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Crosslinking of gelatin-based drug carriers by genipin induces changes in drug kinetic profiles in vitro

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Abstract

Hydrogels are extensively studied as carrier matrices for the controlled release of bioactive molecules. The aim of this study was to design gelatin-based hydro-gels crosslinked with genipin and study the impact of crosslinking temperature (5, 15 or 25^{o}C) on gel strength, microstructure, cytocompatibility, swelling and drug release. Gels crosslinked at 25^{o}C exhibited the highest Flory–Rehner crosslink density, lowest swelling ratio and the slowest release of indomethacin (Idn, model anti-inflammatory drug). Diffusional exponents (n) indicated non-Fickian swelling kinetics while drug transport was anomalous. Hydrogel biocompatibility, in vitro cell viability, cell cycle experiments with AH-927 and HaCaT cell lines indicated normal cell proliferation without any effect on cell cycle. Overall, these results substantiated the use of genipin-crosslinked hydrogels as a viable carrier matrix for drug release applications.

1. Introduction

The use of natural polymers (e.g., gelatin [1], chitosan [2], and silk proteins [3]) for the development of hydrogels is gaining in importance due to their inherent biocompatibility. However, a common disadvantage of such matrices is their structural and thermal lability, which may be improved by using crosslinkers such as formaldehyde [4] and glutaraldehyde [5]. As these common crosslinkers are cytotoxic and may initiate calcification of implants, there remains the need for crosslinkers from natural sources with improved biocompatibility. Genipin is one such crosslinker, obtained from the fruits of Gardenia jasminoides, which is 10,000 times less

toxic and degrades more slowly when compared with glutaraldehyde [6–9]. Apart from improved stability, crosslinking also alters swelling or degradation-induced diffusion and thus the release of incorporated bioactive agents from hydrogels [10]. Gelatin is a natural polymer derived from collagen that has been used for various pharmaceutical and biomedical applications [11]. Given its biodegradability and biocompatibility in physiological environments, it has been extensively used in the design and application of various drug carriers [12]. In the present study, we developed a genipin-crosslinked gelatin-based matrix for the controlled release of bioactive agents. Apart from tailoring the release properties of indomethacin (Idn, used as a model drug), genipin was employed to improve the hydrogels mechanical stability. Cytotoxicity of the crosslinked gel constructs was also evaluated. Drug release patterns and kinetics demonstrated the suitability of genipin-crosslinked gelatin gels for drug delivery.

2 Materials and methods

2.1 Materials

Porcine gelatin (Type A; bloom strength 300), Idn (99% TLC) and glycine were obtained from Sigma-Aldrich (Oakville, ON, Canada). Genipin was purchased from Challenge Bioproducts (Taipei, Taiwan, PRC). Ninhydrin was procured from SRL (Mumbai, India). All other chemicals were of reagent or analytical grade. Deionized water (DI) was used for all experiments. Feline fibroblast AH-927 and human keratinocyte HaCaT cells (National Center for Cell Sciences, NCCS, Pune, India) were chosen for cell culture studies, as these cell lines were highly stable, fast-growing and represented well-defined internal cytoskeleton components. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2.4 g sodium bicarbonate, antibiotics (10,000 U/l penicillin and 10 mg/l streptomycin) (Himedia, Mumbai, India) and 10% (v/v) fetal bovine serum (FBS) (HyClone, New Delhi, India). Cells were incubated at 37^{o}C in 5% CO_2 and 95% humidity air. Cells in their logarithmic growth phase were used for viability and cytotoxicity experiments.

2.2 Preparation of gelatin hydrogels

A 10% (w/w) gelatin sol was prepared by dissolving the gelatin powder in DI water at 60^{o}C with stirring at 100 rpm. The final pH of the solution was 6.2. Evaporative losses were corrected for

on a weight basis by addition of DI water. The above dispersion was poured into Petri-dishes and set at 5^{o}C.

2.3 Crosslinking with genipin

A genipin solution was prepared by dissolving 400 mg of genipin in 100 ml of DI water, yielding a 0.4% (w/v) solution [13]. Following gelation, a 10 ml volume of this solution was added to each Petri dish and incubated at 5, 15 or 25^{o}C for 24 h to promote crosslinking of the gelatin polymer chain. Afterwards, the genipin solution was drained off slowly, a 1% (w/v) glycine solution (10 ml) was added to the crosslinked gel and subsequently incubated for 24 h to bond unreacted genipin. After 24 h, the glycine solution was drained and the gels were washed with DI water several times.

