Adipic Acid Interaction Enhances the Mechanical and Thermal Stability of Natural Polymers

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ABSTRACT: Application of biopolymers for biomedical applications limits with the functional and structural properties of the materials. Crosslinkers are used to provide the requisite properties. The question on biocompatibility of the existing crosslinkers necessitates the need for alternative crosslinkers. In the present study, adipic acid was chosen as crosslinker and evaluated the mode of interaction with chitosan and type-I collagen. A 3D scaffold biopolymer material was prepared using chitosan at 1.0% (w/v) and adipic acid at 0.2% (w/v), similarly collagen 0.5% (w/v) and adipic acid 0.2% (w/v) displayed an improved mechanical strength and in addition found biocompatible for NIH 3T3 fibroblast cells. The chemistry behind the interaction and the characteristics of the biopolymer material obtained upon crosslinking suggests that noncovalent interactions play the major role in deciding the property of the said materials and their suitability for biomedical applications. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: noncovalent interaction; adipic acid; chitosan; collagen; mechanical strength; biocompatible; thermal stability

INTRODUCTION

Recent research on biomaterial development suggests that biopolymers of natural origin find immense clinical applications. Most of the research publications discussed the significant role of natural polymers, chitosan, and collagen in biomedical applications.[1–4]

Both of these materials, chitosan and collagen, after extraction did not have much stability to act as biomaterial for clinical applications and the use of stabilizers in the form of crosslinkers is in practice. Diisocyanates, resimene,[5] *N,N*-disuccinimidyl subarate,[6] epichlorohydrin,[7] Genipin,[8] and glutaraldehyde (GA)[9] were, the stabilizers for chitosan and chromium,[10] aldehydes,[11] hexamethylene diisocyanate,[12] carbodiimide,[13] acylazides,[14] citric acid, maleic acid derivatives,[15] and various other physical treatments such as UV[16] and gamma irradiation[17] were the stabilizers for type-I collagen. All the said exogenous crosslinkers were crosslinked with chitosan or with type-I collagen through (i) covalent amide/imine linkage, (ii) metal–protein complex formation (chromium crosslinking with type-I collagen), (iii) H-bond formation (between polyphenolic —OH group with different types of amino acids of type-I collagen molecule and amine group of chitosan), and so on. The stability of the biomaterial upon crosslinking with these agents though highly appreciable, the complete utilization of these materials was restricted because of low mechanical strength. In addition, the biocompatibility of the resultant biopolymer material is questionable because of the release of toxic components from some of the crosslinkers upon usage.

In general, mechanical property of any biomaterial depends on the interaction between the crosslinkers/stabilizers and the parent molecule (here it is chitosan and collagen of type-I). As summarized above, the reports on bonding interaction of said crosslinkers suggest that only the covalent interactions predominate which ultimately restrict the molecule to attain the desired mechanical strength.

Thus, to obviate the problems on mechanical property and the biocompatibility of biomaterials, we attempted to crosslink the parent molecule with suitable crosslinker through noncovalent interactions. In the present study, we have chosen adipic acid (AA) to crosslink with natural polymers. AA (hexanedioic acid), C-4 dicarboxylic acid is slightly soluble in water with the formula (HOOC $(CH_2)_4$COOH). It is used in manufacturing plasticizers and lubricant components and it is also used in making nylon 6,6 fibers, polyester polyols for

Additional Supporting Information may be found in the online version of this article.

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polyurethane systems. Food-grade AA is used as gelling aid, an acidulant, leavening and buffering agent. The reason for selecting AA is because of its nontoxicity.[18]

The present study emphasizes that the preparation of biopolymers using AA as crosslinker for the natural polymers, chitosan, and type-I collagen and demonstrated the crosslinking chemistry between the molecules using suitable bioinformatic tools. Further, the study also explores the thermal, mechanical properties, and the biocompatibility of the resultant biopolymers.

EXPERIMENTAL

Materials

Chitosan from shrimp shells (≥75% deacetylated), AA, and picrylsulfonic acid (2,4,6-trinitrobenzene sulfonic acid [TNBS]) were obtained from Sigma-Aldrich, India. 3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide (MTT), dexamethasone was purchased from Hi-Media, India. All the other reagents were of Analytical Reagent grade and used without further purification.

Type-I collagen from bovine skin was extracted according to the procedure followed by Mitra et al.[19]

Preparation of biopolymers

The method of preparation of biopolymers of chitosan and collagen with AA was developed in our laboratory and in this method the use of acetic acid was completely avoided; instead, the crosslinking agent (AA) itself acts as a dissolution agent and the resulting homogenous solution was used for the preparation of 3D scaffold material. In brief, the powder form of chitosan (1%) and type-I collagen (0.5% w/v) was individually added to 20 mL of water taken in the glass beaker and stirred vigorously to ensure the uniform distribution. To that dispersed mixture, AA was added and the stirring was continued for an hour. Concentration of AA was varied between 0.05 and 0.5% (w/v). For biopolymers of collagen samples, reaction was proceeded at 4°C, whereas ambient temperature was given for chitosan samples. Followed by stirring, the samples were subjected to centrifugation and a clear solution obtained upon centrifugation at 5000 rpm for 10 min was poured in Tarson (India) vial of an inner diameter of 4.5 cm and frozen at −4°C for 2 h, −20°C for 12 h and −80°C for another 12 h. The frozen samples were lyophilized for 48 h at vacuum of 7.5 mTorr (1 Pa) and a condenser temperature of −70°C (PENQU CLASSIC PLUS, Lark, India). The resultant 3D scaffold biopolymer material was neutralized with repeated washings with 0.05*N* NaOH/ethanol mixture followed by washings with water/ethanol mixture (to remove the unreacted chemicals) and finally again lyophilized for 24 h. The scaffold obtained in this procedure was designated as AA crosslinked chitosan (AACCH) and AA crosslinked collagen (AACC). For comparative analysis, GA crosslinked chitosan scaffold (GACCH) and GA crosslinked collagen scaffold (GACC) were prepared according to the method described using 0.2% GA.

The morphology of the biopolymers of AA crosslinked chitosan and collagen was assessed by scanning electron micrograph. Scanning electron microscope (SEM) micrograph analysis was made using F E I Quanta FEG 200—High-Resolution SEM instrument under high voltage at 20 kV.

Analysis of functional groups

Functional group analysis (Fourier transform infrared, FTIR) for AA, chitosan, type-I collagen, AACCH, and AACC biopolymers was made by spectrum one (Perkin-Elmer, USA model) FTIR instrument. All spectra were recorded with the resolution of 4 cm^{-1} in the range of 400–4000 cm^{-1}.

Estimation of percentage of crosslinking degree (TNBS assay)

Degree of crosslinking was quantified using TNBS assay according to the procedure summarized by Bubnis and Ofner.[20] The absorbance of the resulting mixture was measured at 345 nm.

