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Plumbagin caged silver nanoparticle stabilized collagen scaffold for wound dressing

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The present work describes the development of a novel wound dressing material based on nano-biotechnological intervention by caging plumbagin on silver nanoparticle (PCSN) as a multi-site cross-linking agent of collagen scaffolds with potent anti-microbial and wound healing activity. Cross-linking of collagen with PCSN enhanced the physical, thermal, mechanical properties along with the kinetics of micro structural fibril assembly of collagen molecule. FTIR and CD analysis revealed that cross-linking of collagen using PCSN did not induce any structural changes in the collagen molecule. Further, cross-linking of collagen with PCSN resulted in uniform alignment of collagen fibrils to form orderly aligned porous structured scaffolds with potent anti-bacterial activity that in turn enhanced its ability to promote cell proliferation and wound healing. The cross-linking ability, biochemical and therapeutic properties of plumbagin caged silver nanoparticles was observed to be attributed by the cumulative effect of plumbagin and silver nanoparticle since individual molecules had minimal effect on these parameters.

Keywords: Collagen, Plumbagin, Silver nanoparticles, Cross-linking, Wound dressing.

Introduction

Collagen is the major structural protein present in the extracellular matrix providing mechanical strength and structural integrity to the cells and tissues. Collagen provides requisite strength and flexibility to cells and tissues enabling them to perform their normal physiological functions.[1] Hemocompatibility, low antigenicity, anti-inflammatory and cytotoxic properties of collagen makes it a desirable molecule for developing biomaterials for regenerative medicine and tissue engineering.[2,3] Furthermore collagen enhances cell attachment, cell growth and possesses advantageous characteristics of the natural extracellular matrix making it the most preferred candidate for biomaterial and biomimetic material.[4]

However, the use of native collagen as a biomaterial for tissue repair is hindered due to its low biomechanical stiffness and rapid biodegradation.[5] Further high enzymatic turnover rate of natural collagen *in vivo* and increased activation of MMPs by collagen necessitate stabilization of collagen for biomaterial applications.[6] Cross-linking of collagen is a commonly employed strategy to achieve desired mechanical properties in collagen for implant and other biomaterial applications.[7] Enzymatic and chemical agents are currently used cross-linking agents of collagen.[8] Glutaraldehyde is one of the most widely used chemical cross-linking agent. Glutaraldehyde cross-links collagen by bridging amino groups in the collagen molecule.[9] However, the presence of unreacted functional groups and release of those groups during enzymatic degradation from glutaraldehyde cross-linked collagen is associated with significant cytotoxicity *in vivo* that prevents its application as cross-linker for biomaterial development.[1,10] Chemicals that allows cross-linking of collagen without physical incorporation of the cross-linker are currently explored to overcome the setbacks associated with glutaraldehyde. 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide (EDC) or acylazide were developed to cross-link collagen without EDC getting incorporated into the collagen molecule.[11-14] EDC along with N-hydroxysuccinimide (NHS) generate peptide-like bonds in collagen enhancing the cross-links in collagen.[6, 15] Although the use of EDC and NHS for cross-linking collagen yield collagen matrix with good cyto-compatibility but unreacted carbodiimide leads to several side reactions.[16]

Biocompatible, ecologically safe cross-linking agents are therefore in great demand. Nutraceuticals and phyto-chemicals are gaining much current attention as cross-linking agents. In this direction there are reports using plant extracts from Myrica rubra as well as active components from plant sources like polyphenols (tannic acid,[17] catechin proanthocyanidin,[18] aldehydes (furfural),[19] aglycones (genipin)[20] etc. for stabilization of collagen.

Plumbagin is one such nutraceutical possessing several pharmacological properties namely anti-microbial, anti-malarial, anti-inflammatory, anti-carcinogenic, cardio-tonic, immune-suppressive, anti-fertility, neuroprotective and anti-atherosclerotic.[21,22] Many of the above mentioned properties which plumbagin possess reflect that it can have potent therapeutic application as a wound healing agent. The benefit of identifying such molecules is that it would result in obtaining molecules with both therapeutic properties as well as cross-linking ability. The beauty of such a molecule would be their

biocompatibility and the therapeutic properties that they possess. In case during collagen metabolism, if these nutraceuticals leach out from the biomaterial they may provide beneficial effects rather than inducing harmful side effect that is usually seen with chemical cross-linking agents. Further with two active functional keto group this molecule may be a promising candidate that may have good cross-linking capability. However use of plumbagin alone may lead to only a two point connections between the collagen molecules. It has been reported that multiple way cross-linking leads to formation of collagen scaffolds exhibiting novel properties. One of the possible ways to achieve multiple points cross-linking is through nano-biotechnological intervention where the nutraceutical caged on to the nanoparticle provides multiple sites for cross-linking with collagen molecule. In addition the physico-chemical properties of such conjugates will have the cumulative properties of both the nutraceutical and the nanoparticle.[23-27] In this manuscript we evaluated the effect of nano-caged plumbagin silver nanoparticles for the development of novel cross-linked collagen scaffolds for wound dressing applications.

Experimental Section

Synthesis of plumbagin caged silver nanoparticle

Plumbagin caged silver nanoparticles (PCSN) were prepared by oxido-reduction method as reported earlier using plumbagin and silver nitrate as the precursor.[28] 10mM of silver nitrate solution in sterile double distilled water was reacted with plumbagin (10mM) dissolved in 0.5M KOH at 25ºC. 0.5M KOH without plumbagin treated with 10mM silver nitrate solution served as control. Nanoparticles were collected by centrifugation at 8500rpm for 10 minutes. Nanoparticles were freeze dried using a lyophilizer to obtain fine structured nanoparticles.

Characterization of Nanoparticles

Powder XRD

X-ray diffraction pattern of the PCSN was measured using Bruker D8 advance diffractometer instrument with Cu κα 1.54Å radiation and the X-Ray diffraction pattern was detected using a Brukerlyrix eye detector. Measurement temperature and a slit size were set at 25ºC and 0.6 respectively for all measurements. The X-Ray diffraction spectra were recorded in the range 2θ from 10.0 to 60.0 with a stepwise increment of 0.02º and count time of 5s.

Electron microscopic analysis

The morphology of control nanoparticle and PCSN was determined by the Quanta 200 FEG scanning electron microscope (SEM). 1mg of synthesized nanoparticle was placed on the stub and coated with gold by sputtering for 2-3 mins. The samples were then scanned with high resolution electron beam at high vacuum mode.