2.4 Colour analysis

Colorimetric analysis of the gels was performed using a Hunterlab colorimeter (Hunter Associates Laboratory, Reston, Virginia, USA). This instrument provided L*, a* and b* values, where L* is the lightness component ranging from 0 (black) to 100 (white), and parameters a* (from greenness to redness), and b* (blueness to yellowness) are the two chromatic components. Based on these, the whiteness index (WI) was calculated as follows: [14]

$$WI = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{0.5} \qquad (1)$$

2.5 Crosslinking index

The crosslinking degree of each test group was determined with the ninhydrin assay [13, 15]. Samples were weighed (~20 mg) and subsequently heated with a ninhydrin solution (2% w/w) at 100^{o}C for 20 min. The optical absorbance of each solution was recorded with a Lambda 20 UV–VIS spectrophotometer (Perkin-Elmer, Bridgeville, PA, USA) at 570 nm. Gelatin matrices prepared without genipin were used as a control. The number of free amino groups measured in the test samples was proportional to optical absorbance [8]. Each experiment was performed in triplicate. The crosslinking index of the samples was determined as previously established [15, 16].

2.6 Microstructure

The microstructure of the hydrogels was analyzed with an upright Zeiss LSM-510 confocal laser scanning microscope (CLSM) (Zeiss Inc., Toronto, ON, Canada). Gel microstructure was observed at 100x and image contrast and brightness were optimized with the LSM-510 software. A 488 nm laser was used to excite the genipin-crosslinked structures and the emission wavelength was set at 515 nm [17, 18]. Numerous images were captured and those shown herein are representative.

2.7 Mechanical properties

The hydrogels' mechanical properties were analyzed with a puncture probe mated to a Texture Analyzer (TA-XT2i, Texture Technologies Corp, Scarsdale, NY, USA). The polymethyl methacrylate piercing probe (D = 6.27 mm) was driven through the gels at 0.5 mm/s and a 5 N load cell was used for calibration. The force–displacement curves were recorded, with the puncture strength measured at the hydrogel's yield point [19]. All samples used for this test were free from imperfections.

2.8 Swelling

Hydrogels were cut into circular discs (D = *25 mm, h = *3.4 mm) and swelled in a beaker containing 50 ml of DI water. Sample weights at t = 0 (W0) and at 30 min intervals (Wt) were recorded. Swelling ratio (SR) was calculated as [13]:

$$SR = \left(\frac{W_t - W_o}{W_o} \right) \tag{2}$$

The equilibrium water content (EWC) of the hydrogels was measured at SR = 24 h.

Equation 3, which was derived for the swelling of ahydrogel prepared in the presence of a solvent, [20–22] was used to determine crosslinking density:

$$\frac{V_1}{4I}\left(\frac{v_{2,s}}{\bar{v}}\right)^2\left(\frac{K_b}{10^{pOH}-K_a}\right)^2 = \left[\ln(1-v_{2,s})+v_{2,s}+\chi_1 v_{2,s}^2\right] + v_{2,r}\left(\frac{V_1}{\bar{v}\overline{M}_c}\right)\left(1-\frac{2\overline{M}_c}{\overline{M}_n}\right)\left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3}-\left(\frac{v_{2,s}}{2v_{2,r}}\right)\right] \tag{3}$$

Here, Mn is the MW of the polymer without crosslinking, Mc is the number-average polymer MW between two adjacent crosslinks, v is the specific volume of the hydrogel prior to swelling, V_1 is the molar volume of the solvent water, $v_{2,s}$ is the polymer volume fraction in the swollen state determined as roughly the inverse of the equilibrium swelling ratio, $v_{2,r}$ is the polymer volume fraction in the relaxed state (the state of the polymer immediately after crosslinking but before swelling), I is the ionic strength, Ka and Kb are the dissociation constants for the acidic and basic moieties on the polymer, and χ_1 is the Flory–Huggins parameter describing the polymer–solvent interaction [23, 24]. Using Mc; the crosslink density, q, can be determined from [25]:

$$q = \frac{\overline{M}_n}{\overline{M}_c} \tag{4}$$

The parameter v2,s was determined from the volumeswelling ratio, qv [26]:

$$v_{2,s} = \frac{1}{q_v} \tag{5}$$

where the volume-swelling ratio was calculated as [26]:

$$q_v = 1 + \frac{(q_w - 1) \times \rho_2}{\rho_1} \tag{6}$$

where q_2 and q_1 are the densities of the polymer network and solvent, respectively. The weight-swelling ratio, qw, was determined from [26]:

$$q_w = \frac{m_s}{m_o} \tag{7}$$

where m_o and m_s are the mass of the unswollen gel and the mass of the swollen gel at equilibrium, respectively. Table 1 provides the parameters used to calculate M_c and q.

2.9 In vitro drug release

One gram of Idn was dispersed in 99 g of the gelatin solution and mixed at 1,000 rpm for 4 h at 40°C. This dispersion was then allowed to gel and subsequently crosslinked at 5, 15 or 25 °C as

discussed earlier. Gel discs (D = ~25 mm; h = ~3.4 mm) of known weights were placed in 50 ml of DI water in a beaker stirred with a magnetic stirrer at 100 rpm. At 60 min intervals, the contents of the beaker were replaced with 50 ml of fresh DI water to maintain sink conditions. Aliquots (2 ml) were withdrawn and analyzed spectrophotometrically (Perkin- Elmer Lambda 20 UV–Vis spectrophotometer, Bridgeville, PA, USA) at 317 nm for the determination of Idn concentration. Cumulative Idn release was calculated from the amount of Idn released in the DI water at set time intervals over 24 h.