Analysis of mechanical properties of GACCH, GACC, GADCCH, and GADCC biopolymers

Mechanical properties, viz., young's modulus, ultimate tensile strength, stiffness and percentage of elongation of the dried scaffold biopolymers were measured using Universal Testing Machine (INSTRON model 1405) at a crosshead speed of 5 mm min^{-1} at 25°C and 65% relative humidity. Length and width of the dumbbell-shaped test sample was maintained as 20 and 5 mm, respectively. All the mechanical tests were performed with dried samples and were examined in triplicate way.

Thermal analysis (thermogravimetric analysis and differential scanning calorimetry)

Thermal decomposition analysis of AA, native, and crosslinked biopolymers (chitosan, type-I collagen, AACCH, AACC, GACCH, and GACC) was carried out under nitrogen flow (40 and 60 mL min^{-1}) with ramp 20°C min^{-1} using thermogravimetric analysis (TGA) Q 50(V20.6 build 31) instrument. For differential scanning calorimetry (DSC) analysis, model-DSC Q 200(V 23.10 Build 79) with standard mode at

nitrogen (50 mL min^{-1}) atmosphere with ramp 10°C/min was employed.

Circular dichroism spectroscopy

With regard to circular dichroism (CD) analysis, the spectrum of native collagen and AACC solutions were recorded at 25°C using a Jasco 715 Circular Dichroism spectropolarimeter. A scan speed of 20 nm min^{-1} was used with an average of three scans per sample. A slit width of 1 nm and a time constant of 1 s were used. A 1-mm cell was used for the experiments. A reference spectrum of water was also recorded. The CD spectra of the samples were obtained after subtracting the reference spectrum. Change in the conformation of collagen on addition of AA was recorded.

Binding energy calculations using bioinformatic tools

For the docking study, chemical structures of chitosan and AA were generated using ACD/ChemSketch[21] (ACD/ChemSketch Version 12, Advanced Chemistry Development, Toronto, ON, Canada, 2009) and according to Madhan et al.[22] The 3D structure of type-I collagen was generated using gencollagen program.[23] Docking technique is useful to find out the binding efficiency with ligand and a chemical compound. To find out the interaction between chitosan and type-I collagen with AA, AUTODOCK has been used and AutoDock 4.2 used to calculate[24] the free energy of binding of AA with chitosan and type-I collagen.

In vitro assessment on cell compatibility of the biopolymers GACCH and GACC

Biocompatibility in terms of cytotoxicity, cell proliferation, live cell detection, and cell attachment on the prepared scaffold biopolymers was analyzed using NIH 3T3 fibroblast cell line. According to Trentani et al.[25] This cell line is a robust and durable platform for investigating common cellular functions: attachment, viability, proliferation, cellular properties, etc.

Cell proliferation study (MTT assay)

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic was incubated at 37°C in 5% CO_2-humidified atmosphere. Polystyrene 24-well culture plates (Tarson, India) were coated with native chitosan, type-I collagen, AACCH, and type-I collagen (AACC) biopolymers. The plates were dried under laminar air flow hood followed by UV sterilization. The cells were seeded at the density of 1.5×10^4 per well and incubated at 37°C in a humidified atmosphere containing 5% CO_2. At scheduled time points of 24, 48, and 72 h, the supernatant of each well was replaced with MTT diluted in serum-free medium and the plates incubated at 37°C for 4 h. After removing the MTT solution, acid isopropanol (0.04*N* HCl in isopropanol) was added to each well and pipetted up and down to dissolve all of the dark-blue crystals and then left at room temperature for 5 min and the absorbance was measured at 570 nm using UV spectrophotometer.[26] Each experiment was performed at least three times. The sets of three wells for the MTT assay were used for each experimental variant.

Cell tracker assay to detect live cells

Cell viability was measured using 5-chloromethylfluorescein diacetate probe (CMFDA) (Invitrogen, India). NIH 3T3 cells were subjected to respective treatment conditions. Cells were probed with 5 μM CMFDA and incubated for 2 h. Cells were then washed with sterile PBS and images were taken using DP71 camera adapted to an Olympus IX71 microscope.[27]

Cell growth and morphology of NIH 3T3 cells in GACCH and GACC biopolymers

AACCH and AACC scaffolds ($2 \times 2 \times 1$ cm) were placed individually in six-well culture plates (Tarson, India) and ethylene oxide sterilized. Culture media were added to the scaffolds for overnight. NIH 3T3 fibroblast cells were seeded on to the scaffolds at a density of 5×10^4 cells and incubated in an atmosphere of 5% CO_2 at 37°C. The medium was changed every 24 h. Morphology of the cells was examined after 12 days according to the following procedure. The cell-scaffold constructs were fixed in 2.5% GA and dehydrated through graded ethanol series.[28] The dried cell scaffold were coated with gold (E-1010 Ion sputter, HITACHI) and examined under SEM (S-3400 N SEM, HITACHI).

RESULTS AND DISCUSSION

As described in the **INTRODUCTION** section, crosslinkers are used to stabilize the natural polymer materials for biomedical applications. However, the use of these materials primarily depends on the mechanical strength and biocompatibility. Achieving good mechanical strength without compromising the biocompatible nature of the material is a challenging task. However, the nature of the crosslinkers and the interaction chemistry may provide biopolymer materials with requisite properties. In the present study, we have chosen two natural polymers to understand

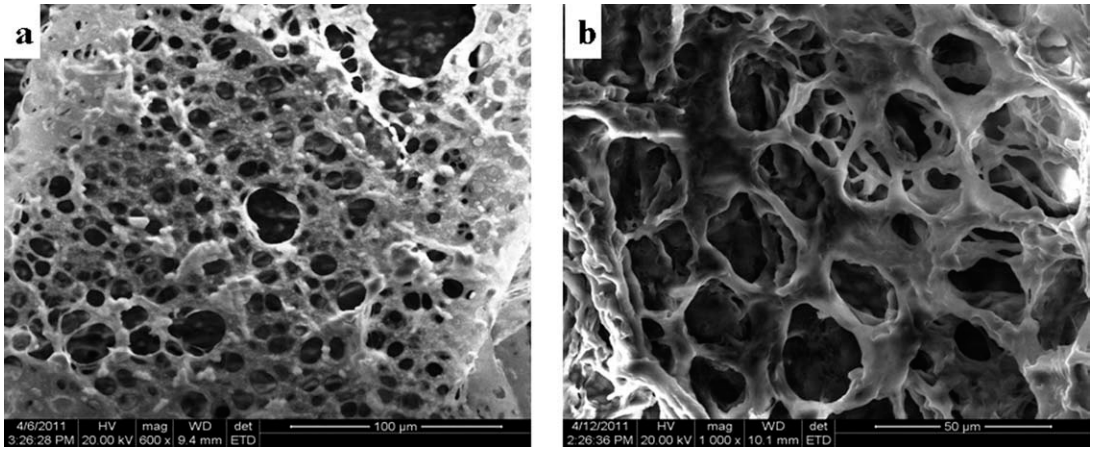
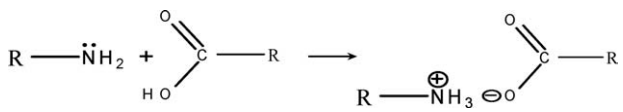


Figure 1 SEM micrographs of (a) AACCH and (b) AACC biopolymers.

the chemistry behind the interaction of the chosen crosslinker namely, AA.

