas Red®-phalloidin were used to stain the nuclei and actin filaments, respectively. The gels were then mounted on microscope slides for confocal imaging.

#### 2.6.2. Live/dead assay

Fibroblasts were cultured on the gels and controls for 24 h. The gels were then washed thoroughly and 300 μl of live/dead assay was added to 9 samples at a time. The samples were incubated in the staining solution for 15 min followed by immediate fluorescence imaging. Doing the assay for 9 samples at a time was essential to avoid long cellular exposure to the staining solution and avoid unwarranted cell death. ImageJ was used to count the number of dead and live cells and viability was calculated using Eq. (3).

$$\frac{\text{Live cells}}{\text{Total \# of cells}} \quad (3)$$

#### 2.6.3. Ki67 staining

Fibroblasts were cultured on the different chitosan gels for 72 h. The gels were then washed thoroughly with PBS and fixed in Parafolmaldehyde for 30 min. The gels were washed three times and incubated in PBS + 0.3% tritonX 100 for 5 min to permeabilize the cells. Cells were washed twice with PBST, and incubated in a 3% BSA PBST solution for 5 min for blocking. After another washing step with PBST followed by PBS, anti-Ki67 antibodies (1/500 dilution in PBS) was added to the cells and left overnight at room temperature. Fluorescently tagged secondary antibodies (GαR-Dylight 650) were diluted 100 times and added to each well for 60 min in the dark. The cells were washed, nuclei were stained with Hoescht and the gels were mounted on microscope slides for fluorescence microscopy. ImageJ was used to count the number of nuclei that expressed Ki67.

#### 2.6.4. Statistical analysis

All statistical analysis was performed using OriginPro 8.6. One-way ANOVA Tukey test was used to compare statistical difference between groups with a 95% confidence. All experiments were done in triplicates ($n = 3$).

## 3. Results and discussion

In this study we report on the fabrication and characterization of genipin-crosslinked chitosan/PLL 2D gels for potential use in regenerative medicine applications, particularly for soft tissue engineering. Even though there were previous reports on the fabrication of chitosan/PLL blends, there were no reports that employed genipin crosslinking for stabilization of these compositions in an aqueous environment. Crosslinking was deemed essential, especially in chitosan/PLL blends since both polymers are cationic in nature and therefore, will repel each other in an aqueous environment.

### 3.1. Effect of crosslinking on stability of 2D Chitosan/PLL gels in an aqueous environment

PLL leached out of uncrosslinked chitosan/PLL gels after a 2 h immersion in PBS as demonstrated in Fig. 1A. Genipin added to PBS after removing the gels caused a change in color that increased in