2.10 Swelling and drug release kinetics

Drug release was modelled as follows [27, 28]:

$$\frac{M_t}{M_\infty} = kt^n \tag{8}$$

where Mt/M∞ is the fractional release at time t, k is a rate constant and n is the diffusional exponent. The exponent n provides an indication of the release mechanism and generally ranges from 0.5 to 1. It was calculated from the slope of the natural logarithmic values (ln) of fractional release as a function of time. An n value of 0.45 indicates Fickian diffusion [29] whereas a value of 0.45–0.89 indicates anomalous transport, as there exists an influence of swelling and/or erosion. Finally, a value ≥0.89 indicates Case II relaxation [30].

2.11 In vitro cell viability

Gels were sterilized prior to cell culture studies by immersing them in 70% (v/v) ethanol for 1 h followed by 3–4 thorough rinses with sterile phosphate buffer saline (PBS) (pH 7.4) to remove residual alcohol. For cytotoxicity evaluation, portions of the gels crosslinked at each temperature were incubated in complete DMEM medium for 24 h in 12-well cell culture plates. This conditioned media was used for subsequent cell viability tests, determined by MTT-dye reduction assay [31] using AH-927 and HaCaT cells. The cell suspensions were dispensed (2 ml) in triplicate into 12-well tissue culture plates at 5 x 10^4 cells/well in complete DMEM. Twenty-four hours after cell seeding, the media was removed and the cells were treated with conditioned media from each well, or with a control (media sans gel) and kept 48 h for evaluation of cell viability. Cell viability was measured at 540 nm using a microplate reader (BioRad Lab-550, BioRad, Gurgaon, Haryana, India). Cell viability and cytotoxicity were assessed using a

LIVE/DEAD kit (Molecular Probes, Invitrogen, Paisley, UK) following the manufacturer's instructions. Cells were seeded in 60-mm tissue culture plates at 5 x 10^5 cells/plate. Cells were treated with conditioned media preincubated with gel construct or with the control, and stored 48 h. After treatment, the cells were trypsinized, collected and washed in Dulbecco's PBS and resuspended in 1 ml saline. To this were added 2 ll of 50 mM calcein–acetoxymethyl solution and 4 µl of 2 mM ethidium homodimer-I per ml of cell solution. After incubation for 30 min with gel construct or with the control, and stored 48 h. After treatment, the cells were trypsinized, collected and washed in Dulbecco's PBS and resuspended in 1 ml saline. To this were added 2 µl of 50 mM calcein–acetoxymethyl solution and 4 µl of 2 mM ethidium homodimer-I per ml of cell solution. After incubation for 30 min in the dark, samples were analyzed with a flow cytometer (Becton–Dickinson FACSCaliber, Gurgaon, Haryana, India). Acquisition and analysis was performed at 488 nm, measuring green fluorescence (FL1) emission for calcein (live cells) and red fluorescence (FL2) emission for ethidium homodimer I (dead cells) using CellQuest software (Becton–Dickinson, Gurgaon, Haryana, India). Cells were gated (to exclude debris), and voltage and compensation were adjusted before final acquisition and analysis. Acetoxymethyl ester derivatives of calcein are used to study live cells as it fluoresces when absorbed by viable cells.

2.12 Cell cycle and apoptosis

Cells were seeded in 60-mm tissue culture plates at 5 x 10^5 cells/plate. Cells were treated with conditioned media pre-incubated with the gel constructs or the control, and stored 48 h. The cells were then trypsinized, collected and washed in PBS and incubated in 70% ethanol for 45 min at 4^oC or kept at -20^oC overnight for fixation. Cells were centrifuged, washed and then incubated with propidium iodide (PI) solution (40 µg/ml PI, 100 µg/ml RNase A in PBS) at 37^oC for 1 h. Cells were analyzed using the Becton–Dickinson flow cytometer and data acquisition was performed using the CellQuest software. Apoptotic cells were determined by their hypochromic subdiploid staining profiles. The distribution of cells in the different cell-cycle phases was analyzed from the DNA histogram using Mod Fit software (Topsham, ME, USA).

2.13 Statistical analyses

All quantitative experiments were performed in triplicate and the results were expressed as arithmetic means ± standard deviation (SD) for n = 3, except for the MTT test results (n = 6). Statistical analyses were performed by GraphPad Prism 5 software (La Jolla, CA, USA), using one-way analysis of variance (ANOVA) and t-tests. Tukey's multiple comparison tests among all groups was performed to demonstrate significant differences amongst groups. Differences between groups at $P<0.05$ were considered statistically significant and at $P<0.01$ and $P<0.001$, highly significant.