Understanding the dissolution chemistry

For the preparation of any biopolymer materials, the solution form of parent compound/polymer is required to proceed further. However, in the case of chitosan and collagen, two natural polymers chosen for the present study were insoluble in water and acetic and formic acids were used for dissolution.[29,30] The "proton exchange" between —COOH groups of acid molecule and free $-NH_2$ groups of chitosan and collagen shown in the following scheme could be reasoned for the dissolution in the said acids.



Therefore, it has been expected that like acetic acid, AA also possible to provide protons to dissolve chitosan and type-I collagen. Further, alike interaction of tripolyphosphate[31] with chitosan, AA also interacts with both the natural polymers through ionic interaction. Because of the said proton exchange, chitosan and type-I collagen get dissolved in the presence of AA in water. The detailed schematic explanation for the dissolution as well as interaction between AA and chitosan/collagen is shown in Supporting Information file S1.

Because of interaction, both the natural polymers were completely dissolved in water in the presence of AA. With the resulting solution, scaffolds were prepared and subjected to characterization studies. Figure 1 shows the morphological features of the crosslinked biopolymers (AACCH and AACC). The 3D biopolymer material was highly porous and the pore structures of the membranes were well-distributed and interconnected. It was obvious that most of the volume of the membranes were taken up by the interconnecting pore space. The high porosity suggests the suitability of this biopolymer for biomedical applications, including serving as absorption sponges and matrices for cell proliferation.

FTIR studies were conducted to monitor the chemical modifications in chitosan and type-I collagen structures upon crosslinking with AA. Figure 2 shows the FTIR spectral details of AA, chitosan, type-I collagen, GACC, AACCH, and AACC. In FTIR spectrum of GACC, peaks observed at 1645 and 1547 correspond to C=N (the imine bond) and reduction in free amino groups.

FTIR spectrum of AACCH displayed significant changes compared to the parent molecules. A broad, strong absorption in the region of 3508–2685 cm^{-1} was resulting from superimposed —OH and $-NH_3^+$ stretching band. Peak absorption at 1665 and 1579 cm^{-1} corresponds to the presence of asymmetric N—H ($-NH_3^+$) bend and asymmetric $-COO^-$ stretching, respectively. Peak observed at 1534 and 1407 cm^{-1} was owing to symmetric N—H ($-NH_3^+$) bend and symmetric $-COO^-$ stretching, respectively. Other absorption peaks around 1257, 1157, and 897 cm^{-1} observed in AACCH spectrum were similar to the native chitosan spectrum and suggest no change in main backbone of chitosan structure.

From AACC spectrum, compared to native type-I collagen few changes were observed, owing to the crosslinking of type-I collagen with AA. A broad, strong absorption in the region of 3491–2958 cm^{-1} was resulting from superimposed —OH and $-NH_3^+$ stretching band. In native type-I collagen spectrum, a sharp intense amide-I band was observed at around 1658 cm^{-1} which was disappeared with the appearance of two new bands at 1672 and 1626 cm^{-1} upon crosslinking with AA and these peaks were owing to $-NH_3^+$ and $-COO^-$, respectively, and one weak absorption was observed at 1400

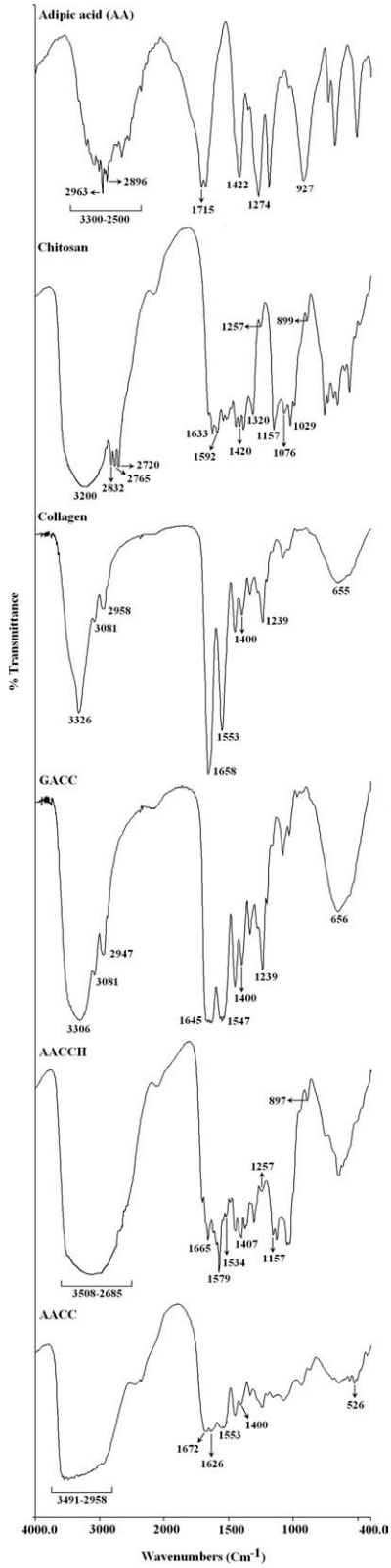


Figure 2 FTIR spectrum of AA, chitosan, type-I collagen, GACC, AACCH, and AACC biopolymers.

cm^{-1} owing to —COO^-. Moreover, when compared with native type-I collagen spectrum, in AACC spectrum, there was a reduction in the region of 1553 cm^{-1} (overlapped band of amide II and free primary amines) which could be owing to the reduction of free —NH_2 group in AACC. In AACC spectra, band around 526 cm^{-1} was observed which was ascribed to the N—H oscillation of —NH_3^+. Results from FTIR analysis reflected that AA was ionically crosslinked with chitosan and type-I collagen.[32]

Though FTIR analysis displayed the ionic interaction between the crosslinker and the natural polymers, results on the percentage of crosslinking degree suggest that increasing the concentration of AA increases the degree of crosslinking upto 0.4% and confirmed the interaction. Table I lists the percentage of crosslinking degree for chitosan and collagen in the presence of increasing concentration of AA and suggested that the observed increase was insignificant. About 60–66% crosslinking was observed with 0.2% AA with chitosan and collagen. However, in the case of experiments with GA, about 88–93% of crosslinking was observed with 0.2% concentration of GA.