The particle size and morphology of the nanoparticle was further confirmed by Transmission Electron Microscopy. For TEM analysis the samples were prepared by air drying the homogenous suspension of nanoparticles. The air dried samples were placed under electron beam in nitrogen atmosphere to acquire the high resolution TEM images.

Isolation of collagen

Acid soluble collagen was isolated by salt precipitation method. [29, 30] Briefly, the 6 month old Wistar rat tail tendons were teased out and lipid content was removed by diethyl ether : chloroform extraction. The tendons were then allowed to swell in 0.5 M acetic acid overnight. The tendons were ground and centrifuged to collect the supernatant. The supernatant was subjected to salt precipitation by gentle addition of 5% NaCl and centrifuged to collect the crude collagen. The crude collagen was dialyzed against 0.05M acetic acid to remove the excess salts to get pure collagen solution. The collagen solution was freeze dried using lyophilizer and stored at -20ºC.

Kinetics study of collagen fibril self-assembly

Collagen fibril formation is the kinetic process of self-organizing collagen fibrils to form helical structure. The assay was performed by treating and incubating overnight 3mg/ml collagen with PCSN, plumbagin, control silver nanoparticle and glutaraldehyde (reference group). Collagen fibrillation was initiated by adding the following components for 1000 µL of total reaction volume; collagen with and without cross linkers (750 µL), phosphate buffer (100 µL), 2M NaCl (75 µL), distilled H_2O (15 µL). The pH was adjusted to 7.2± 0.2 with 1% NaOH (60 µL). The mixture was immediately transferred into a quartz cuvette having 10 mm path length and the turbidity was measured using Perkin Elmer UV spectrophotometer at 313nm using time drive scanning mode for 100 mins at 32ºC. The rate of fibril formation was represented as $t_{1/2}$, where the turbidity was half at log phase of fibrillation curve. $t_{1/2}$ values were obtained by plotting optical density against time in minutes.

Degree of cross-linking

Cross-linking efficiency of PCSN on collagen scaffolds was determined by estimating the free amino group present in collagen after cross-linking with PCSN. The free amino group was estimated by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay. Cross-linked and native collagen samples (1-3 mg) were incubated for 30 min in 1 ml of 4 % (w/v) solution of $NaHCO_3$. To this mixture 1 ml of a freshly prepared solution of TNBS (0.5 wt %) in 4 % (w/v) $NaHCO_3$ was added. The reaction was allowed to proceed for 2h at 60°C. After two hours of incubation HCl (3 ml, 6 M) was added to the TNBS treated samples to solubilise the solid components in the reaction mixture. The temperature was set at 40ºC for solubilisation of the samples. The samples were diluted with distilled water and the absorbance was measured at 345nm using a Perklin Elmer UV-vis-spectrophotometer. The percentage cross-linking was calculated by the following formula

$$\text{Amount of cross linking (\%)} = 1 - \left[\frac{\text{Absorbance of cross linked Collagen}}{\text{Absorbance of native collagen}}\right] * 100$$

Biophysical analysis

Tensile strength

The tensile strength of the collagen films was measured using Instron tensile tester instrument. Collagen samples were prepared by cutting collagen films of uniform thickness having a length of 5 cm and width of 1 cm. The thickness of the sample was determined by digital vernier prior to tensile strength, elongation at break and modulus measurement. A load of 2N was applied and measuring speed was set at 5 mm/min with a relative humidity of 65% at 20°C.

Hydrodynamic study

Shear viscosity and shear stress were measured using Brookefield R/S+ Rheometer with coaxial measuring at various shear rates at 20ºC. Briefly, 0.3mg lyophilized collagen was dissolved in 1ml of 50mM acetic acid and treated with PCSN and glutaraldehyde. The solutions were kept overnight with continuous stirring at 4ºC to allow cross-linking. Collagen without any treatment was used as control and glutaraldehyde cross-linked collagen solution was used as a positive reference.

Water uptake study

Water uptake study was performed to determine the degradation rate and water holding capacity (permeability) of collagen scaffolds with and without cross-linkers. Briefly, GTA cross-linked collagen and collagen scaffolds cross-linked with 2.5µM PCSN were separately immersed in distilled water at room temperature for various time periods namely 1hr, 3hr and 6hr. After various time intervals the samples were removed from water and weighed. The water uptake of the matrices was calculated by the following equation

$$\text{Water uptake } (\%) = \left[\frac{(Ws - Wd)}{Wd}\right] * 100$$

where, W_d is the weight of the dry matrix and Ws is the weight of the wet matrix.

Conformational Analysis

Circular dichroism measurements (CD)

The influence of the PCSN on triple helical conformation of collagen was studied using Circular Dichroic spectropolarimeter using a 0.1 cm rectangular quartz cell in N_2 atmosphere at 25ºC. The samples for CD measurements were prepared by treating 0.5 ml of collagen solution (0.3mg/ml) with PCSN (2.5µM) and glutaraldehyde (50µM). The solutions were stirred at 4ºC for 4 hrs. The CD spectra of these solutions were recorded from 300 to 190 nm with scanning rate of 100nm/min. The band width of 1 nm, data pitch of 0.5 nm, and the response time of 1s, was set prior to the measurement. CD spectra were also recorded for collagen and 0.05 M acetic acid as control and blank respectively. The solvent spectrum (blank) was used as baseline that was normalised by subtracting it from the other collagen CD spectra. The spectra reported are the average of three scans. The CD spectra in mean degree units were converted into mean residual ellipticity (MRE) to calculate the Rpn ratio.

FTIR measurements

FTIR spectra of native collagen, plumbagin caged silver nanoparticle cross-linked collagen and glutaraldehyde cross-linked collagen sponges (served as a positive control) were recorded at transmission mode using a Spectrum Two Perkin-Elmer spectrophotometer. All the above mentioned samples were ground with KBr in the ratio of 1:100 and made into a pellet by Atlas Manual 15T Hydraulic Press pellet maker. Each sample was scanned from 800 to 4000 cm^{-1} with 8 scans per sample and a resolution of $1cm^{-1}$. The FTIR spectra were baseline corrected and normalized with the solvent spectrum (50mM acetic acid).

Morphology and permeability of collagen scaffolds

SEM analysis of collagen scaffold

0.3mg lyophilized collagen was weighed and solubilized in 1 mL of 50mM acetic acid. The collagen solution was then treated with PCSN and freeze dried to obtain collagen sponge. The collagen sponge was cut into small pieces (2mm) to analyze the structural organization of collagen by SEM analysis. Collagen without any treatment served as control.

