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Bonding interactions and stability assessment of biopolymer material prepared using type III collagen of avian intestine and anionic polysaccharides

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Abstract The present study demonstrate bonding interactions between anionic polysaccharides, alginic acid (AA) and type III collagen extracted from avian intestine used for the preparation of thermally stable and biodegradable biopolymer material. Further the study describes, optimum conditions (pH, temperature and NaCl concentration) required for the formation of fibrils in type III collagen, assessment on degree of cross-linking, nature of bonding patterns, biocompatibility and biodegradability of the cross-linked biomaterial. Results revealed, the resultant biopolymer material exhibit high thermal stability with 5–6 fold increase in tensile strength compared to the plain AA and collagen materials. The degree of cross-linking was calculated as 75%. No cytotoxicity was observed for the cross-linked biopolymer material when tested with skin fibroblast cells and the material was biodegradable when treated with enzyme collagenase. With reference to bonding pattern analysis we found, AA cross-linked with type III collagen via (i) formation of covalent amide linkage between –COOH group of AA and ε-NH_2 group of type-III collagen as well as (ii) intermolecular multiple hydrogen bonding between alginic acid –OH group with various amino acid functional group of type-III collagen. Comparisons were made with other cross-linking agents also. For better understanding of bonding pattern, bioinformatics analysis was carried out and discussed in detail. The results of the study emphasize, AA acts as a suitable natural cross-linker for the preparation of wound dressing biopolymer material using collagen. The tensile strength and the thermal stability further added value to the resultant biopolymer.

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1 Introduction

Collagen, the most abundant (20–30%) protein, exists in different types. About 28 different types of collagen identified and grouped under collagen super family [1]. Fibril-forming collagen, type I, II, and III [2] constitute the majority of the internal structure. Type III collagen comprises three α1 (III) chains and expressed in early embryos and involved throughout embryogenesis and often associated with tissues with ordered fibril diameters [3]. In addition, it was co-localized with the most abundant member of the family, type I collagen, in such tissues as blood vessels and skin [4]. Compared to type I, the length of the triple helix in type III collagen is slightly longer with slightly short telopeptides [5].

Though all collagen types found essential for the cellular and other related biochemical activities, the strength, flexibility and movement of organelles was solely depends on the fibrillar collagen. The ability to self-assemble was an intrinsic property of the fibril-forming collagen [6]. Formation of fibrils involves, aggregation and alignment of collagen molecule [7], which ultimately improve the thermal behavior of the biomaterial [8]. The rate of fibril formation and the nature of fibril produced depend on experimental variables such as pH, ionic strength, nature of micro-ions, dielectric constant of the solvent used, temperature of reaction and collagen concentration [9]. The

introduction of cross-linkers among collagen fibrils during fibril formation would increase the thermal stability of collagen fibrillar gel, thereby providing fibrillar gels as biomaterials. Cross-linking analysis reveals connectivity between the C-terminal telopeptides of type III collagen and the N-terminal helical region of another type III molecule [10]. With regard to cross-linkers for the preparation of wound dressing materials, a recent realization on toxicity problems with a well established cross-linker, glutaraldehyde, demand, a new and natural cross linker for the preparation of biopolymer material.

Alginic acid (AA), a linear anionic copolymer of 1,4 linked β-D-mannuronic acid (M) with α-guluronic acid (G) arranged as homopolymeric (GG or MM) or heteropolymeric (GM) block, constitutes major structural polysaccharide of brown seaweeds (*phaeopyta*), found nontoxic, non-carcinogenic, biocompatible, sterilizable and offers cheap processing technique [11]. AA and its sodium/calcium salts have long been used in food, cosmetics, drugs, drug delivery, and tissue engineering, etc. [12].

Based on the above said merits of AA, and a high need for natural cross-linker, in the present study, we made an attempt on evaluating the cross-linking ability of a anionic polysaccharides, AA with type III collagen. The stabilized material received upon cross-linking was further characterized for its physical, chemical, mechanical, biocompatible, biodegradable properties using instrumental techniques. Further more, the study demonstrate, the various possible interactions between AA and type III collagen assessed using binding energy calculations obtained from bioinformatics tool and finally emphasize the reason behind the stability and degradability of the biopolymer material prepared. In addition, comparisons on tensile strength, thermal stability were made with biopolymer material prepared using glutaraldehyde as cross-linker.

2 Materials and methods

Alginic acid, picrylsulfonic acid [2,4,6-trinitrobenzene sulfonic acid (TNBS)], glutaraldehyde, coomassie brilliant blue (CBB) R 250 were obtained from Sigma-Aldrich (USA) and S.D. Fine-Chem Limited, Mumbai, India respectively. Avian intestine obtained from slaughterhouse was used as a source material for type III collagen. All other reagents were of Analytical Reagent grade and used without any further purifications.