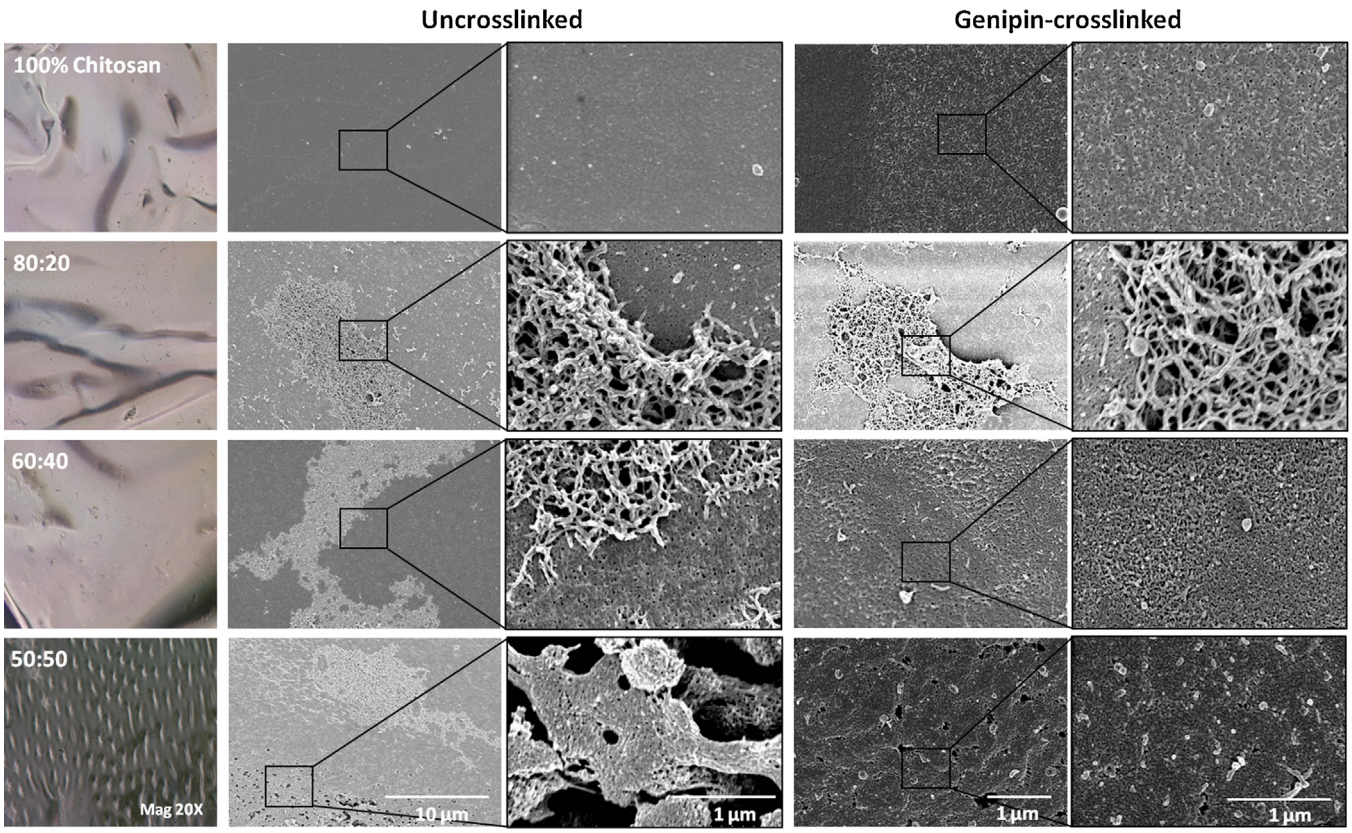


**Fig. 2.** Light microscopy images (first column) of the 2D gels showing the 'polka dots' pattern observed on the 50:50 chitosan/PLL blend. Scanning Electron Microscopy (SEM) images of uncrosslinked gels demonstrate the formation of PLL fibrous patches on a homogenous layer of chitosan. Moreover, cracks in the gels that are responsible for the 'polka dots' pattern is shown in the 50:50 gels. SEM images of genipin-crosslinked gels (24 h) show a more homogenous surface in 60:40 and 50:50 compositions.

intensity with increasing PLL content. In fact, 100% chitosan did not undergo any color change (Fig. 1A), while 50:50 had the darkest brown color formation. It was thus evident that crosslinking is needed to stabilize the chitosan/PLL gels.

Genipin crosslinks amine groups available in both chitosan and PLL. Therefore, it can be deduced that the genipin-crosslinking produced intra- and intermolecular crosslinks within the chitosan and PLL chains to produce stable 2D gels (Fig. 1B). All gels containing

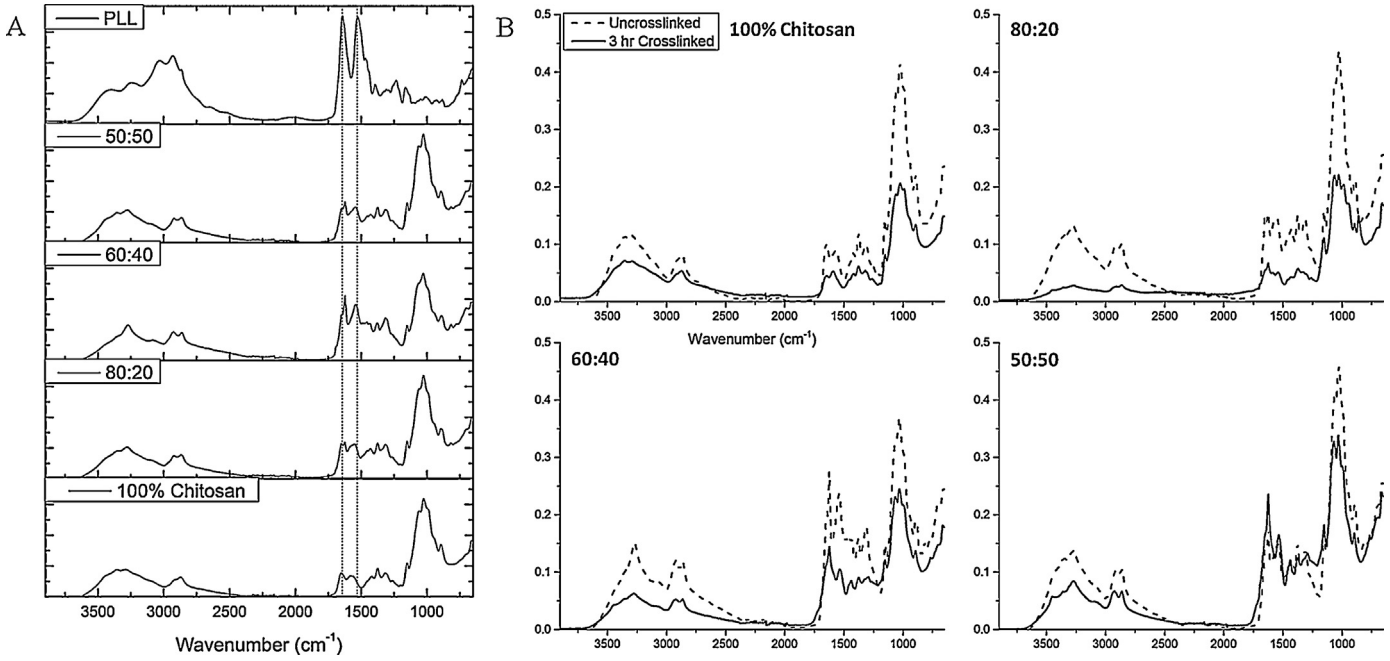


**Fig. 3.** (A) FTIR spectra of uncrosslinked gels with different compositions to illustrate the successful incorporation of PLL. This can be observed by the increase in the amide I and II peaks (dotted lines); (B) the difference in FTIR spectra for each 2D gel before and after 3 h crosslinking.