Substance permeability assay

In vitro substance (Rhodamine B) permeability of collagen scaffolds were carried out to determine the porosity of cross-linked and non cross-linked collagen scaffolds. Permeability of various collagen scaffolds was determined by calculating the amount of Rhodamine B passed across the collagen scaffolds at various time intervals. The amount of dye in the medium that passed across the scaffolds was calculated by taking the absorbance of Rhodamine B at 550nm in Biorad Elisa Plate reader at different time interval.

Biological Applications

Cell compatibility assay

Collagen reconstituted with various concentrations ranging from 1µM to 5µM of PCSN was tested for cell viability by MTT assay.[31] Collagen after gelation was allowed to air dry in 48 well tissue culture plates to obtain a monolayer of collagen sheets. The collagen sheets were washed extensively with distilled water to remove acetic acid and other salts. The culture plates were exposed under UV light for surface sterilization. Approximately 12,000 Swiss 3T6 (mouse fibroblasts) cells were seeded into the culture well containing cross-linked and non cross-linked collagen scaffolds. The culture plates were then incubated in a CO_2 incubator. After 1, 3 and 7 days of incubation the culture medium was removed and the cells were treated with 0.5 mg/ml of 3- (4, 5dimethylthiazolyl – 2) - 2, 5-diphenyltetrazolium bromide (MTT) salt in PBS and incubated in dark at 37º C. After 4 hrs of incubation, the blue/ purple formazan crystals formed were solubilized using DMSO (dimethyl sulphoxide) and the absorbance was read at 570nm in Bio-Rad ELISA plate reader. The cell viability was calculated by the following formula

$$\%\text{ of Cell viability } = \left[\frac{\text{OD of treated culture}}{\text{OD of control}}\right] * 100$$

Antimicrobial activity

a. Disc diffusion method:-

Anti-microbial activity of collagen cross-linked with plumbagin and PCSN was assessed using disc diffusion method. Briefly, both plumbagin crosslinked collagen and plumbagin caged silver nanoparticles cross-linked collagen was coated on filter paper disc at concentrations (corresponding to plumbagin) ranging from 1 to 5 µM and allowed to air dry. The air dried samples were then incubated overnight by placing it into petri dishes containing E. *coli* and B. *subtilis* cultures. After incubation the inhibitory effect of the samples were calculated by measuring the clear zone formed around the sample discs.

b. Broth dilution method

Anti-microbial activity of collagen scaffolds was also tested using E. *coli* and B. *subtilis* by broth micro dilution assay using LB broth. Briefly the PCSN, plumbagin, silver nanoparticles and ampicillin (Positive control) with varying concentrations ranging from 1µM to 5µM was mixed with the 90 µL of LB broth. The culture inoculums (10µL) having cell density at $1x10^5$ cells were added to the samples in LB broth and incubated for 12 hours. After incubation the cell growth was calculated by measuring the turbidity (OD) at 630nm spectrophotometrically. The OD of the treated cultures was

normalized with blank (medium alone). The percentage growth inhibition was determined by the following formula

$$\text{Inhibition of growth}(\%) = \left[\frac{\text{OD of treated culture} - \text{OD of blank}}{\text{OD of control} - \text{OD of blank}}\right] * 100$$

Wound healing

Wound healing experiments were carried with prior approval of Institutional Animal Ethical Committee (CSIR-Central Leather Research Institute, Chennai) and were done in agreement with the guidelines for the proper use of animals for biomedical research. Male Wistar rats (body weight ranging from 100-150 g) were used for the study. Animals were acclimatized under standard animal laboratory condition for 7 days prior to the experiment. Animals were anesthetized by intra-peritoneal injection of ketamine/xylazine. The dorsal hair was shaved off and disinfected with spirit. Open excision wound was created by gently cutting off the dorsal side of the skin. The wound size was traced and photographed to measure the wound area. The wounds were then treated with plumbagin caged silver nanoparticle cross-linked collagen (biocomposite/scaffold -Test group) and the other group consisting of 5 rats were treated with collagen (reference group). The 3rd group containing 5 rats were used as untreated controls (control group). The test materials were applied on excised wounds that was then covered and tied with absorbent sterile gauze. The dressing material was replaced every 3rd day after cleaning the wound with sterile saline solution. The experiment was performed for 21 days. The reduction of wound size was marked and photographed. The wound area was calculated using imageJ software. The percentage wound reduction was calculated according to the following formula.[32]

$$Cn = \left[\frac{(So - Sn)}{So}\right] * 100$$

where, Cn is the percentage of wound size-reduction on days 0, 8 and 15 after treatment, So – initial wound size, Sn – wound size on day 0, 8 and 15 after treatment.

Statistical Analysis

All the experiments were carried out in triplicates and the data given are expressed as mean ± standard deviation (S.D). Statistical analysis was performed using SPSS 12.0 software. A value of $P<0.05$ was considered significant.

Results and Discussion

Characterization of plumbagin caged silver nanoparticle

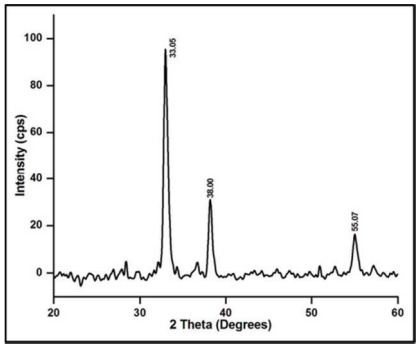


Figure 1 X ray diffraction pattern of PCSN.

X ray diffraction studies were performed to characterize PCSN. Figure 1 shows the XRD pattern of PCSN. The XRD spectra showed characteristic peaks at 33.05, 38.00 and 55.07 degrees representing the presence of silver in crystalline phase

The morphology and size of the silver nanoparticle was analyzed by the scanning electron microscopy with a magnification of 100,000 X. Figure 2a shows the scanning electron microscopic images of control silver oxide nanoparticle and Figure 2b represents plumbagin caged silver nanoparticle. The SEM images clearly indicated that the morphology of the nanoparticles were observed to be spherical and compared with the control nanoparticle the plumbagin caged silver nanoparticle was smaller in size. The result clearly demonstrated that the conjugation of plumbagin on silver nanoparticle did not change the morphology of the nanoparticles. Further it was observed that the size of the control nanoparticles was quite larger than plumbagin caged silver nanoparticles. The diversity or dispersity was observed to be greater in plumbagin caged silver nanoparticle when compared with control silver nanoparticle.