2.1 Extraction of collagen from avian intestine

Avian intestine obtained from slaughter house was cleaned with running tap water and again washed with chloroform for complete removal of fat materials and then chopped into pieces. Chopped pieces were soaked in 0.5 (M) sodium acetate buffer with continuous stirring at 4°C for 12–24 h. After 24 h, the soaked material was homogenized with 0.5 (M) acetic acid and kept at 4°C for 12–24 h under stirring condition. The homogenized solution was then centrifuged at 10,000 rpm for 20 min at 4°C and collected the supernatant. Sodium chloride (5%) was added to the collected supernatant for salting out the protein (collagen). Followed by the salting out, the obtained collagen was dialyzed against 0.005 (M) acetic acid at 4°C for 24–72 h and then lyophilized. Since collagen was extracted using acetic acid, the resultant collagen was designated as acid soluble collagen (ASC) and all the fibril formation and cross-linking studies were carried out with ASC.

2.2 Characterization of collagen (SDS-PAGE)

Acid soluble collagen obtained from the above procedure was subjected to SDS-PAGE analysis to assess the molecular profile. In brief, electrophoresis was carried out with 8% polyacrylamide gel according to the procedure of Laemmli [13]. Followed by electrophoresis, gel was stained with CBB solution containing −0.5% CBB R-250 in 30% methanol and 10% acetic acid. Mixture of methanol, acetic acid and water in the following ration 3:1:6 was used for destain the gel. Molecular weight marker from Sigma (USA) was used to measure the molecular weight of the bands appeared.

2.3 Kinetics of collagen fibril self assembly

Collagen self-assembly was performed according to Nomura et al. [14] with slight modifications. In brief, reaction mixture containing 0.2% collagen concentration in 70 mM phosphate buffer at different pH's and with varied concentration of NaCl, incubated at different temperatures and measured the turbidity change at 310 nm using Shimadzu UV–Visible spectrophotometer. Phosphate buffer pH's at 5.0, 5.5, 6.0, 6.5, 7.0, 7.4, and NaCl concentrations at 0, 35, 70 and 140 mM and temperatures at 20, 25, 30 and 37°C were the variables chosen for the study.

2.4 Preparation of biopolymer material

Concentration of 1.5% homogenized solution of low viscous AA of molecular weight 200 was prepared by dissolving the required quantities in 70 (mM) sodium phosphate buffer (pH 6.0). About 0.5% type III ASC [dissolved in 0.005(M) acetic acid] mixed with AA at 3:1 ratio respectively and incubated for overnight at 4°C. Followed by incubation the reaction mixture was transferred to polypropylene plate (Tarson, India) and air-dried at 37°C for 12 h. Experiments with other cross-linking

agent for comparison was made with glutaraldehyde. In brief, glutaraldehyde at 1.5% (v/v) was mixed with 0.5% of collagen and sheet was prepared according to the procedure summarized above. Sheets obtained from the above processes were designated as alginic acid cross-linked collagen (AACC) and glutaraldehyde cross-linked collagen (GCC).

2.5 Characterization of biopolymer material

Morphology of native and cross-linked biopolymer material AACC was tested for physical appearance, smoothness, transparency by physical and feel observations and thickness using screw gauge. Functional group analysis (FT-IR) was made by cutting the sheets into pieces (1 × 1 cm) and placed in the sample compartment and analyzed using spectrum one (Perkin-Elmer Co., USA model). To assess the mechanical property, dumbbell shaped specimens of 5 mm wide and 10 mm length and thickness of 0.04 mm were punched out from the prepared sheets using dye. Tensile strength (MPa) was measured using Universal Testing machine (INSTRON model 1405) at a crosshead speed of 25 mm/min. Thermal decomposition analysis of uncross-linked collagen (ASC), AACC, GCC and AA was carried out under nitrogen (40 & 60 ml/min) atmosphere using TGA Q 50 (V20.6 build 31) instrument. All the samples were heated from 37 to 600°C at a heating rate of 20°C/min. Similarly, melting temperature measurements were made using differential scanning calorimeter (DSC), model-DSC Q 200(V 23.10 Build 79) with standard mode at nitrogen (50 ml/min) atmosphere with the heating rate maintained constant at 10°C/min over a temperature range of 0–250°C. Scanning electron micrograph (SEM) analysis of native and AACC material was made by using JEOL JSN 6360 (Japan) instrument after sputter coated with gold.

Further, degree of cross-linking was measured quantitatively using TNBS assay according to the procedure summarized by Bubnis and Ofner [15]. In brief, 6.0 mg of sample (AACC and GCC) was immersed in 2 ml solution [1 ml of 4% (w/v) di-sodium hydrogen orthophosphate and 1 ml of 0.5% (v/v) TNBS] and incubated at 40°C for 2 h and the reaction was terminated by adding 3 ml of 6 M (V/V) HCl and continued the incubation at 60°C for 90 min and measured the absorbance at 345 nm and the percentage of cross linking was calculated according to the standard procedure.