Supporting Information file S2 demonstrates the FTIR spectrum of AACCH and AACC with varied concentrations of AA (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 w/v). The spectral details suggested the existence of NH_3^+ and COO^- (1665–1670, 1575–1580, 1530–1535, and 1405–1410 cm^{-1}).

With regard to mechanical property of the biopolymer materials, the mechanical property is a fundamental property for any biopolymer in application point of view. From the results, we observed that the mechanical strength of the biopolymer was increased with an increase in AA concentration up to 0.2%. Further increase in AA concentration leads to the decrease in mechanical strength (results not shown). Table II summarizes tensile strength, young's modulus, stiffness of native, and AA (0.2%) crosslinked biopolymers. High-tensile strength (MPa) values were observed for both the crosslinked biopolymers (AACCH-26.47 and AACC-3.05) compared to the native biopolymers (chitosan-0.37 and collagen-0.13). Moreover, the young's moduli of

TABLE I
Measurement of Crosslinking Degree of AACCH and AACC Prepared Using Different Concentrations of AA (0.05–0.5%)

Concentration of AA (%, w/v)	Percentage of crosslinking degree of AACCH (%)[a]	Percentage of crosslinking degree of AACC (%)[a]
0.05	51 ± 0.5	53 ± 0.5
0.1	57 ± 0.6	58 ± 0.2
0.2	66 ± 0.8	65 ± 0.7
0.3	72 ± 1.0	74 ± 0.9
0.4	75 ± 0.4	78 ± 0.7
0.5	79 ± 0.6	82 ± 1.0

[a] Mean ± SD.

TABLE II
Assessment of Mechanical Properties of Chitosan, AACCH, Collagen, and AACC in Terms of Tensile Strength, Elongation at Break, Young's Modulus, and Stiffness

Sample name	Maximum load (*N*)[a]	Tensile strength (MPa)[a]	Elongation at break (%)[a]	Extension at maximum load(mm)[a]	Young's modulus (MPa)[a]	Stiffness (κ) (N/mm)[a]
Chitosan	1.32 ± 0.08	0.37 ± 0.02	8.33 ± 0.16	1.67 ± 0.22	4.43 ± 0.46	0.79 ± 0.04
AACCH	9.27 ± 0.12	26.47 ± 1.8	22.3 ± 1.02	4.48 ± 0.86	118.16 ± 4.66	2.069 ± 0.08
Collagen	0.37 ± 0.02	0.13 ± 0.02	6.17 ± 0.62	1.23 ± 0.04	2.114 ± 0.48	0.3 ± 0.02
AACC	3.2 ± 0.06	3.05 ± 0.02	23.58 ± 2.04	2.36 ± 0.12	2.36 ± 0.22	1.35 ± 0.06

[a] Mean ± SD.

AACCH and AACC were 118.16 and 2.36, respectively. The stiffness values (AACCH 2.069 and AACC 1.35*N*/mm) were also greater than that of the native biopolymers (chitosan 0.79 and collagen 0.3*N*/mm).

All these observations on mechanical properties suggest that AA crosslinked biopolymer materials demonstrated appreciable mechanical strength compared to GA, where we observed brittleness. Schiffman and Schauer[33] reported brittle nature of the biomaterial upon crosslinking with GA. Further, when the concentration of AA was increased to >0.2%, a decrease in mechanical strength was observed and this could be reasoned to high degree of crosslinking of AA with the biopolymers which is clearly proved from the results of TNBS assay.[34] In the same manner, with the high crosslinking degree, GA displays materials with brittle nature.

Figure 3 shows the crosslinking chemistry of GA with the chosen natural polymers and reasons out the brittle nature. The schemes suggested that GA could covalently be crosslinked with chitosan and collagen through the formation of double bond (C=N, imine bond) between —CHO group of GA and —NH_2 group of polymers (chitosan and collagen), results with the large energy barrier for rotation of associated groups linked by a double bond (C=N) and finally provided brittle nature to the biopolymer material.

TGA for the experimental samples AA, chitosan, type-I collagen, AACCH, AACC, GACCH, and GACC are shown in [Fig. 4(a,b)]. From the results, we observed that incorporation of AA with chitosan and type-I collagen tends to shift the thermal region to higher temperature and such a shift is attributed to an increase in thermal stability.

DSC studies were performed to understand the behavior of AACCH and AACC on application of thermal energy. The thermograms of AA, chitosan, type-I collagen, AACCH, AACC, GACCH, and

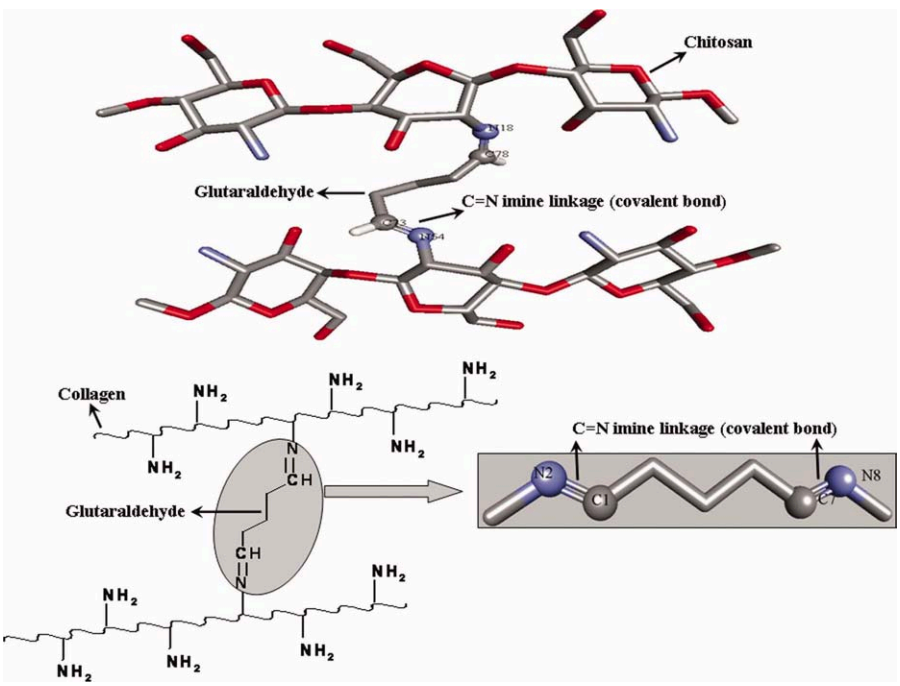


Figure 3 Schematic representation of crosslinking of GA with chitosan/collagen (demonstrating the imine linkage). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