PLL demonstrated a gradual color change from light brown to dark green as the crosslinking time increased. Pure chitosan gels, on the other hand, had a very slight color change after 48 h of crosslinking (Fig. 1C). The color change that took place after crosslinking is typical of genipin crosslinking, and even though there were different colors based on PLL content, all gels turned blue when immersed in PBS, which is in agreement with previous literature (Mekhail et al., 2010; Song et al., 2004).

### 3.2. Physicochemical characterization

Light microscopy images of the 2D gels demonstrated plain surfaces with the exception of the 50:50 gels that consistently demonstrated a 'polka dot' pattern formation (Fig. 2). SEM images of gels containing PLL showed patches of fibrous PLL on the surface of a homogenous chitosan layer. Moreover, 50:50 gels had cracks on the surface that created the 'polka dot' pattern. The authors believe that these cracks occurred due to the low concentration of chitosan in 50:50 gels, which was not enough to provide a homogenous layer and instead formed cracks during film casting (Fig. 2). Genipin crosslinking for 24 h formed a more homogenous gel surface, with less observed PLL patches, by initiating crosslinking of chitosan and PLL polymeric chains (Fig. 1B). A homogenous surface is desirable since it provides a consistent cellular response throughout the entire gel.

Uncrosslinked chitosan/PLL gels were characterized using FTIR to determine whether the blending was successful. The peaks in chitosan have been assigned as follows: 1027 $cm^{-1}$ is attributed to the C—O stretch of —$CH_2OH$; 1376 $cm^{-1}$ is the —$CH_3$, —$CH_2$ bending; 1571 $cm^{-1}$ is the N—H bending (amide II); 1654 $cm^{-1}$ is the C=O stretching (amide I); 2877 $cm^{-1}$ is the C—H stretching and 3330 is the O—H and N—H stretching. After blending with PLL, the amide I and II peaks increased and shifted slightly to the right (1627 and 1539 $cm^{-1}$, respectively). This illustrates the increase in amine groups on the surface of the gels after blending, which is due to the presence of PLL (Fig. 3A). Genipin crosslinking was also evident from the FTIR spectra (Fig. 3B). There was an increase in the ratio between the stretching vibration of the amide bond (C=O) at 1630–1680 $cm^{-1}$ and the bending vibration of the primary amine group (N—H) at 1550–1640 $cm^{-1}$ in all 2D gel compositions except for 100% chitosan after 3 h of genipin crosslinking. Also, the ratio between the stretching vibration of the amide bond (C=O) and the stretching vibration of the primary amine group (N—H) at 3100–3500 $cm^{-1}$ was higher for all crosslinked 2D gels except for 100% chitosan. Both of these observations, along with the reduction in the intensity of the primary amine peaks after genipin crosslinking are strong indications for increasing amide bonds, and a reduction in free amine groups after genipin crosslinking (Chiono et al., 2008).

In terms of the physical properties, there was no significant change in gel swelling using different PLL concentrations or crosslinking times (Fig. 4A). Gels swelled between 10 and 25 times their own weight, which demonstrates their hydrophilicity. Moreover, there was no significant difference in swelling using the different crosslinking time points (Fig. 4A). This has been previously demonstrated (Song et al., 2004), and is attributed to the poor protonation of amine groups present in chitosan at pH 7, which limits swelling. Moreover, since there was no difference in the degree of crosslinking after 3 h, the swelling was also not significantly changed with increasing crosslinking time.

The ninhydrin assay revealed that within the uncrosslinked gels, 100% chitosan had the highest degree of stability ($P < 0.001$). However, for genipin-crosslinked gels there was a significant increase in degree of crosslinking after 3 h ($P < 0.001$), which did not significantly increase in any of the gel compositions beyond that point (Fig. 4B). This is not unusual, since even shorter crosslinking times