Skin fibroblasts cells were used for cell toxicity assay. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum and 1% antibiotic. Fibroblast cells were incubated at 37°C in 5% CO_2 humidified atmosphere. Cells were trypsinized after reaches confluence and seeded on to a specimen with 0.5×10^6 cells in 200 µl (96 well plate) of growth medium and allowed to proliferate at 37°C. For MTT assay, AACC at different concentrations (0.5–1.5%) in solution state was prepared and followed by cell proliferation; respective concentration of the sample was implemented and the incubation was continued. At scheduled time points of 2, 4, and 6 h, growth medium containing test solution was removed and replaced with fresh medium and to this added 50 µl of MTT solution and incubated further for the period of 4 h in a humidified atmosphere at 37°C. The MTT-formazan crystals obtained after the removal of the medium was dissolved in 200 µl of dimethyl sulfoxide (DMSO) and 25 µl of glycine buffer. The optical density (OD) of resulting solution was read at 570 nm. All the tests were performed in triplicates and repeated twice.

For cell adhesion assessment, biopolymer of AACC was developed on cover slip separately under sterile condition and was carefully transferred to 24 well plates and again UV sterilized to avoid further contamination. Followed by UV sterilization, cells obtained from the above step was seeded (1.5×10^6) and incubated along with growth medium for the period of 3 days. The medium was changed at regular intervals (every 24 h). Samples were with drawn from 24 well plates at 1, 2 and 3 days and viewed for cell adhesion and the images were photographed accordingly.

For docking studies, chemical structures were generated using ACD/ChemSketch [16]. The three dimensional structure of collagen was generated using gencollagen program at The University of California, San Francisco [17]. Docking techniques were useful to find the binding efficiency with ligand and a chemical compound. To assess the interaction between collagen and AA, AUTODOCK has been used and AutoDock 4.2 was used to calculate (http://www.cgl.ucsf.edu./cgi-bin/gencollagen.py) the free energy of binding between the AA and collagen.

The biodegradability assessment was made by two different procedures. The first procedures demonstrate the measurement of release of hydroxy proline by the enzymatic treatment of AACC and native sheets [18] and the second procedure emphasizes the release of amino acids in terms of leucine upon enzymatic treatment [19].

3 Results

Extraction procedures followed in the present study provided ASC of type III collagen. Molecular profile study demonstrates (Fig. 1A) presence of α-1 (III) chains (100 KDa) and β chains (200 KDa). With reference to physical observations made for the biopolymer material prepared in the form of sheets, we obtained a clear transparent, smooth surface with 0.06–0.08 mm thickness (Fig. 1B).

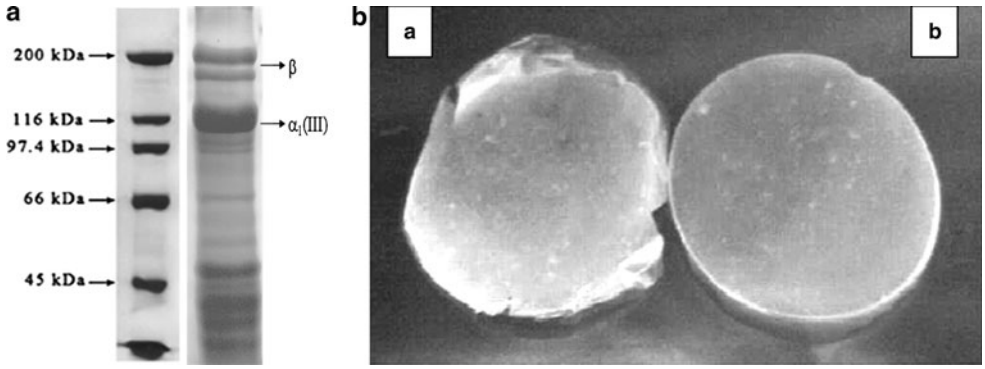

Fig. 1 **A** Molecular profile of ASC obtained from avian intestine (*Lane 1* molecular marker; *Lane 2* ASC from avian intestine). **B** Physical appearance of biomaterial made from (*a*) type III collagen alone and (*b*) AACC

With regard to the results on fibril formation and fibril assembly studies, Fig. 2A illustrated pH optimization and Fig. 2B (a–d) illustrated temperature optimization at different NaCl concentration. The absorbance versus incubation time curves showed the typical sigmoid curves with three stages of molecular rearrangement. Progressive aggregation of collagen increases the absorbance of solution. The first stage, lag phase, without any change in the absorbance corresponds to the nucleation of collagen fibrils followed by the second stage, growth phase, with rapid absorbance corresponding to growth of fibrils and then the final stage, maturity phase, with constant value of absorbance including three dimensional networks of fibrils [20]. The optimum condition for fibril formation was observed as 20°C, pH 6.0 and 35–70 mM NaCl concentration.

Figure 3A illustrated FT-IR spectral details of AA (a), native (ASC) (b) and AACC (c) respectively. With regard to mechanical property, about 5–6 fold increase in tensile strength was observed after cross-linking with AA. The tensile strength measurements of native (ASC), AACC and GCC were taken and the ultimate tensile strength (MPa) and Young's modulus (Gpa) values were represented in Table 1.