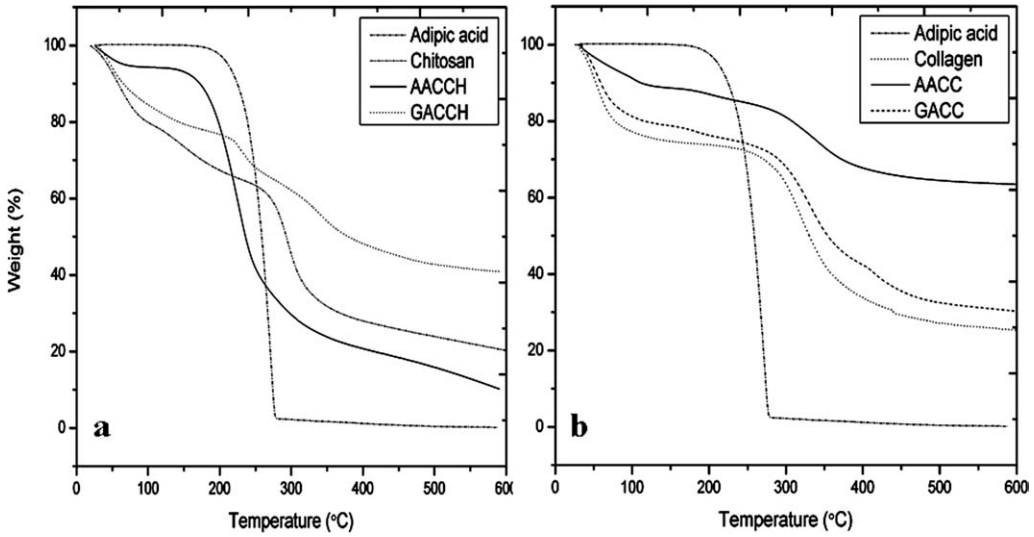


Figure 4 (a) TGA of AA, chitosan, AACCH, and GACCH biopolymers. (b) TGA of AA, collagen, AACC, and GACC biopolymers.

GACC are shown in [Fig. 5(a,b)]. DSC studies recorded exothermic peak differences among AA (153°C), chitosan (107°C), collagen (99°C), AACCH (189°C), and AACC (123°C), whereas in GACCH and GACC it was observed at 149 and 151°C, respectively. The shifting of exothermic peak of AACCH and AACC to the higher temperature region suggested the requirement of more energy to degrade the crosslinked biopolymers. Similar kind of observation was reported by Bhumkar and Pokharkar.[31]

Further, to ascertain any change in the secondary structure of collagen upon interaction with AA, CD spectral analysis was made for native collagen and AACC. Collagen exhibits a unique CD spectrum with a small positive peak between 220 and 225 nm and a large negative peak at 197 nm.[35,36] The CD spectrum of native collagen and all AACC are shown in Fig. 6. Furthermore, calculations on the Rpn values (the parameter Rpn denotes the ratio of positive peak intensity over negative peak intensity and the parameter can be used to differentiate triple helical conformation from other nontriple helical conformations[22]) suggested no change in the triple helical structure of collagen upon interaction with AA. The Rpn value for native collagen was 0.063 and for all AACC it was observed as 0.063 (±0.004).

Results on binding energy calculations based on bioinformatics tool for the crosslinking of AA with chitosan and type-I collagen using Auto Dock

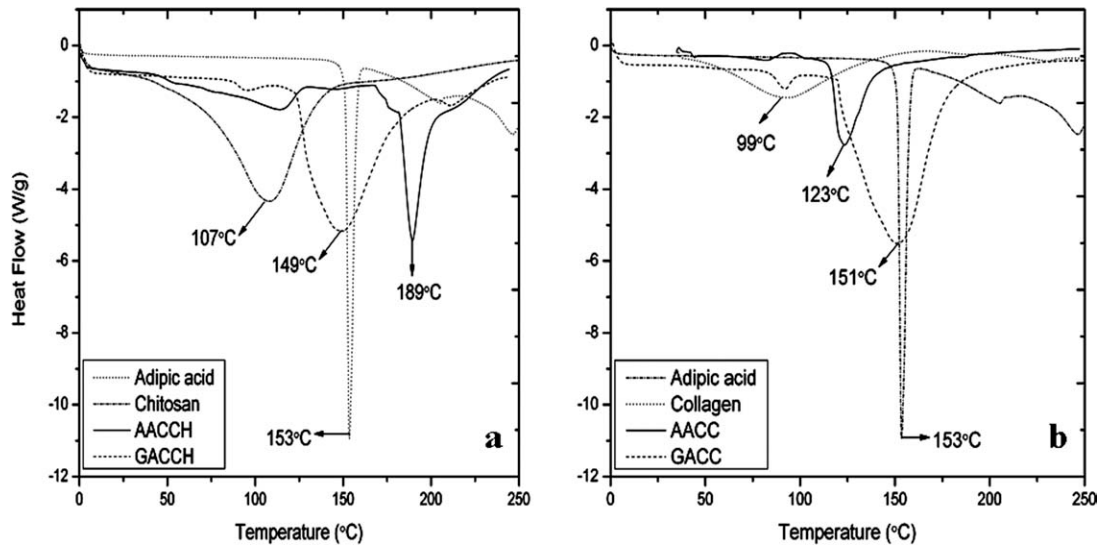


Figure 5 (a) DSC analysis of AA, chitosan, AACCH, and GACCH biopolymers. (b) DSC analysis of AA, collagen, AACC, and GACC biopolymers.

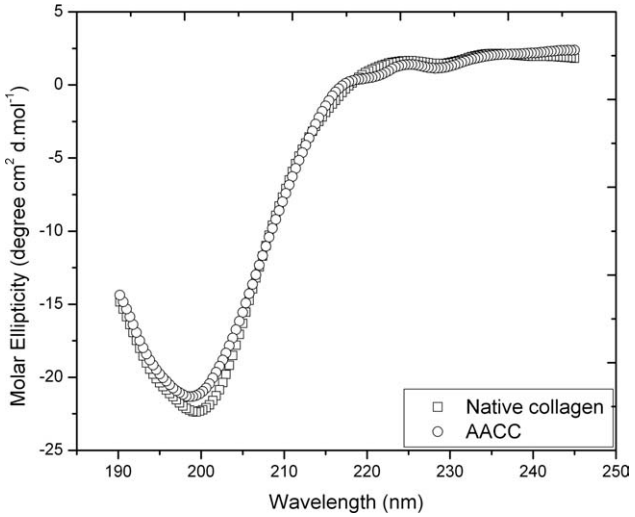


Figure 6 CD analysis of native collagen and AA-interacted collagen biopolymer.

software proved that chitosan and type-I collagen can crosslink with AA not only with ionic interaction but also through multiple intermolecular hydrogen bonding. Autodock is an automated procedure for predicting the interaction of ligands with biomacromolecular targets. Hundred runs were given for docking AA with chitosan and type-I collagen. The best binding energy values and their corresponding rank and run numbers are listed in Table III. The binding energy of −4.43 and −4.43 (kcal/mol) was observed when AAs were interacted with chitosan and type-I collagen, respectively. These interactions were made by multiple intermolecular hydrogen bonds between —COOH group of AA and —NH_2 group of chitosan and free ε-NH_2 group of lysine from type-I collagen [Fig. 7(a,b)]. In addition to ionic crosslinking, hydrogen bonding interaction can also