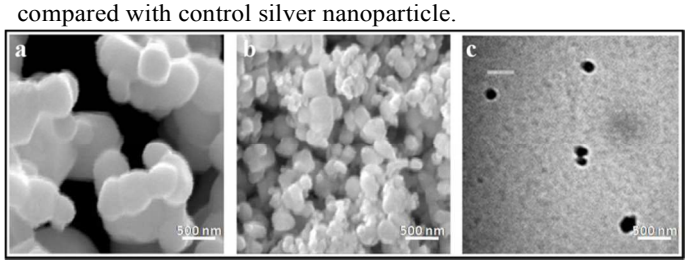


Figure 2 Electron microscopy analysis of nanoparticles. a) SEM images of control silver nanoparticle and b) SEM images of plumbagin caged nanoparticle at 100000X magnification. c) TEM images of PCSN at 65000X magnification. Scale bar represents 500 nm.

Figure 2c shows the transmission electron microscopic image of the plumbagin caged silver nanoparticle. The results indicated that the nanoparticles were having spherical morphology and the size of the particle was observed to be 60nm. The TEM results were consistent with SEM and XRD analysis. Further, appearance of translucent covering on the surface of the nanoparticles indicated a uniform capping of plumbagin onto silver nanoparticle surface.

Kinetics of self-assembly process of collagen for scaffold fabrication and cross-linking efficiency

In order to elucidate the cross-linking efficiency of PCSN nanoparticle, the *invitro* fibrillogenesis assay was performed.

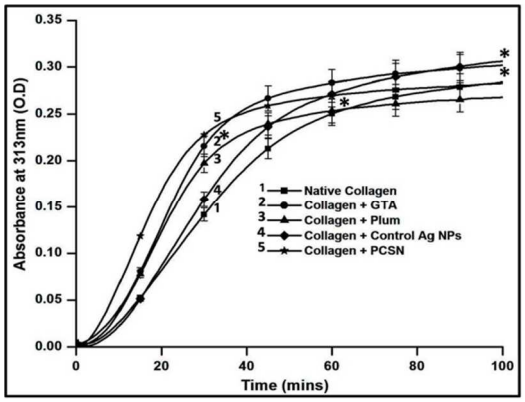


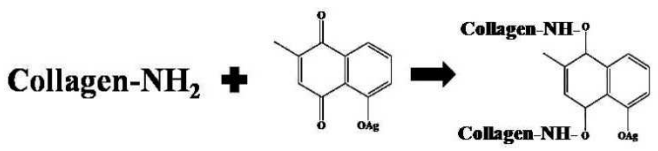
Figure 3 Turbidometric analysis to determine the kinetics of fibril formation of collagen with various cross-linking agents like glutaraldehyde (GTA), plumbagin (plum), control silver nanoparticles (Control Ag NPs) and plumbagin caged silver nanoparticels (PCSN). * Statistically significant compared to Native Collagen.($p<0.05$)

Figure 3 depicts the rate of fibril formation in presence of various cross linking agent. The rate of fibril formation was evaluated based on the measurement of turbidity at the time $t_{1/2}$. $t_{1/2}$ represents half the time required for fibril formation at log phase of fibrillation curve. In figure 3, Curve 1 showed that the collagen without any cross-linking had lesser turbidity as indicated by a lower absorption maxima at 40 min whereas PCSN cross-linked collagen bio-composites (curve 5) showed higher absorption maxima at 40 min when compared with native collagen as well as control silver nanoparticle cross linked collagen scaffolds (curve 4). Curve 2 showed that the collagen cross-linked with glutaraldehyde (considered as a positive control or reference) exhibited extensive fibril formation at $t_{1/2}$ that was almost similar to PCSN cross linked collagen scaffolds. Further the degree of cross-linking was assessed by estimating the free amino group present in the cross-linked and uncross-linked collagen scaffolds by TNBS assay. The results are given in Table 1.

	Absorbance at 345nm (Mean ± S.D)	Free amino group	% of Free amino group	Reactive amino group	% of reactive amino group
Native Collagen	0.33±0.04	1	100	0	0
Collagen + GTA	0.17±0.04	0.51	51	0.49	49
Collagen +1.25µM PCSN	0.16±0.01*	0.48*	48*	0.52*	52*
Collagen +2.5µM PCSN	0.1±0.03*#	0.28*#	28*#	0.72*#	72*#
Collagen +5µM PCSN	0.05±0.01*#	0.15*#	15*#	0.85*#	85*#

Table 1. Degree of cross linking of native collagen, glutaraldehyde (GTA) and different concentrations of plumbagin caged silver nanoparticles (PCSN). * Statistically significant compared to native collagen $p<0.05$. # Statistically significant compared to glutaraldehyde cross linked collagen ($p<0.05$)

The results showed a concentration dependent decrease in the amount of free amino group in collagen scaffolds cross-linked with various concentrations of PCSN. The amount of free amino group present in collagen scaffolds cross-linked with 1.25µM, 2.5µM and 5µM PCSN was observed to be 48%, 28% and 15% respectively. The decrease in free amino content clearly suggested that the cross-linking of PCSN involved the amino group coupling with the side chain of plumbagin. The mechanism of cross-linking is proposed to be as given below.



Cross-linking indicates that the monomeric collagen molecules get aggregated to form multimeric.[33] Numerous reports suggested that metal complexes support the cross-linking by forming intermolecular hydrogen bridges with free residual amino groups of the collagen molecule.[34,35] Our results are consistent with above observation since we observed enhanced cross-linking of collagen in presence of plumbagin caged silver nanoparticle. PCSN enhanced inter and intra molecular interactions with free amino groups of collagen fibrils to form the quarter staggered arrangement aiding in establishing the collagen triple helical structure. The results from TNBS assay indicated that the crosslinking may be due to the Schiff's base formation involving the amino group in collagen molecules with the keto group in plumbagin.[9] Further the possibility of electrostatic interaction between the silver nanoparticles and hydrogen bonding may also contribute to enhance cross-linking of collagen in presence of PCSN. It has been reported that metal nanoparticles enhanced crosslinking via electrostatic interactions.[36-39] The cross-linking efficiency of plumbagin and control silver nanoparticles were lower when compared to PCSN treated collagen scaffolds but higher than collagen control. The increased cross-linking of collagen observed in presence of PCSN may be due to the cumulative effect of plumbagin and silver nanoparticles where the transition metal silver in the PCSN acts as promoter or catalyst to speed up the cross-linking of collagen and the plumbagin caged on PCSN would participate in the extensive fibril formation. Further caging of plumbagin on silver nanoparticle would provide multiple sites for interacting with collagen molecule.

Water uptake study was carried out to reveal the cross-linking levels and water holding capacity of the scaffolds. The assay was performed to assess the level of crosslinking since it was essential for optimisation of cross-linking density for comparison of other biophysical and biological properties. The results are given in table 2.