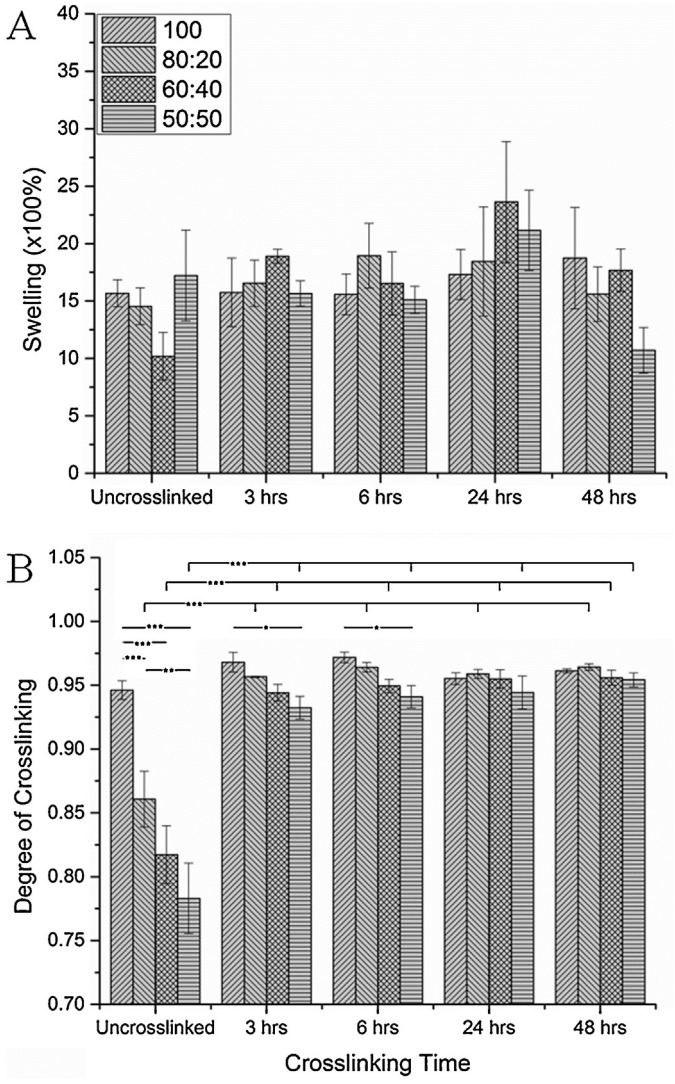


**Fig. 4.** (A) Gel swelling at pH 7 for uncrosslinked and crosslinked gels ($n = 3$); (B) degree of crosslinking of the different gel compositions measured using the ninhydrin assay ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

(e.g. 30 min) were previously used to crosslink chitosan films (Silva, Caridade, Mano, & Reis, 2013). In order to eliminate the possibility that genipin was exhausted from the crosslinking solution after 3 h, the crosslinking solutions used to crosslink the gels for 48 h were examined for the presence of genipin by adding lysine and observing color change. Genipin crosslinking solutions used to crosslink all four chitosan compositions turned brown upon the addition of lysine, indicating the presence of genipin in the solution even after 48 h of crosslinking. Therefore, for cell studies only 3 h crosslinking was used.

### 3.3. Fibroblasts' viability, adhesion and proliferation

Confocal microscopy images taken after 48 h of 3T3 fibroblast culturing indicated that fibroblasts adhered on both crosslinked and uncrosslinked gels. However, attachment was more pronounced on crosslinked gels (Fig. 5A). This confirmed that the genipin-crosslinked gels were more cytocompatibile compared to uncrosslinked gels. Martín-López et al. demonstrated that chitosan/PLL mixtures inhibited growth and proliferation of various cell types (Martin-Lopez, Nieto-Diaz, & Nieto-Sampedro, 2012);