3 Results and discussion

3.1 Degree of crosslinking

The formation of a blue pigment may be qualitatively linked with the extent of hydrogel crosslinking with genipin (Fig. 1A) [32–35]. Gelatin crosslinked at 5 ^{o}C showed a significantly higher WI (~19) when compared to those crosslinked at 15 ^{o}C (~14) and 25 ^{o}C (~7) ($P<0.0001$) (Fig. 1B), which strongly suggested a lower crosslinking density at lower temperature. The ninhydrin assay showed that the initial light yellow colour of the solution gradually turned blue depending on the extent of reaction with the free amino group present in solution. Gelatin crosslinked at 25 ^{o}C demonstrated the highest crosslinking index (~88) compared to 15 ^{o}C (~77) and 5 ^{o}C (~21) ($P<0.0001$) (Table 2). Table 3 shows that an increase in crosslinking temperature resulted in decrease in Mc and an increase in q, with the gels crosslinked at 5 ^{o}C demonstrating the lowest Flory–Rehner crosslink density ($P<0.0001$).

3.2 Microstructure

As genipin autofluoresces when excited at 488 nm, no dye was needed to analyze gel structure using CLSM [17] (Fig. 2). Crosslinking temperature had a significant impact on gelatin hydrogel microstructure. At 5 ^{o}C, the microstructure consisted of heterogeneously and sparsely dispersed pores (Fig. 2A). The pore size of the gels crosslinked at 15 ^{o}C was ~40–60 μm in diameter and generally <50 μm for the gel crosslinked at 25 ^{o}C. As the crosslinking temperature was raised, a more compact microstructure resulted (Fig. 2B, C).

3.3 Mechanical analysis

Gelatin sols are transformed into viscoelastic gels via gelation with the network stabilized mainly by hydrogen bonding [36]. Addition of a fixative such as genipin further stabilizes the network and reduces chain relaxation by the formation of thermally-stable chemical crosslinks. The gels crosslinked at 25 ^{o}C showing the highest puncture strength (~0.40 MPa) followed by those crosslinked at 15 ^{o}C (~0.32 MPa) and at 5 ^{o}C (~0.08 MPa) ($P<0.0001$) (Fig. 3). Gels crosslinked at 25 (Fig. 3). Gels crosslinked at 25 ^{o}C demonstrated a puncture strength ~89 higher than an uncrosslinked control ($P<0.05$), which was comparable to other studies [11, 37–39].

3.4 Swelling behaviour

Upon immersion in water, all gels imbibed water rapidly and reached equilibrium within 24 h, with swelling highlydependent on crosslinking temperature (Fig. 4). Gels crosslinked at 5 ^{o}C showed an EWC of ~0.9 followed by ~0.5 and 0.4 for those crosslinked at 15 and 25 ^{o}C, respectively (Table 4) ($P<0.05$). Swelling kinetics were non-Fickian, with all n values between 0.61 and 0.65 ($P<0.05$) irrespective of temperature (Table 5), implying anomalous transport with coupled diffusion and biopolymer relaxation. Swelling-related kinetic constant (k) values (Table 5) decreased with an increase in crosslinking temperature ($P<0.05$), which was presumably related to the more highly-crosslinked and rigid gelatin polypeptide
mesh slowing water ingress [40].

3.5 Drug release and kinetics

After 24 h, cumulative Idn release was ~96, 56 and ~47% for the gels crosslinked at 5, 15 and 25 ^{o}C, respectively (Fig. 5) ($P<0.05$), with release reaching a plateau for the gels crosslinked at 15 and 25 ^{o}C, but not at 5 ^{o}C. The kinetic constant k was highest for the gels crosslinked at 5 ^{o}C (~0.13) followed by 15 ^{o}C (~0.11) and 25 ^{o}C (~0.10) ($P<0.05$) (Table 6) whereas the diffusional exponent n values were ~0.72, 0.82 and 0.86, respectively ($P<0.05$), indicating non-Fickian release [30, 41]. With many compounds incorporated within hydrogels, release is swelling-dependent in that a critical pore/mesh size must first be attained for release to occur. As Idn is a small molecule (MW: 358 g/mol), [42] its initial release rate was not greatly influenced by the extent of crosslinking. Rather, crosslinking-independent passive transport through the gels initially occurred, as suggested by the nearly identical k values. Secondly, Idn is a weakly

ionizable acidic drug (pKa = 4.5) drug with poor aqueous solubility (0.72 mg/ml at pH 7.0) [43]. With the gelatin gel at pH ~6, Idn was partly ionized, but not fully solubilized. Thus, Idn existed as both crystals dispersed within the gel and dissolved in the continuous aqueous gelatin phase. It has been reported that the solubility of lipophilic drugs in gelatin solutions increases when compared with their corresponding aqueous solubility [44], though this is highly concentration-dependent. This suggests that there may have been some Idn affinity for the gelatin in solution, increasing its solubilization. Nevertheless, the Idn release observed was likely related to both solubilized Idn diffusion and crystalline Idn partitioning.