	Collagen +GTA	Collagen + 2.5 µM PCSN
1hour (Wt%)	248±14	216 ±0
3hours (Wt%)	452±7	408 ±0
6hours (Wt%)	848±14	896±70

Table 2. Water uptake of glutaraldehyde (GTA) and plumbagin caged silver nanoparticles (PCSN) cross linked collagen scaffolds.

The results indicated that the weight percentage increase in PCSN cross-linked scaffolds and GTA cross-linked collagen was almost similar. The water uptake percentage in PCSN cross-linked collagen and GTA cross-linked collagen at 1, 3 and 6 hours were 216%, 408%, 896% and 248%, 452%, 848% respectively. These results indicated a sustained uptake of water and prolonged incubation enhanced the water uptake due to the establishment of hydrogen linkages in the micro structural assembly in PCSN and GTA cross-linked collagen constructs. The results further suggested that the cross-linking levels of GTA and PCSN cross-linked collagen were similar. The thermo gravimetric analysis results were also found to be consistent with water uptake assay.[40, 41] The results are given in supplementary figure 1.

Biomechanical properties of bio-composites

Table 3 summarizes the biomechanical parameters of native collagen, glutaraldehyde (positive control) cross-linked collagen and collagen scaffold cross-linked with different concentrations of PCSN.

	Tensile Strength (Mpa)	Elongation at Break (%)	Extension at Max. Load (mm)	Modulus (MPa)
Native Collagen	1.65±0.12	5.25±2.2	1.15±0.07	5.67±2.04
Collagen + GTA	7.83±0.50	8.16±3	1.75±0.2	17.75±8.70
Collagen + 1.25µM PCSN	8.25±0.30*	5.33±0.7	1.77±0.035	14.24±3.86*
Collagen + 2.5µM PCSN	8.69±1.70*	8.39±0.8*#	2.24±0.007*#	23.5±0.70*#
Collagen + 5µM PCSN	17.54±1.20*#	10.33±4.24*#	3.10±1.28*#	33.5±0.70*#

Table 3. Biomechanical properties of native collagen, glutaraldehyde (GTA) and various concentrations of Plumbagin caged silver nanoparticles (PCSN) cross linked Collagen scaffolds. * Statistically significant compared to native collagen $p<0.05$. # Statistically significant compared to glutaraldehyde cross linked collagen $p<0.05$.

The results indicated a concentration dependent increase in the tensile strength and modulus of collagen scaffolds cross-linked with PCSN which clearly demonstrated that the PCSN cross-linking enhanced the biomechanical properties of the collagen scaffolds in a concentration dependent manner. PCSN cross-linked collagen showed higher biomechanical property with respect to tensile strength, elongation at break, extension at maximum load and Young's modulus which indicated that the hydrogen bonding in collagen is conserved. It has been reported that the hydrogen bonds in collagen molecule is so important and a good cross-linking agent should avoid the loss of hydrogen linkages.[42,43] The dehydration process may change the structure of collagen network by deducing the space between molecules as well as affecting the inter- and intra-molecular chemical bonds.[44] PCSN facilitates to establish hydrogen bridges, covalent and electrostatic interactions stronger enough by introducing linkages with their side chain and collagen.[9] We assume that this could be the plausible reason for PCSN having higher tensile strength and elasticity.

Hydrodynamic properties of PCSN cross-linked Collagen

Hydrodynamic properties of the native collagen, PCSN cross-linked collagen and glutaraldehyde cross-linked collagen was studied by analyzing its rheological properties namely shear stress and shear viscosity over shear rate with an interval of 1 second from 0 to 500s. Figure 4a represents the rheogram of shear stress versus shear rate. It was observed that the shear stress increased with increase in shear rate in all groups namely native collagen, PCSN cross-linked collagen and glutaraldehyde cross-linked collagen but the shear stress (i.e pressure, Pa) varied among native collagen, PCSN cross-linked collagen and glutaraldehyde cross-linked collagen. Glutaraldehyde cross-linked collagen and PCSN cross-linked collagen was more resistant to shear stress than native collagen. Figure 4b represents the relative viscosity of native collagen, collagen cross-linked with PCSN and glutaraldehyde. The results showed that the viscosity of PCSN cross-linked collagen was higher when compared with the native collagen.

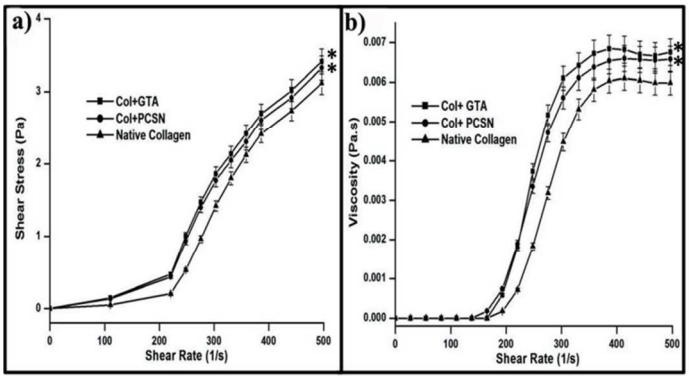


Figure 4 Rheological analysis. a).Shear Stress Vs Shear rate. b). Viscosity Vs Shear Rate of native collagen, glutaraldehyde (GTA) and plumbagin caged silver nanoparticles (PCSN) cross-linked collagen.* Statistically significant compared to native collagen. ($p<0.05$)

The increased shear stress and shear viscosity may be attributed to the cross-linking ability of PCSN. The shear stress and shear viscosity of glutaraldehyde cross-linked collagen was more or less equal to that of PCSN cross-linked collagen which indicated that the cross-linking ability of PCSN was almost similar to that of glutaraldehyde. Cross-linking of PCSN could be attributed to their ability to induce fibril formation in aqueous medium. Our study showed that PCSN induce rapid cross-linking of collagen when compared with native collagen. The micro-fibrils formation and aggregation significantly contribute to the viscoelastic properties of collagen.[45] PCSN promoted collagen fibril-fibril contact that enhanced fibril cross-linking and aggregation in solution. It is also possible that micro fibrils can form a stabilizing layer around a larger fibers that results in highly dense collagen fibers. It has been reported that highly dense collagen fibers gets converted to branched clusters by intermolecular bonds that results in forming a rigid fibrillar network (viscous medium) that is more resistant to deformation and flow than native collagen.[46] Further increased cross-linking between collagen micro fibrils and cross-linker would cause dehydration since new linkages would make the collagen more rigid.[47]