Figure 3B illustrated thermo gravimetric analysis (TGA) and the corresponding derivative peaks for all the experimental samples obtained in the present study. Thermal stability of AA was observed as 252°C, whereas for native collagen (ASC) it was 322°C. When type III collagen was cross-linked with AA and glutaraldehyde, thermal stability increased up to 370 and 337°C respectively. DSC measurements of native (ASC), AACC and GCC were illustrated in Fig. 3C. The melting temperature of 88, 96.98, 147, and 151°C respective to AA, native collagen (ASC), AACC and GCC was observed. Figure 3D showed SEM analysis of uncross-linked collagen and AACC sheets. Followed by cross-linking, the biopolymer material exhibit more compact, porous and highly denser structures. With regard to analysis of degree of cross-linking, Fig. 4 depict, the percentage of cross-linking degree with reference to increasing concentration of AA. The maximum of 75% degree of cross-linking was observed with 1.5% concentration of AA with 0.5% of ASC. However, the cross-linking degree of GCC was observed as 83%. Cell toxicity studies (MTT assay) revealed, skin fibroblast cells showed live cells with an increased OD measured at 570 nm for the increased concentration of AA cross-linked biopolymer (Fig. 5A). Figure 5B (i–iv), demonstrate, growth of fibroblast cells in the presence of AACC viewed on day 2 and day 3. Though proliferation was observed with control cells (Fig. 5B (i–ii)), however, confluence in growth was observed only after incorporation of AACC (Fig. 5B (iii–iv)), suggests, AACC, promote cell proliferation and also the morphology of fibroblast cells were maintained.

With regard to biodegradability assessment, biopolymer material obtained upon cross-linking of AA with ASC found degradable in the presence of collagenase enzyme with in the period of 4–20 h of incubation as assessed in terms of release of hydroxy proline and other amino acids as leucine (Fig. 6a, b). Further increase in incubation period did not increase the release of hydroxy proline (data not shown) and leucine. When compared to native, the release of both hydroxy proline and leucine was significantly less ($P < 0.05$) in AACC.

Results on binding energy calculations made based on bioinformatics tools for the cross-linking of AA and ASC using AutoDock software, (Fig. 7a, b) and Table 2 depict the values for the binding energy, interaction sites, hydrogen bond sites and bond distance respectively. Binding energy of −7.14 observed when Alanine (11) residue of A chain of collagen interacting with AA through Nitrogen of alanine and forming three hydrogen bond with bond distance of about 3.29, 3.04 and 2.85. Alanine (9) of C chain of collagen interacted with AA through oxygen (of –OH group) and forming two hydrogen bonds with the bond distance of 3.54 and 2.78. Similarly glycine (7) of C chain of collagen also forms one hydrogen bond through Nitrogen with bond distance of 2.88 and 2.68.

4 Discussions

Preparation of stable, biocompatible and biodegradable collagen based biopolymer materials reduces most of the