3.6 Cytocompatibility

Figure 6A presents micrographs of HaCaT cells cultured for 48 h after treatment with the conditioned medium. The control group (gelatin hydrogels without crosslinking) [Fig. 6A(a)] dissolved completely when placed in the culture medium. The gels crosslinked at 5 ^{o}C dissolved to some extent, but not at 15 or 25 ^{o}C [Fig. 6A(b–d)]. All images indicated that contact with the crosslinked gels had no toxic effects on seeded cell viability. The cellular viability and mitochondrial activity of viable cells on the crosslinked gelatin gels was determined using an MTT assay. Figure 6B shows cell viability based on formazan produced by the mitochondria of actively dividing cells [45, 46]. Feline fibroblast AH-927 and human keratinocyte HaCaT cell viability was comparable to that of the control ($P>0.05$).

Figure 6C shows flow cytometric analysis using a LIVE/DEAD kit. After incubating 48 h, 94% of the cells on the negative control [Fig. 6C(a)] were viable while the experimental groups [Fig. 6C(c–e)] showed 91, 93 and 92% cell viability in the presence of gels crosslinked at 5, 15 and 25 ^{o}C, respectively. For comparison, a positive control [Fig. 6C(b)] showed no viable cells. These results supported the cell photomicrographs, as cell morphology, and in particular the nucleus of both the control and experimental constructs, remained unaffected. All images confirmed cellular integrity.

Figure 6D displays the DNA histogram of different phases of the cell cycle of AH-927 cells treated with media containing the different genipin-crosslinked gels. No noticeable cell cycle changes were observed with different gel constructs compared to the control. In all cases, ~60,

20 and 21% gated cells were observed in the G0/G1, S and G2/M phases, respectively, suggesting normal cell cycle patterns without any visible cell cycle arrest. In most cases, apoptosis was <5%, indicating little or no cytotoxicity associated with the genipin-crosslinked gels.

4 Conclusion

This work reports on the cytocompatibility, swelling and drug release properties of gelatin gels crosslinked with genipin, a natural fixative. Three temperatures (5, 15 and 25 ^{o}C) were used to modulate the extent of crosslinking, which significantly impacted the hydrogels' mechanical properties, swelling and drug release behaviour. The gels crosslinked at 25 ^{o}C showed a puncture strength 5x higher than those crosslinked at 5 ^{o}C. Modelling of the swelling and release indicated non-Fickian swelling kinetics while drug transport was anomalous. Together, these results demonstrated that crosslinking temperature was a significant tuning parameter in regards to the possible utility of these hydrogels. Biocompatibility, in vitro cell viability, cell cycle experiments with AH-927 and HaCaT cell lines all indicated normal cell proliferation without any effect on cell cycle demonstrating the appropriateness of genipin as a crosslinker and genipin-crosslinked hydrogels as drug delivery systems for transdermal, and other, applications.

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Table 1 Parameters used for the determination of the molecular weight between crosslinks and crosslinking density

Parameter	**Value**
$\overline{M_1}$ (polymer average MW)	75,000 g/mol [47]
$\bar{v}$ (hydrogel specific volume)	0.910 cm^3/g
V_1 (molar volume of water)	18 ml/mol
$v_{2,s}$ (polymer volume fraction post-swelling)	Determined experimentally
$v_{2,r}$ (polymer volume fraction pre-swelling)	2.121 cm^3
I (ionic strength)	2.20 9 10-5 mol/cm^3
K_a (gelatin acid dissociation constant	3.981 9 10^{-4} [47]
K_b (gelatin base dissociation constant)	2.512 9 10^{-7} [47]
q_2 (density of hydrogel)	1.7591 g/cm^3
q_1 (density of water)	0.998 g/cm^3
v_1 (Flory–Huggins parameter)	0.49518 [24]

Table 2 Gelatin gel crosslinking index expressed as a percentage of free amino groups lost during crosslinking

Crosslinking temperature	**Crosslinking index (%) ± SD**
5 ^oC	20.48 ± 0.50
15 ^oC	76.49 ± 0.40
25 ^oC	88.01 ± 1.47

Data are expressed as arithmetic means ± standard deviation (SD) (n = 3)

Table 3 Flory–Rehner crosslink density q and the number-average molecular weight between crosslinks Mc of the gelatin gels crosslinked at 5, 15 and 25 °C

Crosslinking temperature	**q ± SD**	**Mc± SD**
5 °C	109 ± 6	692 ± 38
15 °C	304 ± 13	248 ± 11
25 °C	430 ± 43	176 ± 18

Values are expressed as means ± standard deviation (SD) (n = 3)

Table 4 Equilibrium water content (EWC) (%) of crosslinked gelatin gels

Crosslinking temperature	**EWS (%) ± SD**
5 °C	89.9 ± 2.6
15 °C	51.0 ± 1.3
25 °C	40.1 ± 1.1

All data are expressed as means ± standard deviation (SD) (n = 3)

Table 5 Swelling kinetics of crosslinked gelatin gels

Crosslinking temperature	**n ± SD**	**k ± SD**	**R^2**
5 ^{o}C	0.61 ± 0.01	0.190 ± 0.003	0.96
15 ^{o}C	0.63 ± 0.01	0.180 ± 0.001	0.98
25 ^{o}C	0.65 ± 0.02	0.160 ± 0.004	0.99