Conformational Studies

FTIR

A good cross-linking agent should conserve the structural and conformational integrity of collagen after cross-linking. It is essential to analyze the structural changes in collagen after cross-linking because the structural and conformational change in collagen could alter the physiological function by reacting with other bio macromolecules when used as a biomaterial. Figure 5 shows the FTIR spectra of native collagen, glutaraldehyde and PCSN cross-linked collagen. We observed peaks at 1650 cm^{-1} characteristic of amide I vibrations of amide carbonyl (C=O) groups along the polypeptide backbone. The FTIR spectra from all the three samples had peaks at similar position. The occurrence of peak at 1650-1655 cm^{-1} indicated that no disruption of alpha helix has occurred after cross-linking collagen with PCSN. The peak at 1553cm^{-1} indicated the amide II bands corresponding to N−H bending. The appearance of peaks at 1650-1655cm^{-1} and 1553cm^{-1} clearly demonstrated that the collagen molecules existed in poly proline type II (PPII) triple helical form. It has been reported

that intact α-helix has absorption maximum at 1650-1655cm^{-1} and disruption of alpha helical structure results in shifting of the absorbance maximum towards higher frequency (1665cm^{-1}).[48]

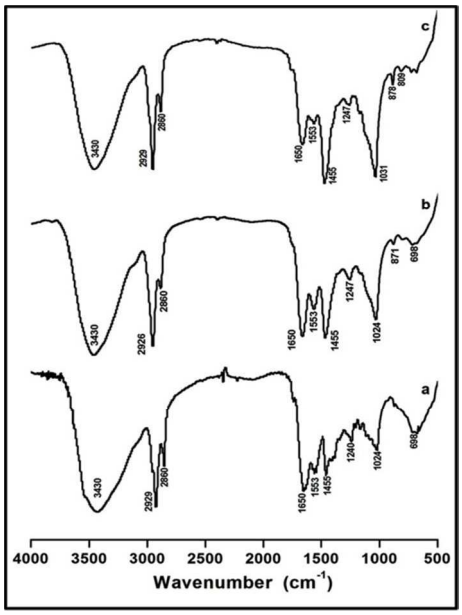


Figure 5 FTIR Spectra of a. Native Collagen, b. PCSN cross-linked Collagen c. Glutaraldehyde cross-linked collagen.

Further, peaks at 3430cm^{-1} and 1247cm^{-1} corresponding to amide A and amide III were also observed in FTIR spectrum of all the three samples. The occurrence of all the significant peaks in PCSN cross-linked collagen demonstrated that the collagen integrity was not disturbed in presence of PCSN. FTIR analysis of collagen indicated that the PCSN did not influence any structural changes in collagen after cross-linking. Amide II band of glutaraldehyde cross-linked and PCSN cross-linked collagen showed a slight change in the intensity when compared with native collagen which may be due to the interaction of aldehyde groups of glutaraldehyde, OH and C=O in plumbagin with collagen respectively.

Circular Dichroism

Circular dichroic measurements further confirmed the conformational integrity of collagen after cross-linking with PCSN.[49] The CD spectra of collagen cross-linked with PCSN as well as native collagen are given in Figure 6.

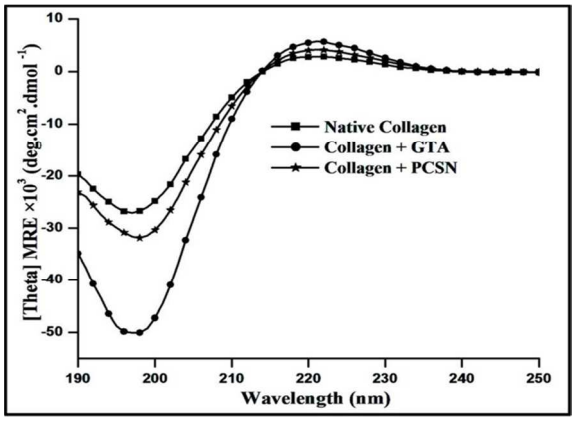


Figure 6 Circular dichroic spectra of native collagen, glutaraldehyde and PCSN cross-linked collagen.

The dichroic spectra showed a negative peak at 198 nm and a positive peak at 222 nm in all the three samples. A positive cross over was observed at 215 nm for all the samples. Proteins and polypeptides have CD bands in far ultraviolet region (178–260 m) that may arise mainly from amide bond of the protein backbone and are sensitive to their conformations. The presence of peaks at 198nm and 222nm characteristic of PPII like secondary structure indicated that the structural integrity of collagen was conserved after cross-linking with PCSN.[50] We further analysed the presence and integrity of triple helical structure of collagen after cross-linking by estimating the Rpn ratio. Rpn ratio is the ratio of positive peak over to negative peak at the peak maxima i.e; 222 and 198 respectively.[34]

	Rpn Ratio
Native Collagen	0.10
Collagen + GTA	0.11
Collagen+ 2.5μM PCSN	0.12

Table 4 Rpn ratio of native collagen, glutaraldehyde (GTA) and plumbagin caged silver nanoparticles (PCSN) cross linked collagen.

The Rpn ratios are provided in Table.4 The results indicated that the Rpn ratio of the collagen, collagen treated with glutaraldehyde and PCSN cross-linked collagen had no significant changes (less than 0.02). It clearly demonstrated that PCSN does not make any conformational changes in collagen when used as a cross-linking agent.

Surface morphology of PCSN cross-linked collagen

Figure 7 shows the scanning electron microphotographs of collagen scaffolds cross linked with PCSN and native collagen.

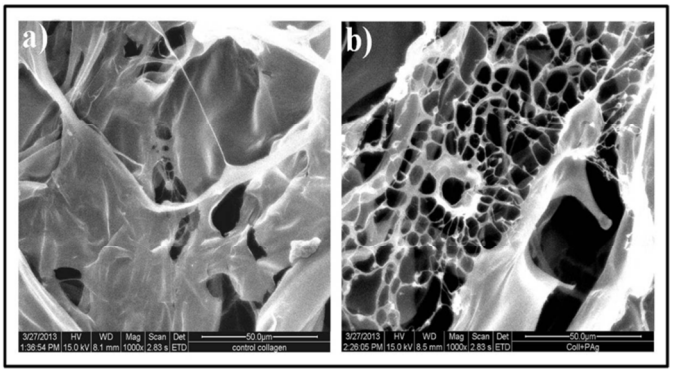


Figure 7 Morphological characterization of native collagen (a), PCSN cross-linked collagen scaffold (b) by Scanning electron microscopy. Scale bar represents 50μm and magnification at 1000X.