TABLE III
Binding Energy Values of AACCH and AACC Biopolymers

Rank	Binding energy (kcal/mol)	No. of runs
The binding energy calculation between chitosan and AA based on autodock tool software		
1	−4.43	73
2	−4.39	43
3	−4.26	87
4	−4.23	33
5	−4.19	99
6	−3.99	25
The binding energy calculation between type-I collagen and AA based on autodock tool software		
1	−4.43	61
2	−4.29	27
3	−3.82	34
4	−3.81	73
5	−3.66	26
6	−3.24	29
7	−3.17	45

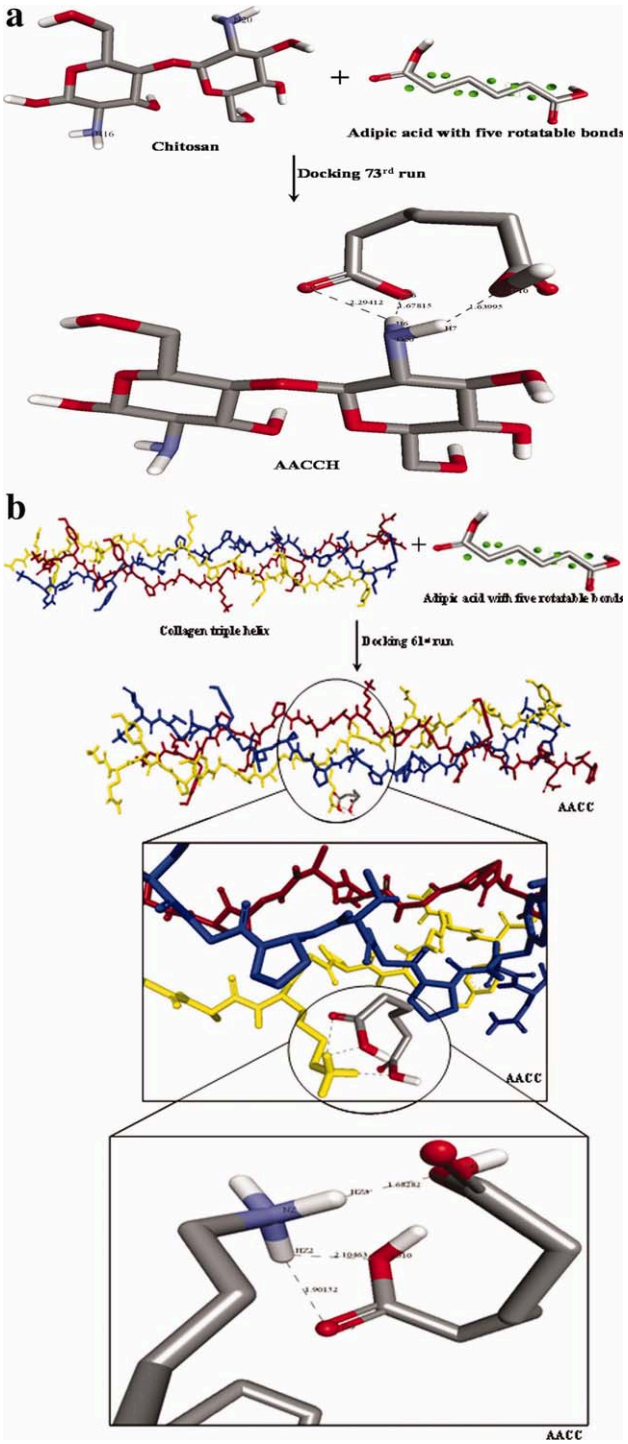


Figure 7 (a) Multiple hydrogen bonding interaction between chitosan and AA. (b) Multiple hydrogen bonding interaction between collagen and AA. (The black dotted line indicates the hydrogen bond. In this figure, white color is for hydrogen atom (H), red color indicates oxygen atom (O), grey color for carbon atom (C), and blue color corresponds to nitrogen atom (N)). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

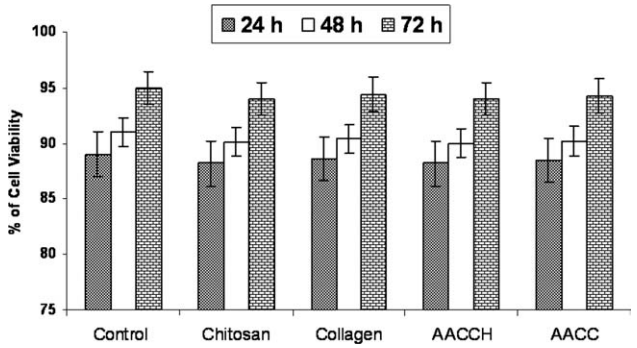


Figure 8 MTT analysis of control, chitosan, collagen, AACCH, and AACC biopolymers at 24, 48, and 72 h time interval.

improve the mechanical property of the biopolymer. The details of these intermolecular hydrogen bonding sites are given below.

Intermolecular hydrogen bond details between AA and chitosan

i. H (6) of chitosan is linked with O (5) of AA with the bond distance of 2.29412,
ii. H (6) of chitosan is linked with O (6) of AA with the bond distance of 1.67815, and
iii. H (7) of chitosan is linked with O (10) of AA with the bond distance of 1.63995.

Intermolecular hydrogen bond details between AA and type-I collagen

i. LYS (12) H2 of type-I collagen is linked with O (9) of AA with the bond distance of 1.90152,
ii. LYS (12) H2 of type-I collagen is linked with O (10) of AA with the bond distance of 2.10463, and
iii. LYS (12) H3 of type-I collagen is linked with O (6) of AA with the bond distance of 1.68282.

(H, hydrogen; LYS, lysine amino acid; O, oxygen; and AA, adipic acid)

With reference to the biocompatibility of the resulting polymers, cell attachment, proliferation assays were carried out. MTT assay was done to check the toxicity of the prepared biopolymers (AACCH and AACC). Only cells that are metabolically viable can turn the tetrazolium salts into purple crystals.