Data are shown as arithmetic means ± standard deviation (SD) (n = 3)

Table 6 Indomethacin release (%), diffusional exponent (n) and release constant (k)

Crosslinking temperature	Idn release (%) ± SD	**n ± SD**	**k ± SD**	**R^2**
5 ^{o}C	96.1 ± 0.46	0.72 ± 0.01	0.13 ± 0.01	0.97
15 ^{o}C	55.96 ± 1.04	0.82 ± 0.01	0.11 ± 0.02	0.99
25 ^{o}C	46.67 ± 0.24	0.86 ± 0.02	0.10 ± 0.01	0.99

Data are presented as arithmetic means ± SD (n = 3)

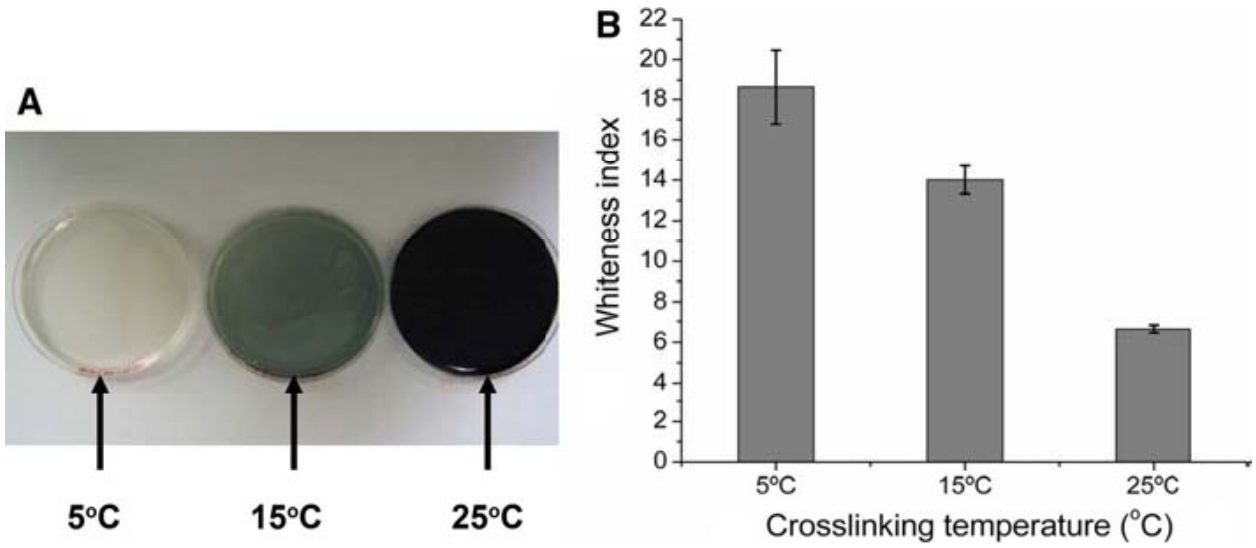

Fig. 1 Appearance (A) and whiteness index (B) of the genipin-crosslinked hydrogels after crosslinking at 5, 15 and 25 °C

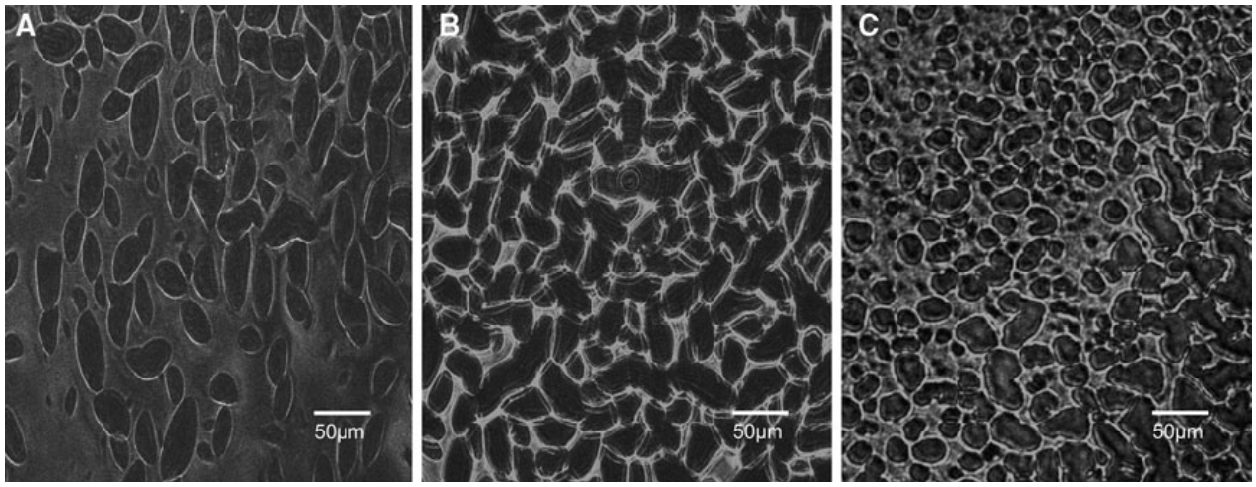

Fig. 2 CLSM micrographs of the genipin-crosslinked gelatin gels at 5 (A), 15 (B) and 25 °C (C). The size bar shown represents 50 μm