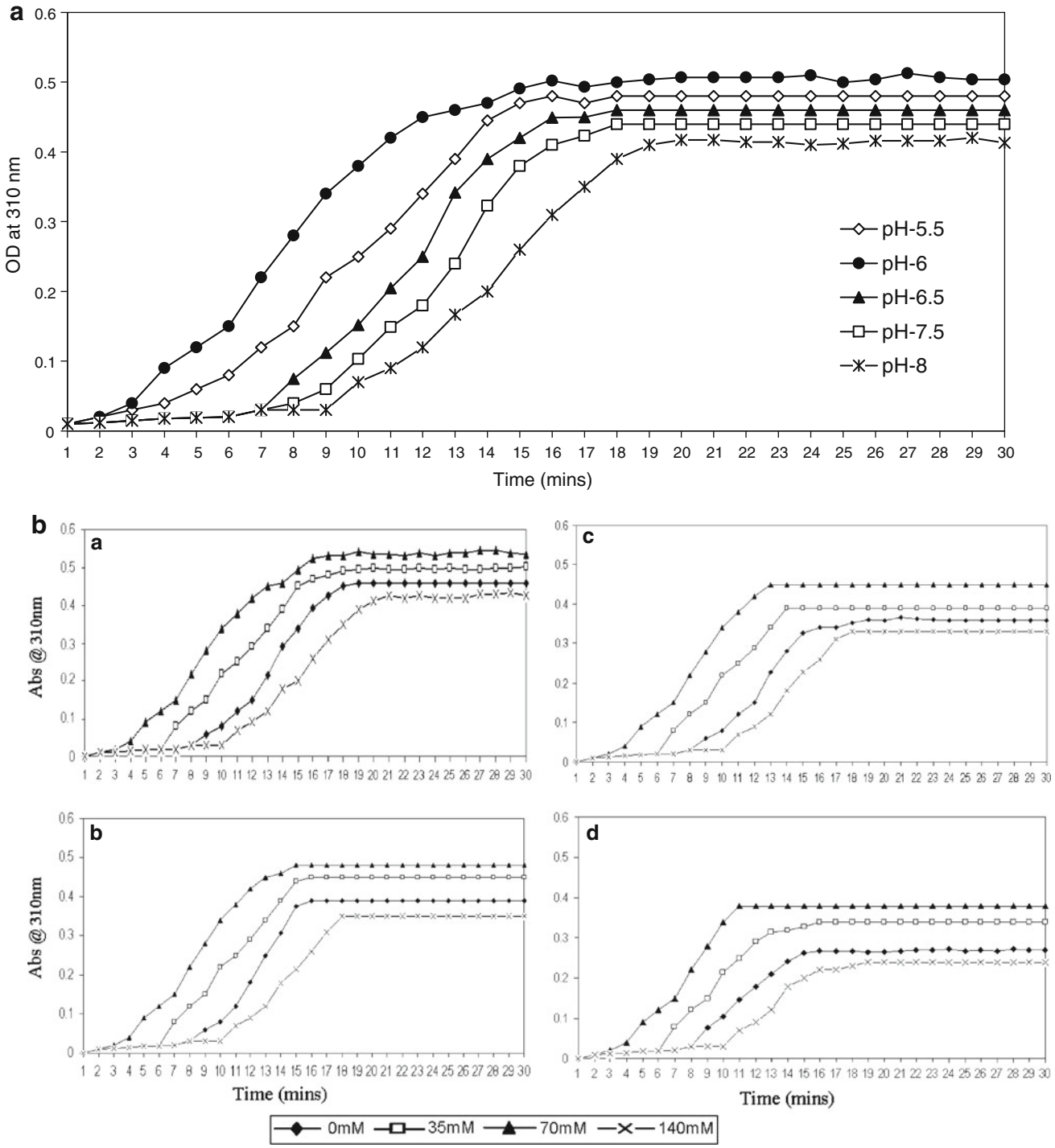

Fig. 2 **A** Optimization of pH on fibril formation of type III collagen. **B** Optimization of NaCl concentration on fibril formation of type III collagen at different temperatures (°C): (*a*) 20, (*b*) 25, (*c*) 30, (*d*) 37

problems associated with the clinical applications of synthetic, metallic and polymeric biomaterials. The present study aimed to prepare a novel biopolymer materials with all the requisite properties (thermal stability, elasticity, biocompatibility, biodegradability) using avian intestine collagen with natural polymer. Avian intestine, are the byproducts of the animal thrown as a waste and or collected by a group of people for the preparation of animal feed [21]. The natural polymer, AA was obtained from seaweeds [22]. The current trends in the replacement therapy requires materials of biocompatible and biodegradable nature, due to the disadvantages like toxicity [23],