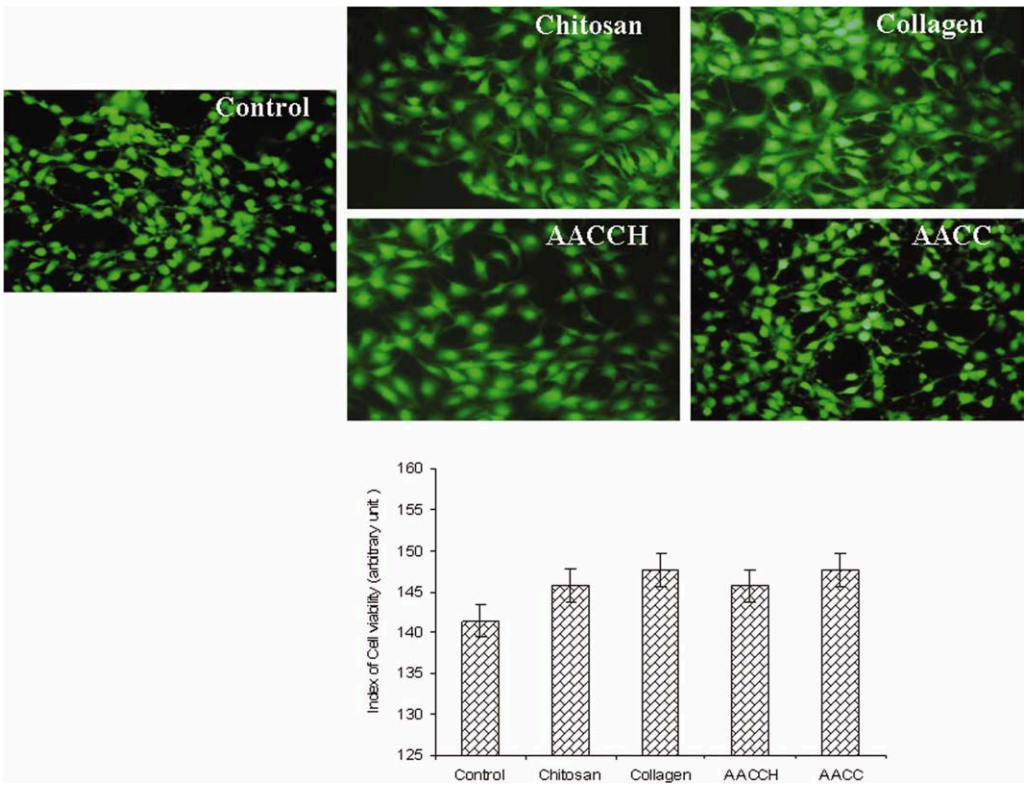


Figure 9 Index of cell viability (arbitrary unit) assessed in AACCH and AACC in comparison with the parent molecules and control. (The assay was carried out using cell tracker kit. NIH3T3 cells were treated on the surface of native and crosslinked biopolymers for 6 h followed by incubation with cell viable dye cell tracker for 30 min. Fluorescence images of the cells were acquired by DP71 camera adapted to an Olympus IX71 microscope. Intensity of green-positive cells was counted and plotted. Next, fluorescence intensities of the images were calculated using Adobe Photoshop version 7.0). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

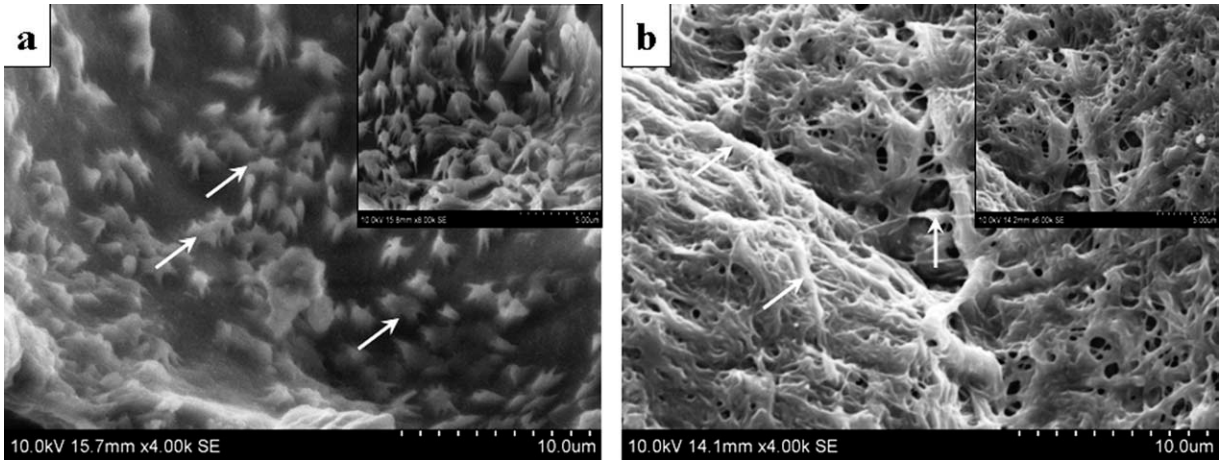


Figure 10 (a) Attachment of fibroblast cells on the AACCH scaffold biopolymer (white arrow indicates the adhered cells on the biopolymer). (b) Attachment of fibroblast cells on the AACC scaffold biopolymer. (Porous AACC scaffold was completely covered by fibroblast cells which are indicated by white arrow in this figure.)

Compared with the native chitosan and type-I collagen, AACCH and AACC showed no significant differences in absorbance (Fig. 8), that is the biopolymers being in direct contact with fibroblast did not lead to apoptosis or necrosis. MTT results clearly indicated that NIH 3T3 cells proliferated well on the surface of the AA crosslinked biopolymers (AACCH and AACC).

The cell viability assay for GACC has not been performed because of the recent realization on the toxicity of GA. GA is the most widely used chemical crosslinking agent[37] because it stabilizes collagen efficiently. The crosslinking is thought to involve the formation of Schiff bases.[38] However, GA-crosslinked biomaterials are poorly biocompatible with some cell lines including human fibroblasts, osteoblasts, Chang cells, and endothelial cells.[39–41] The side effects of GA treatment were attributed to the degradation of the GA-derived crosslinks and the continuous release of aldehydes contributing to prolonged toxic effects.[37,42]

In cell viability assay, we observed intense fluorescence of the cells on the surface of the native and crosslinked biopolymers and suggested the viability of the cells as shown in Fig. 9.

The SEM images of the cell-seeded AACCH and AACC scaffold shown in [Fig. 10(a,b)] demonstrate that after being cultured for prolonged time (12 days), fibroblasts were detected in the scaffolds (AACCH and AACC) with typical spindle-shaped morphology and suggest that the cells were infiltrated into the scaffolds and proliferated there. The higher magnifications of Fig. 10(a,b) were inserted for better observations on the images.

CONCLUSIONS

The present study explicitly demonstrated that AA acts as suitable crosslinkers for the preparation of biocompatible biopolymers from natural polymers (chitosan and collagen) with appreciable mechanical properties. The interaction between AA and chitosan or AA and collagen was identified as noncovalent interactions (ionic and multiple intermolecular hydrogen bonding interactions). These noncovalent interactions provided appreciable mechanical strength to the resulting biopolymers. All the instrumental analyses and bioinformatic tool authenticated the noncovalent interactions. The biopolymer material (scaffold) prepared upon crosslinking of AA with chitosan and or collagen was the green method of preparation. From this study, we conclude that instead of acetic acid, AA can be used as a solvent for dissolving any amino group containing natural polymers. No toxic compounds were involved in this preparation and the resultant material found application as wound dressing material or as an implant in clinical applications.