Source: J Mater Sci: Mater Med (2011) 22:115–123

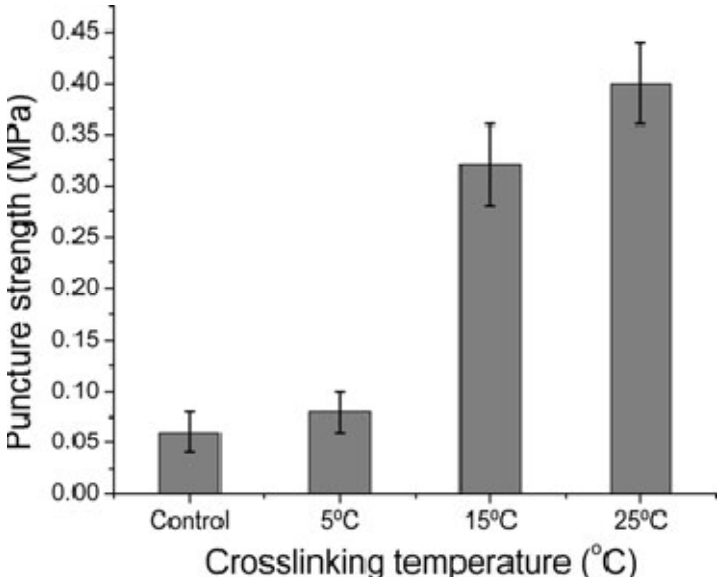

Fig. 3 Puncture strength of the hydrogels crosslinked at 5, 15 and 25 °C as well as a control gelatin gel. The values are expressed as arithmetic means ± SD (n = 3)

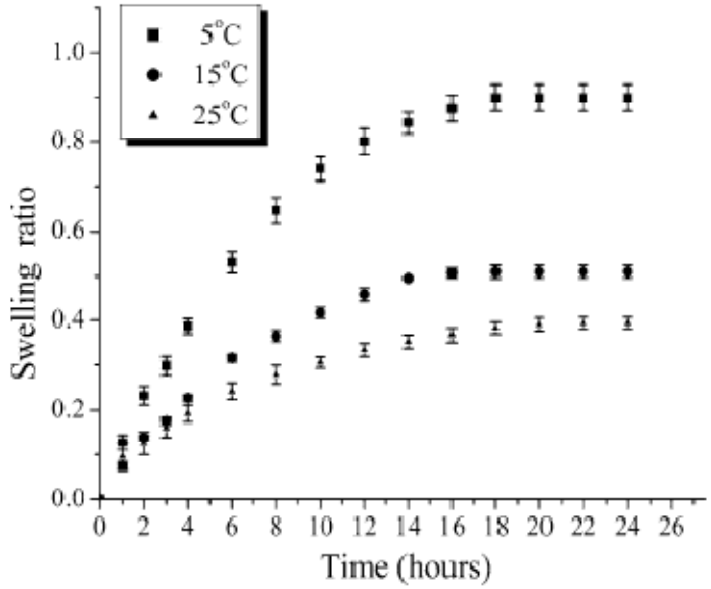

Fig. 4 Swelling ratio of the gelatin gels crosslinked at 5, 15 and 25 °C. Values are shown as arithmetic means ± SD (n = 3)

Source: J Mater Sci: Mater Med (2011) 22:115–123

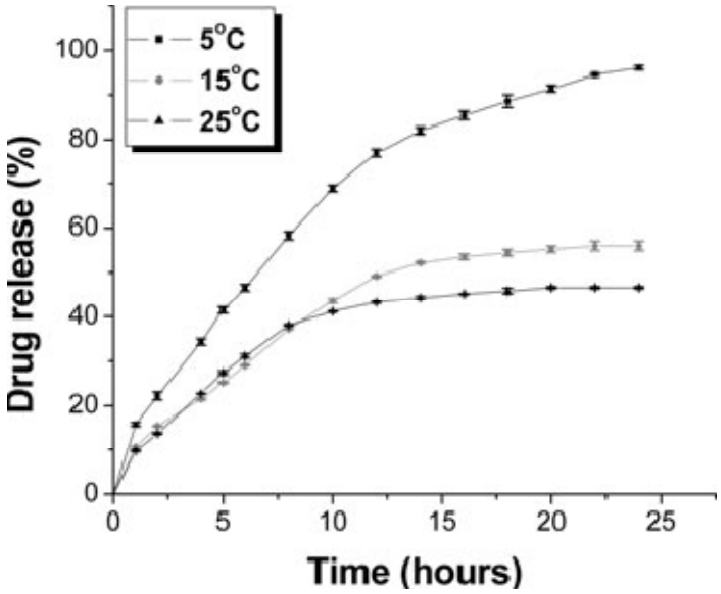

Fig. 5 Indomethacin release from the gelatin gels crosslinked at 5, 15 and 25 °C. Values are expressed as arithmetic means ± SD (n = 3)