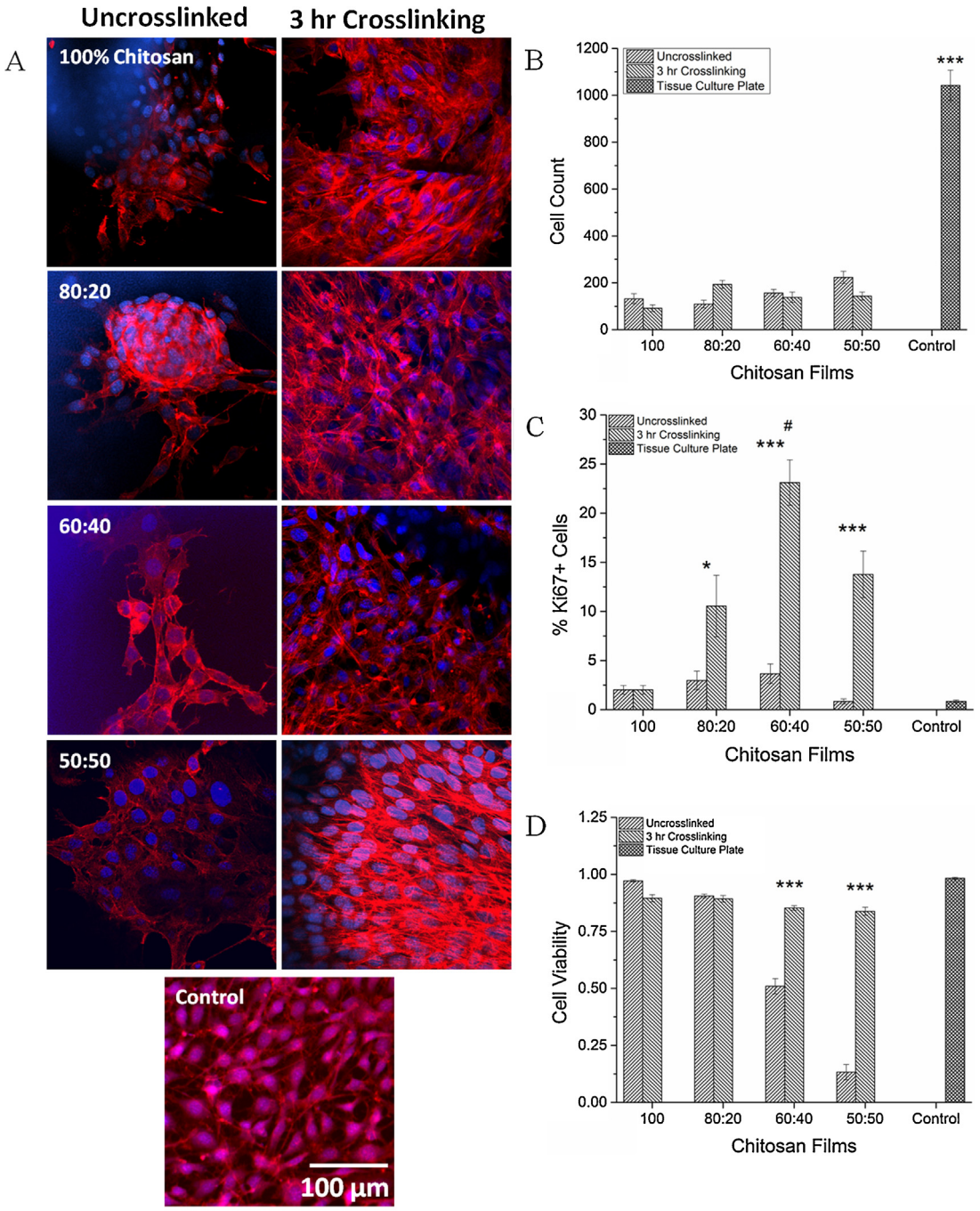


**Fig. 5.** (A) Confocal microscopy images of fibroblasts cultured on uncrosslinked (negative controls), crosslinked chitosan/PLL gels and tissue culture plate (positive control) for 48 h; (B) total cell count of live cells on the different chitosan/PLL gels; (C) percentage of fibroblasts expressing Ki67, which is indicative of proliferation; (D) cell viability assessed using live/dead assay ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

however, genipin-crosslinking presented in this study seems to prevent this effect.

A total cell count demonstrated that the control had a significantly higher cell number than on any of the chitosan gels. This illustrated that proliferation took place more readily on controls than on all gel compositions (Fig. 5B). The proliferation study based on Ki67 expression revealed that fibroblasts divided significantly more on crosslinked 60:40 gels as compared to other gel compositions. Moreover, the controls had the lowest Ki67 expression since fibroblasts reached confluency and ceased to divide (Fig. 5C). The presence of PLL in the crosslinked 60:40 formulation was responsible for the significant enhancement of proliferation of fibroblasts.

Cell viability was significantly enhanced after crosslinking the 60:40 and 50:50 gels; while fibroblast viability on 100 and 80:20 gels was not affected by crosslinking (Fig. 5D). This is in agreement with other groups that reported that genipin improves the biocompatibility of various chitosan formulations (Mathew, Oksman, Pierron, & Harmand, 2013; Norowski, Mishra, Adatrow, Haggard, & Bumgardner, 2012; Tseng, Tsou, Wang, & Hsu, 2013).

## 4. Conclusion

In this manuscript, it was demonstrated that genipin crosslinking of chitosan/PLL gels significantly improved their stability

and biological properties and is thus advantageous in utilizing chitosan/PLL blends for tissue engineering applications. Genipin-crosslinked chitosan/PLL gels were shown to be superior to their uncrosslinked counterparts in terms of enhancing fibroblast attachment, viability and proliferation. Therefore, based on results from two-dimensional chitosan/PLL gels, one can envision making three-dimensional, genipin-crosslinked chitosan/PLL hydrogels as suitable scaffolds for use in a wide range of tissue engineering applications, and particularly for engineering soft tissue.

## Acknowledgements

Authors would like to acknowledge sources of funding from NSERC and CIHR that made this work possible.

## References


Airoldi, C., & Monteiro, O. A. C. (1999). Some studies of crosslinking chitosan-glutaraldehyde interaction in a homogeneous system. *International Journal of Biological Macromolecules*, *26*(2–3), 119–128.

Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N., & Rubini, K. (2002). Stabilization of gelatin films by crosslinking with genipin. *Biomaterials*, *23*(24), 4827–4832.