The result shows the microstructural features of native collagen (a) and PCSN cross-linked collagen scaffold (b). The cross section of freeze dried native collagen showed sheet like morphology. The irregularity and larger pore size was found in native collagen on the other hand PCSN cross-linked collagen showed a network like arrangement of collagen fibrils and uniform pore distribution. The formation of properly aligned uniform porous structure in scaffolds are crucial in determining the ability of the scaffolds for sustained substance transport across the scaffolds that plays a critical role in development of a good wound dressing material. In order to determine the substance transport efficiency of collagen scaffolds cross-linked with PCSN, the permeability of the scaffolds was analysed by dye penetration method using Rhodamine B. The results are given in supplementary figure 2 that represents the permeability

of the collagen cross-linked with 1.25µM, 2.5µM and 5µM PCSN, GTA cross-linked collagen and native collagen scaffolds. The results showed a sustained release of dye with time. Uncross-linked collagen scaffolds showed a rapid release of dyes because of the sheet like morphology as observed in SEM analysis whereas in PCSN cross-linked collagen a sustained release of dye was observed indicating a well organised pore formation in these scaffolds. Surface morphology of PCSN cross-linked collagen showed network like-fibrillar assembly which may be due to the efficient cross-linking of PCSN on collagen by inter and intra molecular linkages with their free functional group especially hydroxyl and carbonyl groups. The ordered micro structural arrangement of PCSN cross-linked collagen bio-composite would enhance the permeability of the scaffolds which attributed to cell proliferation,[51] nutrient transport, waste removal and facilitate the sustained release of therapeutic agents onsite of the wound area which would enhance the wound healing process.[52, 53]

Biological applications

Cell compatibility of scaffold

Cell proliferation and compatibility of the collagen scaffolds are crucial factor for its application in tissue engineering. MTT assay was carried out to assess cell compatibility and the results are given in Figure 8.

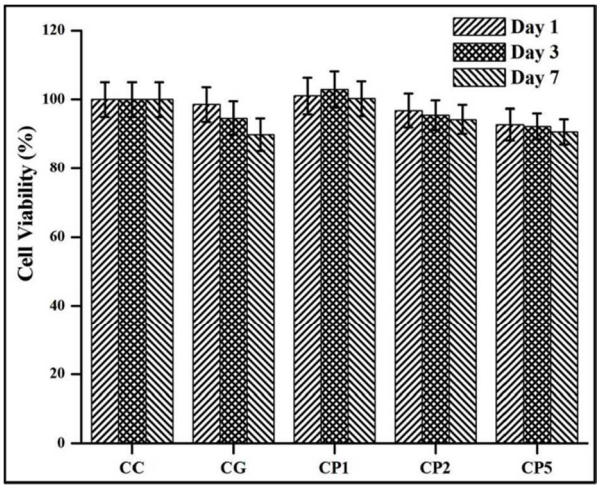


Figure 8 *Invitro* Cell compatibility of collagen scaffolds cross-linked with plumbagin caged silver nanoparticle. Swiss 3T6 mouse fibroblast cells were cultured on collagen scaffolds cross linked with various concentrations of PCSN (1to 5 µM) for 1, 3 and 7 day. CC, CG, CP1, CP2 and CP5 represents native collagen, glutaraldehyde and PCSN cross-linked collagen at 1.25µM, 2.5µM and 5µM concentrations respectively. After incubation the cell viability was analyzed using MTT assay. The bar diagram represents the percentage cell viability.

The results showed that there was no significant toxicity observed in collagen scaffolds cross-linked with various concentrations of PCSN (1.25µM, 2.5µM and 5µM). The cell viability was observed to be above 90% in collagen scaffolds cross-linked with various concentrations of PCSN. (Concentration used was based on the amount of plumbagin solubilized from PCSN). These results indicated that the PCSN cross-linked collagen had no cytotoxic effect and it promoted cell proliferation presumably due to the orderly arranged micro porous structure that facilitated adhesion and 3-dimensional microenvironment for the proliferation of cells.

Antimicrobial activity

Figure 9.b and 9.c shows the anti-bacterial effect of PCSN cross-linked collagen and collagen cross-linked with plumbagin on E. *coli* by Disc diffusion method. The zone of inhibition was observed to be higher in PCSN treated groups. The results showed a significant inhibition of microbial growth in PCSN treated cultures even at concentration as low as 1.25µM. A concentration dependent increase in the inhibitory activity was observed in PCSN cross-linked collagen treated groups. Plumbagin cross-linked collagen treated cultures showed no inhibitory effect at all the concentration tested (1-5 µM). The inhibitory effect of PCSN and plumbagin cross-linked collagen on B. *subtilis* is shown in Figure 9.d and 9.e. The results showed a similar trend as observed with *E. coli*. We observed that B. *subtilis* showed more susceptibility to PCSN when compared with E. *coli* cells. Figure 9.a served as blank.

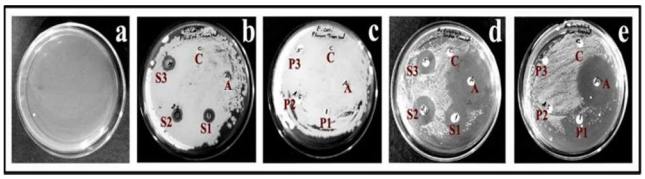


Figure 9 Disc diffusion method for assessing antimicrobial activity on E. *coli* and B. *subtilis*. a) Represents blank, b)-Inhibitory effect of PCSN cross-linked collagen on E. *coli*, c) Inhibitory effect of plumbagin cross-linked collagen on E. *coli*. d) Inhibitory effect of PCSN cross-linked collagen on B. *subtilis*. e) Inhibitory effect of plumbagin cross-linked collagen on B. *subtilis*. C - Control, A - Positive control (Ampicillin) and S1, S2 and S3 - PCSN cross-linked collagen at 1.25µM, 2.5µM and 5µM concentration respectively (With reference to plumbagin). P1, P2 and P3 – plumbagin cross-linked collagen at 1.25µM, 2.5µM and 5µM concentration respectively.

Broth dilution assay was further performed to confirm the antimicrobial activity of the PCSN. Figure 10 shows the percentage microbial growth on treatment with plumbagin and PCSN at various concentrations ranging from 1.25µM, 2.5µM and 5µM against E. *coli* (gram-negative) and B. *subtilis* (gram-positive) strains by micro dilution techniques.