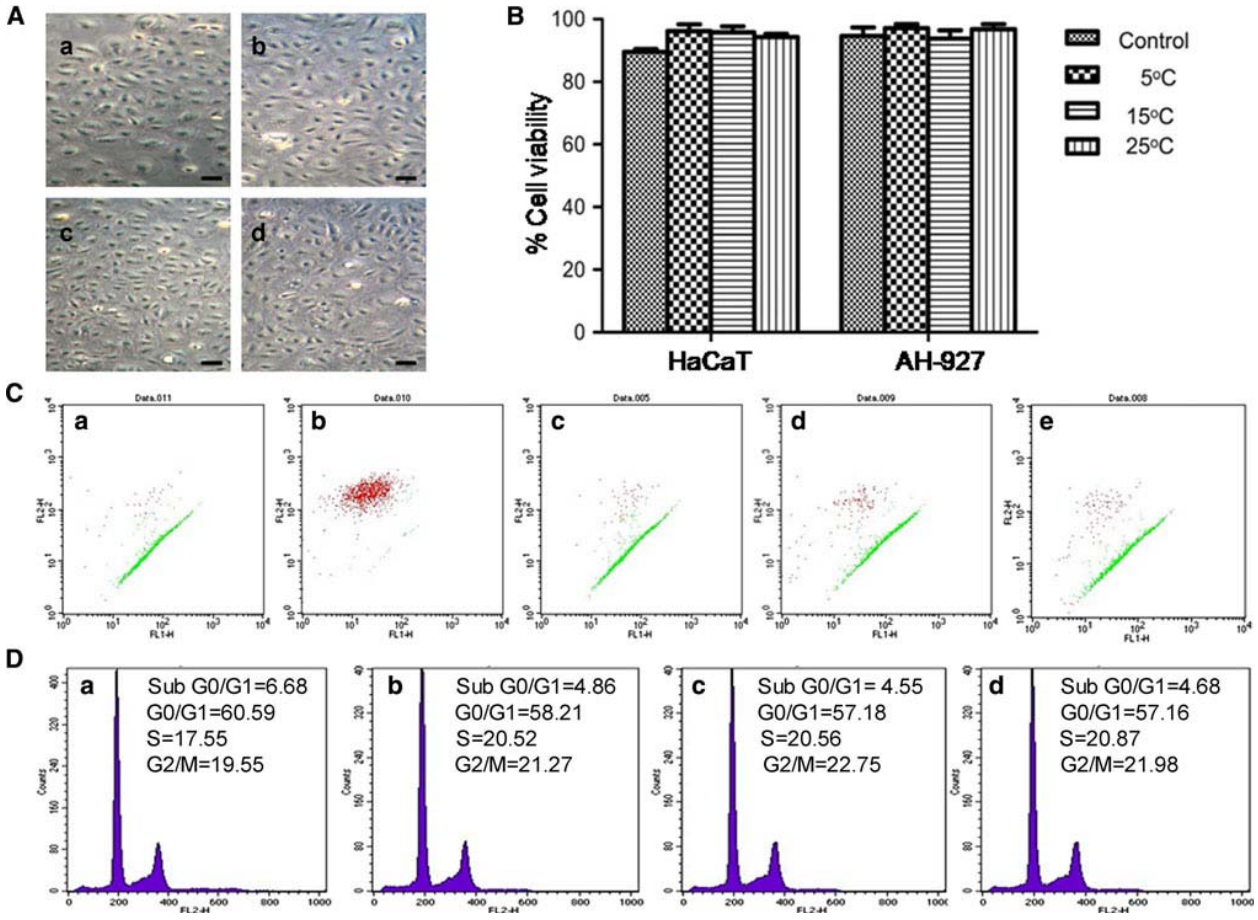

Fig. 6 (A) Cell morphology of HaCaT cells after 48 h of treatment with conditioned media: (a) control and conditioned media of constructs crosslinked at (b) 5, (c) 15 and (d) 25 °C; size bar represents 100 μm. (B) Cell viability using MTT assay on crosslinked gel constructs crosslinked at 5, 15 and 25 °C. Values are expressed as arithmetic means ± SD (n = 6). (C) Flow cytometry showing cell viability on the genipin-crosslinked hydrogels. Live cells in (a) negative control; dead cells in (b) positive control and crosslinked gels at (c) 5, (d) 15 and (e) 25 °C. (D) Cell cycle analysis of AH-927 cells treated with different genipin-crosslinked gels. (a–d) represents 2 days' growth; (a) control, (b–d) gels crosslinked at 5, 15 and 25 °C, respectively. Different cell cycle stages are shown: Sub G0, G0/G1, S and G2/M