Butler, M. F., Ng, Y. F., & Pudney, P. D. A. (2003). Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *Journal of Polymer Science Part A: Polymer Chemistry*, *41*(24), 3941–3953. http://dx.doi.org/10.1002/Pola.10960

Cheng, M. Y., Gong, K., Li, J. M., Gong, Y. D., Zhao, N. M., & Zhang, X. F. (2004). Surface modification and characterization of chitosan film blended with poly-L-lysine. *Journal of Biomaterials Applications*, *19*(1), 59–75. http://dx.doi.org/10.1177/0885328204043450

Chiono, V., Pulieri, E., Vozzi, G., Ciardelli, G., Ahluwalia, A., & Giusti, P. (2008). Genipin-crosslinked chitosan/gelatin blends for biomedical applications. *Journal of Materials Science: Materials in Medicine*, *19*(2), 889–898. http://dx.doi.org/10.1007/s10856-007-3212-5

Cho, Y., Shi, R. Y., & Borgens, R. B. (2010). Chitosan produces potent neuroprotection and physiological recovery following traumatic spinal cord injury. *Journal of Experimental Biology*, *213*(9), 1513–1520. http://dx.doi.org/10.1242/Jeb.035162

da Silva, M. L. A., Crawford, A., Mundy, J. M., Correlo, V. M., Sol, P., Bhattacharya, M., et al. (2010). Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation. *Acta Biomaterialia*, *6*(3), 1149–1157. http://dx.doi.org/10.1016/j.actbio.2009.09.006

Dal Pozzo, A., Vanini, L., Fagnoni, M., Guerrini, M., De Benedittis, A., & Muzzarelli, R. A. A. (2000). Preparation and characterization of poly(ethylene glycol)-crosslinked reacetylated chitosans. *Carbohydrate Polymers*, *42*(2), 201–206.

Fan, D. D., Zhu, C. H., Duan, Z. Z., Xue, W. J., Shang, L. A., Chen, F. L., et al. (2009). Initial investigation of novel human-like collagen/chitosan scaffold for vascular tissue engineering. *Journal of Biomedical Materials Research Part A*, *89A*(3), 829–840. http://dx.doi.org/10.1002/Jbm.A.32256

Feijen, J., Jin, R., Teixeira, L. S. M., Dijkstra, P. J., Karperien, M., van Blitterswijk, C. A., et al. (2009). Injectable chitosan-based hydrogels for cartilage tissue engineering. *Biomaterials*, *30*(13), 2544–2551. http://dx.doi.org/10.1016/j.biomaterials.2009.01.020

Gao, C. Y., Ma, L., Mao, Z. W., Zhou, J., Shen, J. C., Hu, X. Q., et al. (2003). Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. *Biomaterials*, *24*(26), 4833–4841. http://dx.doi.org/10.1016/S0142-9612(03)00374-0

Gorgieva, S., & Kokol, V. (2012). Preparation, characterization, and in vitro enzymatic degradation of chitosan-gelatine hydrogel scaffolds as potential biomaterials. *Journal of Biomedical Materials Research Part A*, *100A*(7), 1655–1667. http://dx.doi.org/10.1002/Jbm.A.34106

Gu, X. S., Ding, F., Wu, J. A., Yang, Y. M., Hu, W., Zhu, Q., et al. (2010). Use of tissue-engineered nerve grafts consisting of a chitosan/poly(lactic-co-glycolic acid)-based scaffold included with bone marrow mesenchymal cells for bridging 50-mm dog sciatic nerve gaps. *Tissue Engineering Part A*, *16*(12), 3779–3790. http://dx.doi.org/10.1089/ten.tea.2010.0299

Hasirci, V., Yilgor, P., Tuzlakoglu, K., Reis, R. L., & Hasirci, N. (2009). Incorporation of a sequential BMP-2/BMP-7 delivery system into chitosan-based scaffolds for bone tissue engineering. *Biomaterials*, *30*(21), 3551–3559. http://dx.doi.org/10.1016/j.biomaterials.2009.03.024

Kavya, K. C., Jayakumar, R., Nair, S., & Chennazhi, K. P. (2013). Fabrication and characterization of chitosan/gelatin/n$SiO_2$ composite scaffold for bone tissue engineering. *International Journal of Biological Macromolecules*, *59*, 255–263. http://dx.doi.org/10.1016/j.ijbiomac.2013.04.023

Kawase, M., Michibayashi, N., Nakashima, Y., Kurikawa, N., Yagi, K., & Mizoguchi, T. (1997). Application of glutaraldehyde-crosslinked chitosan as a scaffold for hepatocyte attachment. *Biological & Pharmaceutical Bulletin*, *20*(6), 708–710.