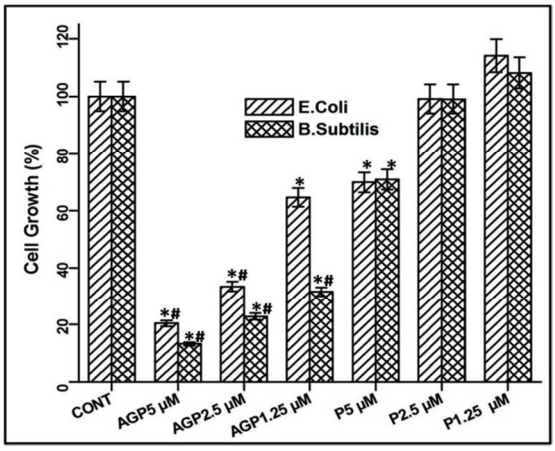


Figure 10 Graphical representations of broth dilution method for assessment of inhibitory effect of PCSN cross-linked scaffolds on E.*coli* and B. s*ubtilis*. * Statistically significant compared to control ($p<0.05$). # Statistically significant compared to plumbagin treatment. ($p<0.05$)

The PCSN treated microbial cultures had lower minimal inhibitory concentration (MIC) values. PCSN treated E. *coli* cells showed a growth rate of 60%, 35% and 20% for concentrations at 1.25µM, 2.5µM and 5µM respectively whereas in case of plumbagin treated cultures the growth rate

was not affected at 1.25µM, 2.5µM treatments but only 70% growth was observed at 5µM concentration. On the other hand PCSN treatment on B. *subtilis* strain showed a growth rate of 30%, 20% and 15% at 1.25µM, 2.5µM and 5µM respectively. Plumbagin treated B. *subtilis* cultures showed the same growth trend that was observed with E. *coli*. The results indicated that the PCSN had significant effect on both gram positive and gram negative bacteria. The inhibitory effect was attributed to the cumulative effect of both plumbagin and silver nanoparticles in PCSN. It has been reported that silver nanoparticle exhibited potent anti-microbial activity.[54]

Wound healing efficiency

Figure 11a shows the microphotographs of the open excision wound assay carried out using male wistar rat as animal model to test the wound healing efficacy of collagen scaffolds cross-linked with PCSN. Figure 11b represents the percentage wound reduction at various time periods from day 1 to Day 15.

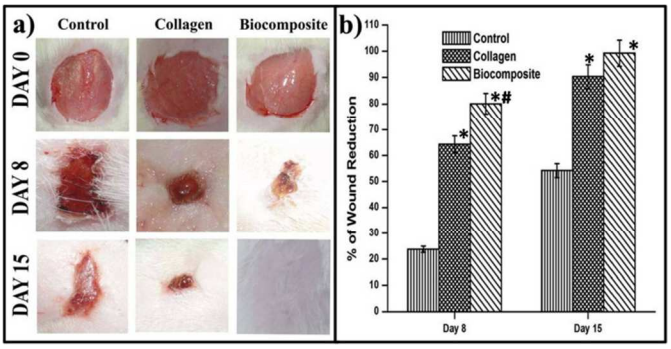


Figure 11a. Microphotographs of open excision wound assay on wistar rats to evaluate the wound healing efficiency of PCSN cross-linked and native collagen. Scale bar represents 10 µm. b. The bar diagram represents the wound reduction rate of native and PCSN cross-linked collagen.* Statistically significant compared to untreated control groups ($p<0.05$). #Statistically significant compared to native Collagen scaffold treated groups ($p<0.05$).

PCSN cross-linked collagen had the better wound healing property than native collagen control groups. On day 8 there was 80% wound reduction in the PCSN cross-linked collagen whereas in collagen and control groups wound size reduction was observed to be 65 and 24% respectively. At day15 in the PCSN cross-linked collagen treated groups a complete wound closure was observed when compared with collagen treated and control groups. The reduction in wound size rate was found to be 85% and 50% in collagen treated and control groups respectively. The increase in wound healing efficacy observed with PCSN cross-linked collagen may be due to the cumulative effects of silver nanoparticle and plumbagin. The enhanced wound healing was attributed to the effect of antimicrobial activity. It has been reported that molecules that cause reduction in the number of pathogens and the inflammatory response in a wound promotes wound healing.[55]

Conclusions

In the present investigation we have attempted to design a cross-linker with the use of nano-biotechnological approach by caging nutraceutical plumbagin on silver nanoparticles. PCSN enhanced the stabilization and cross-linking of collagen by multi-site interaction with collagen molecules and also simultaneously imparted therapeutical properties of both plumbagin and silver nanoparticles on collagen scaffolds that added therapeutic values to the collagen scaffolds as indicated by an increased antimicrobial and wound healing activity. We demonstrated that cross-linking efficiency in terms of biomechanical, thermodynamic, hydrodynamic parameters of collagen significantly increased on cross-linking with PCSN. Conformational studies showed that PCSN did not induce any structural changes in collagen after cross-linking. Further the cumulative expression of therapeutical properties of silver and plumbagin namely anti-bacterial and pro wound healing activities in the PCSN cross-linked collagen scaffold suggest the importance of nano- biotechnological approaches for development of biomaterials for bio medical and tissue engineering applications.

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Notes and references

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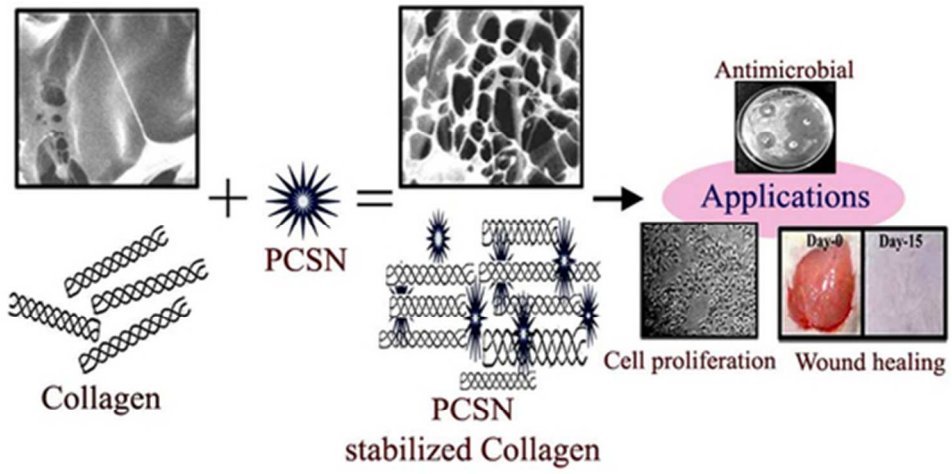

The table of contents entry:

Wound dressing material based on nano-biotechnological intervention by caging plumbagin on silver nanoparticle (PCSN) as a multi-site cross-linking agent of collagen scaffolds with potent anti-microbial and wound healing activity.
47x26mm (300 x 300 DPI)