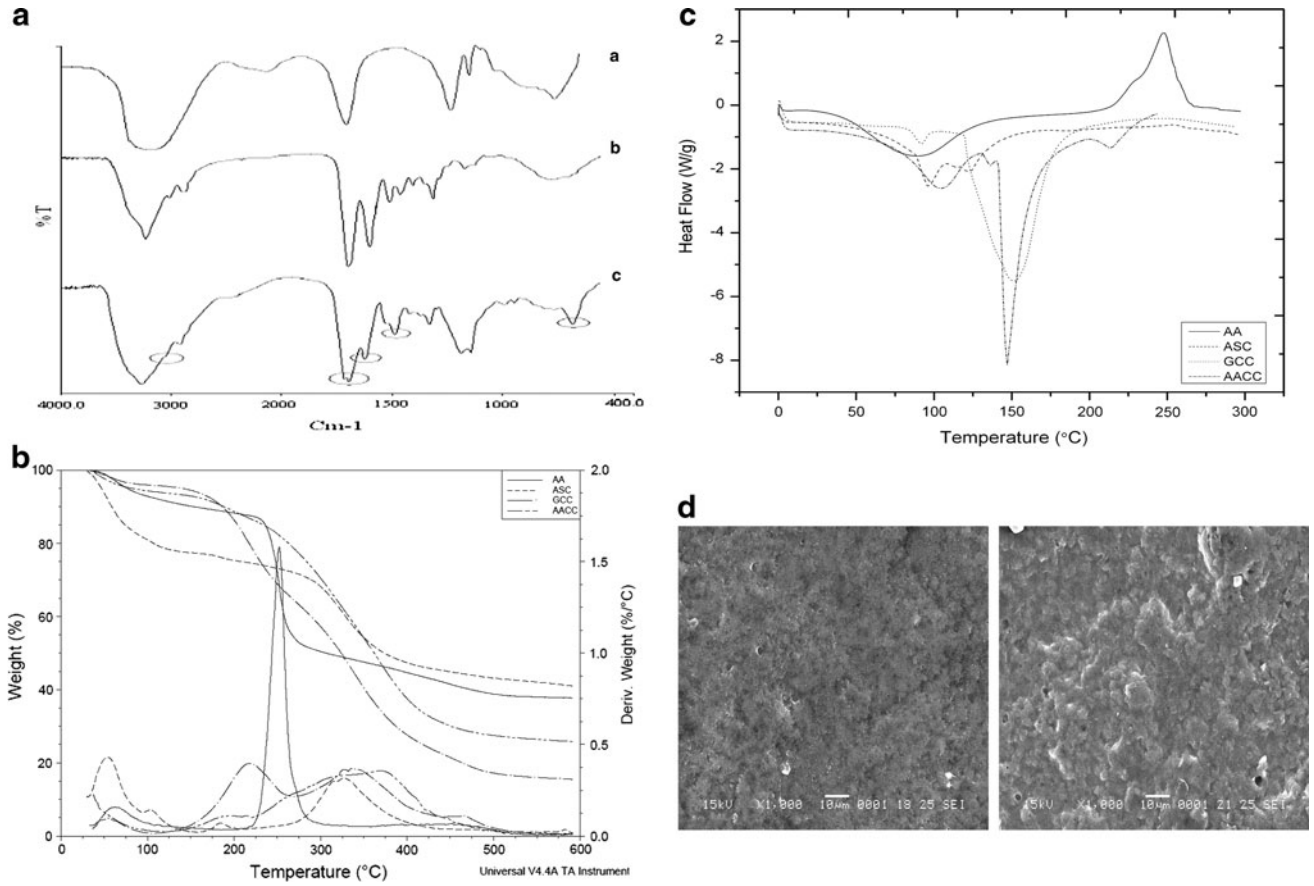

Fig. 3 **A** FT-IR spectrum of (*a*) AA, (*b*) ASC, (*c*) AACC. **B** TGA of ASC, AA and AACC. **C** DSC analysis of ASC, AA and AACC. **D** SEM images of ASC and AACC

Table 1 Tensile strength measurements of native collagen and AACC (type III collagen crosslinked with AA)

Material type	Tensile strength (MPa)	Young's modulus (Gpa)	Elongation at break (%)
Native film	2.110	7.8	31.99
AACC	11.56	47.08	5.31
GCC	9.4	22	3.32

carcinogenicity observed with the current polymeric and other synthetic materials, urges the scientific society to prepare materials of natural origin [24]. Use of natural material alone could not serve the purposes and most of the natural materials need some blends. The choice of blend finally decides the application of the materials. The natural material AA was chosen to cross-link with type III collagen to prepare a biocompatible and biodegradable biopolymer materials. Cross-linked biopolymer materials exhibit all the requisite properties as evidenced through the characteristic analyses made in the present study.

The molecular profile studies on type III collagen was found similar to the report of Kuga et al. [25]. With reference to fibril formation analysis, in general, precipitation of protein with metal salts depends on the binding efficiency of ions with water. Lower binding efficiency favors precipitation, whereas the higher binding efficiency denatures the protein. In other words, addition of NaCl to the mixture containing protein and water, immediate formation of hydrogen bond between NaCl and H_2O, precipitates the protein (salting out). In the case of collagen, the melting temperature of collagen reduced in the presence of NaCl, and at minimum concentration of NaCl, hydrogen bonding preferences to water molecule than collagen, resulting with precipitation or fibrillogenesis of collagen [26], however, at higher concentrations of NaCl, addition to hydrogen bonding with water, bonding with collagen molecule also occurs which ultimately disturb the helical pattern or not allowing the chains to form helix and finally inhibit the fibrillogenesis process [27]. Maximum fibril formation in avian intestine collagen was observed at 70 mM concentration of NaCl, pH 6.0 and at temperature 20°C.

The cross-linked polymer material when subjected to FT-IR analysis, the overlapping region of –C=O and –N–H