Kim, S. K., Venkatesan, J., Qian, Z. J., Ryu, B., & Kumar, N. A. (2011). Preparation and characterization of carbon nanotube-grafted-chitosan – Natural hydroxyapatite composite for bone tissue engineering. *Carbohydrate Polymers*, *83*(2), 569–577. http://dx.doi.org/10.1016/j.carbpol.2010.08.019

Malafaya, P. B., Silva, G. A., & Reis, R. L. (2007). Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. *Advanced Drug Delivery Reviews*, *59*(4-5), 207–233. http://dx.doi.org/10.1016/j.addr.2007.03.012

Marra, K. G., Tan, H. P., Chu, C. R., & Payne, K. A. (2009). Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for cartilage tissue engineering. *Biomaterials*, *30*(13), 2499–2506. http://dx.doi.org/10.1016/j.biomaterials.2008.12.080

Martin-Lopez, E., Nieto-Diaz, M., & Nieto-Sampedro, M. (2012). Differential adhesiveness and neurite-promoting activity for neural cells of chitosan, gelatin, and poly-L-lysine films. *Journal of Biomaterials Applications*, *26*(7), 791–809. http://dx.doi.org/10.1177/0885328210379928

Mathew, A. P., Oksman, K., Pierron, D., & Harmand, M. F. (2013). Biocompatible fibrous networks of cellulose nanofibres and collagen crosslinked using genipin: Potential as artificial ligament/tendons. *Macromolecular Bioscience*, *13*(3), 289–298. http://dx.doi.org/10.1002/mabi.201200317

Matthew, H. W. T., & Madihally, S. V. (1999). Porous chitosan scaffolds for tissue engineering. *Biomaterials*, *20*(12), 1133–1142.

Mekhail, M., Wong, K. K., Padavan, D. T., Wu, Y., O'Gorman, D. B., & Wan, W. (2010). Genipin-cross-linked electrospun collagen fibers. *Journal of Biomaterials Science*, http://dx.doi.org/10.1163/092050610X538209

Mi, F. L., Shyu, S. S., & Peng, C. K. (2005). Characterization of ring-opening polymerization of genipin and pH-dependent cross-linking reactions between chitosan and genipin. *Journal of Polymer Science Part A: Polymer Chemistry*, *43*(10), 1985–2000. http://dx.doi.org/10.1002/Pla.20669

Misra, R. D. K., & Thein-Han, W. W. (2009). Biomimetic chitosan-nanohydroxyapatite composite scaffolds for bone tissue engineering. *Acta Biomaterialia*, *5*(4), 1182–1197. http://dx.doi.org/10.1016/j.actbio.2008.11.025

Moura, M. J., Figueiredo, M. M., & Gil, M. H. (2007). Rheological study of genipin cross-linked chitosan hydrogels. *Biomacromolecules*, *8*(12), 3823–3829. http://dx.doi.org/10.1021/Bm700762w

Muzzarelli, R. A. A. (2009a). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*(2), 167–182. http://dx.doi.org/10.1016/j.carbpol.2008.11.002

Muzzarelli, R. A. A. (2009b). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*(1), 1–9. http://dx.doi.org/10.1016/j.carbpol.2009.01.016

Muzzarelli, R. A. A., Greco, F., Busilacchi, A., Sollazzo, V., & Gigante, A. (2012). Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: A review. *Carbohydrate Polymers*, *89*(3), 723–739. http://dx.doi.org/10.1016/j.carbpol.2012.04.057

Norowski, P. A., Mishra, S., Adatrow, P. C., Haggard, W. O., & Bumgardner, J. D. (2012). Suture pullout strength and in vitro fibroblast and RAW 264.7 monocyte biocompatibility of genipin crosslinked nanofibrous chitosan mats for guided tissue regeneration. *Journal of Biomedical Materials Research Part A*, *100*(11), 2890–2896. http://dx.doi.org/10.1002/jbm.a.34224

Qi, B. W., Yu, A. X., Zhu, S. B., Zhou, M., & Wu, G. (2013). Chitosan/poly(vinyl alcohol) hydrogel combined with Ad-hTGF-beta1 transfected mesenchymal stem cells to repair rabbit articular cartilage defects. *Experimental Biology and Medicine*, *238*(1), 23–30. http://dx.doi.org/10.1258/ebm.2012.012223

Sajesh, K. M., Jayakumar, R., Nair, S. V., & Chennazhi, K. P. (2013). Biocompatible conducting chitosan/polypyrrole-alginate composite scaffold for bone tissue engineering. *International Journal of Biological Macromolecules*, *62*, 465–471. http://dx.doi.org/10.1016/j.ijbiomac.2013.09.028

Sano, M., Hosoya, O., Taoka, S., Seki, T., Kawaguchi, T., Sugibayashi, K., et al. (1999). Relationship between solubility of chitosan in alcoholic solution and its gelation. *Chemical & Pharmaceutical Bulletin*, *47*(7), 1044–1046.

