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

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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,

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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