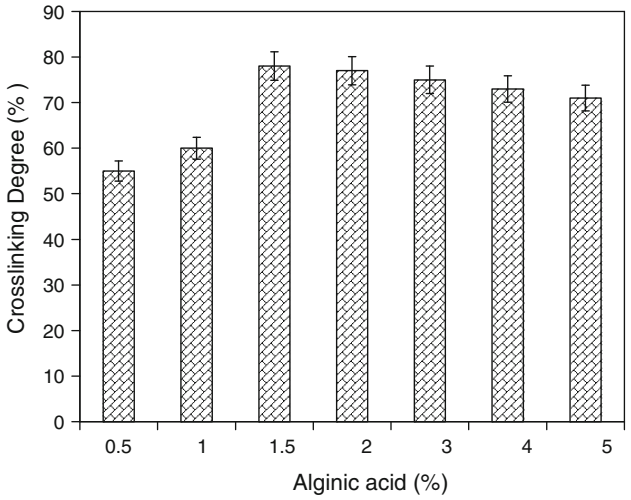

Fig. 4 Measurement of percentage of degree of cross-linking of ASC with AA

bands in the range of 1600–1650 cm^{-1} as shown by the circle marking in Fig. 3A was observed. In addition, the intensity of primary amine –N–H-bending (out-of-plane) broad peak (655 cm^{-1}) changed to sharp peak, and the peak intensity of –C–N-stretch (1400 cm^{-1}) in native collagen found weak, whereas in cross-linked biopolymer material, the intensity of the peak was strong due to the formation of –CONH (amide) bond. The peak intensity of secondary amine –N–H-bending (1553 cm^{-1}) was very strong in native collagen, whereas in AACC, it was very weak due to the absence of $–NH_2$ group in lysine residue of collagen. In native collagen, observed an overtone in the range of 3081 cm^{-1} due to secondary –N–H-bending at 1553 cm^{-1}. However in AACC, the overtone was vanished due to cross-linking of $–NH_2$ group of lysine residue with AA [28].

With regard to the thermal analyses of cross-linked biopolymer material and the uncross-linked (native) material, increase in thermal stability upon cross-linking with AA was observed. Salome Machado et al. [29] observed similar results for the cross-linking of collagen with chitosan. Derivative information's shown in Fig. 3B emphasizes, sharp peak at 252°C corresponds to AA alone and a broad peak at 322°C corresponds to collagen alone. When collagen was mixed with 1.5% of AA, shifting of collagen broad peak towards right side with an increase in peak intensity of AA and a decrease in peak intensity of collagen was observed.

Differential scanning calorimetric studies recorded melting temperature differences among AA, native and AACC. The melting temperature of cross-linked collagen shifts towards right when the concentration of AA is 1.5%.

Collagen reacts with AA through the ester formation in AA due to loss of water [30]. The resultant lactones are biologically active and ready to react with free $–NH_2$ group of lysine in collagen and the final product (AACC) was covalently linked to each other through the formation of amide bond which was reflected in the percentage of cross-linking degree (75%) analysis at 1.5% concentration of AA and FT-IR spectroscopic analysis. Wu et al. [31] observed the covalent linkage between glutaraldehyde and collagen. Further, with reference to chitosan (a natural polycationic polymer) cross-linking with collagen, bond formation was between the $–NH_2$ groups of chitosan with –COOH group of amino acids of collagen. However, in the case of AA (a polyol) [32] having more number of –OH groups, ready to interact with collagen through intermolecular multiple hydrogen bonds as shown in Fig. 7 was similar to the observations made by Madhan et al. [33] in the reaction between collagen and catechin. Further, the binding energy calculations (Table 2) received from the bioinformatics tools software also confirms the intermolecular multiple hydrogen bonding. Thus, both covalent and hydrogen bonding interactions occurs between collagen and AA, which in turn increases the stability and mechanical property (tensile strength) of the resulting biopolymer material appreciably.

Biocompatibility of the stabilized biopolymer is the most important property whenever a new material was prepared. If the prepared material is biocompatible, then most of the problem associated during the application of biopolymer material will be reduced. In general, biocompatibility is assessed using different cell lines. Skin fibroblast cells were used to assess the biocompatibility of cross-linked biopolymer material. Theoretically, if the cross-linked collagen biopolymer material does not have any toxic groups and when tested for cell viability, cells should proliferate well and the OD of the live cells should show an increased value. In the present study, cells treated with the resultant cross-linked material (AACC) showed an increase OD values compared to untreated cells.

In order to study the application of cross-linked collagen biopolymer materials as implants, it is necessary to assess the biodegradability of the biopolymer developed. Since, numbers of enzymes were produced upon various biochemical reactions inside the body, degradation studies using enzymes found reliable. Enzymatic degradation of cross-linked biopolymer material (AACC) and native collagen material were studied and the results showed both the material degraded when treated with collagenase enzyme. However, the rate of release of leucine or hydroxy proline showed significant difference. In native collagen, release of hydroxy proline starts from the minute of exposure to enzymes compared to cross-linked collagen. When we compare the release of leucine and hydroxy proline, we found, amount of other amino acids measured as leucine release was high in comparison with hydroxy proline. This could be due to intermolecular hydrogen bonding between free hydroxyl group of