Sethuraman, S., Dhandayuthapani, B., & Krishnan, U. M. (2010). Fabrication and characterization of chitosan-gelatin blend nanofibers for skin tissue engineering. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *94B*(1), 264–272. http://dx.doi.org/10.1002/Jbm.B.31651

Shoichet, M. S., Kim, H., & Tator, C. H. (2011). Chitosan implants in the rat spinal cord: Biocompatibility and biodegradation. *Journal of Biomedical Materials Research Part A*, *97A*(4), 395–404. http://dx.doi.org/10.1002/Jbm.A.33070

Shyu, S. S., Mi, F. L., & Sung, H. W. (2000). Synthesis and characterization of a novel chitosan-based network prepared using naturally occurring crosslinker. *Journal of Polymer Science Part A: Polymer Chemistry*, *38*(15), 2804–2814.

Silva, S. S., Caridade, S. G., Mano, J. F., & Reis, R. L. (2013). Effect of crosslinking in chitosan/aloe vera-based membranes for biomedical applications. *Carbohydrate Polymers*, *98*(1), 581–588. http://dx.doi.org/10.1016/j.carbpol.2013.06.022

Silva, S. S., Motta, A., Rodrigues, M. T., Pinheiro, A. F. M., Gomes, M. E., Mano, J. F., et al. (2008). Novel genipin-cross-linked chitosan/silk fibroin sponges for cartilage engineering strategies. *Biomacromolecules*, *9*(10), 2764–2774. http://dx.doi.org/10.1021/Bm800874q

Song, M., Jin, J., & Hourston, D. J. (2004). Novel chitosan-based films cross-linked by genipin with improved physical properties. *Biomacromolecules*, *5*(1), 162–168. http://dx.doi.org/10.1021/Bm034286m

Stevens, M. M., & George, J. H. (2005). Exploring and engineering the cell surface interface. *Science*, *310*(5751), 1135–1138. http://dx.doi.org/10.1126/science.1106587

Sun, P., Li, P., Li, Y. M., Wei, Q., & Tian, L. H. (2011). A pH-sensitive chitosan-tripolyphosphate hydrogel beads for controlled glipizide delivery. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *97*(1), 175–183. http://dx.doi.org/10.1002/jbm.b.31801

Sung, H. W., Huang, R. N., Huang, L. L. H., & Tsai, C. C. (1999). In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological

tissue fixation. *Journal of Biomaterials Science: Polymer Edition*, *10*(1), 63–78.

Tsai, W. B., Chen, Y. R., Liu, H. L., & Lai, J. Y. (2011). Fabrication of UV-crosslinked chitosan scaffolds with conjugation of RGD peptides for bone tissue engineering. *Carbohydrate Polymers*, *85*(1), 129–137. http://dx.doi.org/10.1016/j.carbpol.2011.02.003

Tseng, H. J., Tsou, T. L., Wang, H. J., & Hsu, S. H. (2013). Characterization of chitosan-gelatin scaffolds for dermal tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*, *7*(1), 20–31. http://dx.doi.org/10.1002/term.492

Yao, C. H., Chen, K. Y., Liao, W. J., Kuo, S. M., Tsai, F. J., Chen, Y. S., et al. (2009). Asymmetric chitosan membrane containing collagen I nanospheres for skin tissue engineering. *Biomacromolecules*, *10*(6), 1642–1649. http://dx.doi.org/10.1021/Bm900238b

Yu, J. H., Chen, H. L., Huang, J., Liu, S. Y., & Gu, P. (2011). Electrospun chitosan-graft-poly (epsilon-caprolactone)/poly (epsilon-caprolactone) cationic nanofibrous mats as potential scaffolds for skin tissue engineering. *International Journal of Biological Macromolecules*, *48*(1), 13–19. http://dx.doi.org/10.1016/j.ijbiomac.2010.09.019

Zhang, M. Q., Li, Z. S., Ramay, H. R., Hauch, K. D., & Xiao, D. M. (2005). Chitosan-alginate hybrid scaffolds for bone tissue engineering. *Biomaterials*, *26*(18), 3919–3928. http://dx.doi.org/10.1016/j.biomaterials.2004.09.062

Zheng, Z. H., Wei, Y. J., Wang, G., Wang, A. J., Ao, Q., Gong, Y. D., et al. (2009). Surface properties of chitosan films modified with polycations and their effects on the behavior of PC12 cells. *Journal of Bioactive and Compatible Polymers*, *24*(1), 63–82. http://dx.doi.org/10.1177/0883911508099653

Zheng, Z. H., Zhang, L., Kong, L. J., Wang, A. J., Gong, Y. D., & Zhang, X. F. (2009). The behavior of MC3T3-E1 cells on chitosan/poly-L-lysine composite films: Effect of nanotopography, surface chemistry, and wettability. *Journal of Biomedical Materials Research Part A*, *89A*(2), 453–465. http://dx.doi.org/10.1002/Jbm.A.31979

Zhu, A. P., & Chan-Park, M. B. (2005). Cell viability of chitosan-containing semi-interpenetrated hydrogels based on PCL-PEG-PCL diacrylate macromer. *Journal of Biomaterials Science: Polymer Edition*, *16*(3), 301–316.