References

1. Storck, M.; Orend, K. H.; Schmitz-Rixen, T. Vasc Endovasc Surg 1993, 27, 413.
2. Hashimoto, K.; Sudo, M.; Sugimura, T.; Inagaki, Y. J Appl Polym Sci 2004, 92, 3492.
3. Liu, Y.; Guo, L. K.; Huang, L.; Deng, X. M. J Appl Polym Sci 2003, 90, 3150.
4. Engelmayr, G. C.; Hildebrand, D. K.; Sutherland, F. W. H.; Mayerand, J. E.; Sacks, M. S. Biomaterials 2003, 24, 2523.
5. Ligler, F. S.; Lingerfelt, B. M.; Price, R. P.; Schoen, P. E. Langmuir 2001, 17, 5082.
6. Schauer, C. L.; Chen, M.-S.; Chatterley, M.; Eisemann, K.; Welsh, E. R.; Price, R.; Schoen, P. E.; Ligler, F. S. Thin Solid Films 2003, 434, 250.
7. Wei, Y. C.; Hudson, S. M.; Mayer, J. M.; Kaplan, D. L. J. J Polym Sci Part A: Polym Chem 1992, 30, 2187.
8. Jin, J.; Song, M.; Hourston, D. J. Biomacromolecules 2004, 5, 162.
9. Tual, C.; Espuche, E.; Escoubes, M.; Domard, A. J. J Polym Sci Part B: Polym Phys 2000, 38, 1521.

10. Usha, R.; Ramasami, T. Thermochim Acta 2000, 356, 59.
11. Sheu, M. T.; Huang, J. C.; Yeh, G. C.; Ho, H. O. Biomaterials 2001, 22, 1713.
12. Miles, C. A.; Avery, N. C.; Rodin, V. V.; Bailey, A. J. J Mol Biol 2005, 346, 551.
13. Nam, K.; Kimura, T.; Kishida, A. Macromol Biosci 2008, 8, 32.
14. Petite, H.; Rault, I.; Huc, A.; Menasche, P.; Herbage, D. J Biomed Mater Res 1990, 24, 179.
15. Saito, H.; Murabayashi, S.; Mitamura, Y.; Taguchi, T. J Mater Sci Mater Med 2008, 19, 1297.
16. Weadock, K. S.; Miller, E. J.; Bellincampi, L. D.; Zawadsky, J. P.; Dunn, M. G. J Biomed Mater Res 1995, 29, 1373.
17. Olde Damink, L. H. H.; Dijkstra, P. J.; van Luyn, M. J. A.; Van wachem, P. B.; Nieuwenhuis, P.; Feijen, J. J Biomed Mater Res 1995, 29, 149.
18. Horn, H. J.; Holland, E. G.; Hazleton, L. W. J Agric Food Chem 1957, 5, 759.
19. Mitra, T.; Sailakshmi, G.; Gnanamani, A.; Raja, S. T.; Thiruselvi, T.; Mangala Gowri, V.; Selvaraj, N. V; Ramesh, G., Mandal, A. B. Int J Biol Macromol 2011, 48, 276.
20. Bubnis, W. A.; Ofner, C. M. Anal Biochem 1992, 207, 129.
21. ACD, ChemSketch Version 12, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2009.
22. Madhan, B.; Subramanian, V.; Raghava Rao, J.; Nair, B. U.; Ramasami, T. Int J Biol Macromol 2005, 37, 47.
23. Available at: http://www.cgl.ucsf.edu./cgi-bin/gencollagen.py.
24. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. J Comput Chem 2009, 30, 2785.
25. Trentani, L.; Pelillo, F.; Pavesi, F. C.; Ceciliani, L.; Cetta, G.; Forlino, A. Biomaterials 2002, 23, 2863.
26. Mossmann, T. Immunol Met 1983, 65, 55.
27. Majumder, S.; Siamwala, J. H.; Srinivasan, S.; Sinha, S.; Sridharan, S. R. C. Soundararajan, G.; Seerapu, H. P.; Chatterjee, S. J Cell Biochem 2011, 112, 1898.
28. Kim, S. E.; Cho, Y. W.; Kang, E. J.; Kwon, I. C.; Lee, E. B.; Kim, J. H.; Chung, H.; Jeong, S. Y. Fiber Polym 2001, 2, 64.
29. Ohkawa, K.; Cha, D. I.; Kim, H.; Nishida, A.; Yamamoto, H. Macromol Rapid Commun 2004, 25, 1600.
30. EI-Tahlawy, K.; Gaffar, M. A.; El-Rafie, S. Carbohydr Polym 2006, 63, 385.
31. Bhumkar, D. R.; Pokharkar, V. B. AAPS Pharm Sci Tech 2006, 7, 1.
32. Pavia, D. L.; Lampman, G. M.; Kriz, G. S. Introduction to Spectroscopy, 3rd ed.; Thomson Learning, Inc.: USA, 2001.
33. Schiffman, J. D.; Schauer, C. L. Biomacromolecules 2007, 8, 594.
34. Wang, Y.; Ameer, G. A; Sheppard, B. J.; Langer, R. Nat Biotechnol 2002, 20, 602.
35. Venugopal, M. G.; Ramshaw, J. A. M.; Braswell, E.; Zhu, D.; Brodsky, B. Biochemistry 1994, 33, 7948.
36. Sacca, B.; Renner, C.; Moroder, L. J Mol Biol 2002, 324, 309.
37. Jorge-Herrero, E.; Fernandez, P.; Turnay, J.; Olmo, N.; Calero, P.; Garcia, R.; Freile, L.; Castillo-Olivares, J. J. Biomaterials 1999, 20, 539.
38. Olde Damink, L. H. H.; Dijkstra, M.; Van Luyn, M. J. A.; Van Wachem, P. B.; Nieuwenhuis, P.; Feijen, J. J Mater Sci Mater Med 1995, 6, 460.
39. Huang-Lee, L. L. H.; Cheung, D. T.; Nimni, M. J Biomed Mater Res 1990, 24, 1185.
40. Van Luyn, M. J. A.; Van Wachen, P. B.; Olde Damink, L. H. H.; Dijkstra, P. J.; Feijen, J.; Nieuwenhuis, P. Biomaterials 1992, 13, 1017.
41. Gough, J. E.; Scotchford C. A.; Downes, S. J Biomed Mater Res 2002, 61, 121.
42. Schmidt, C. E.; Baier, J. M. Biomaterials 2000, 21, 2215.