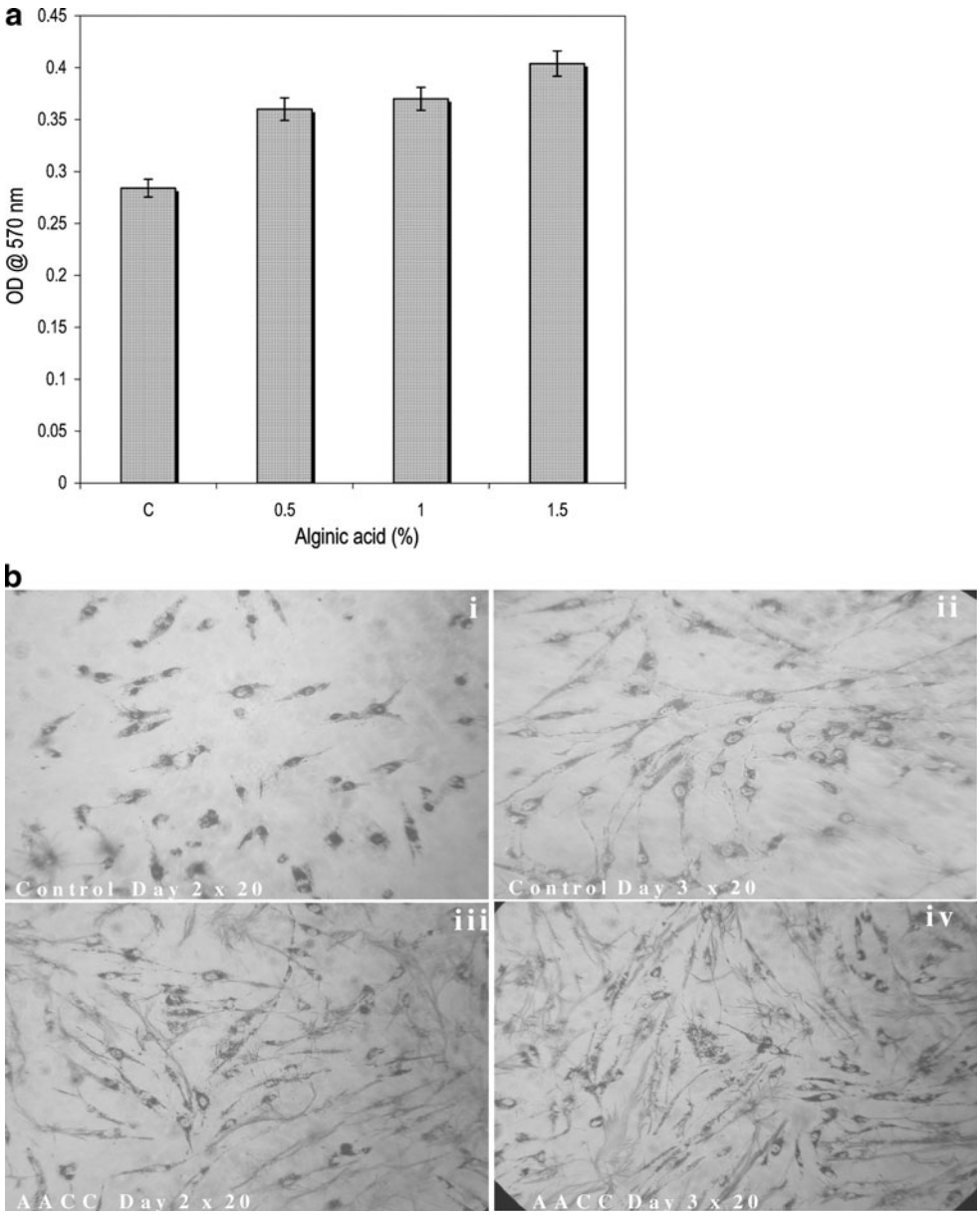

Fig. 5 **A** MTT analysis of collagen (ASC), and different percentages of AACC. **B** Skin Fibroblast cell proliferation observations for control and AACC incorporated cells viewed on day 2 and day 3 of experimental period. *Images i–ii* corresponds to control samples and *iii–iv* corresponds to AACC incorporated cells

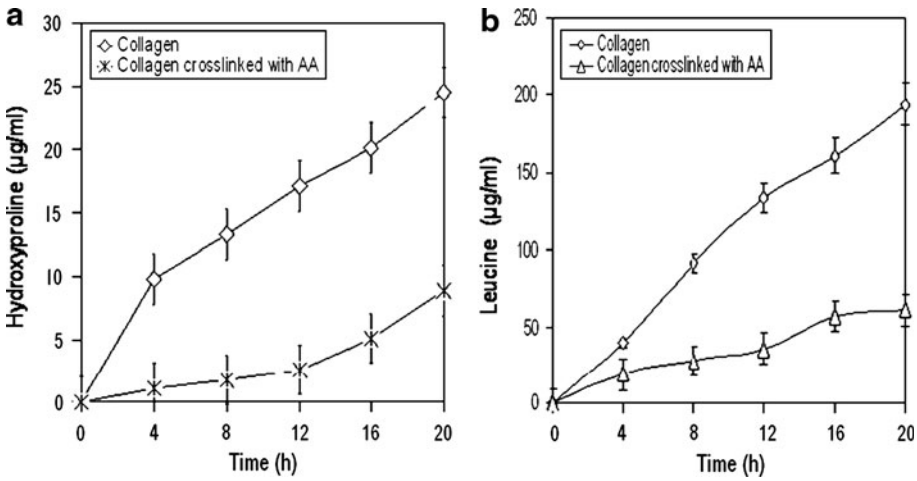

Fig. 6 Biodegradability assessment based on **a** release of hydroxyl proline and **b** release of Leucine from ASC, and AACC

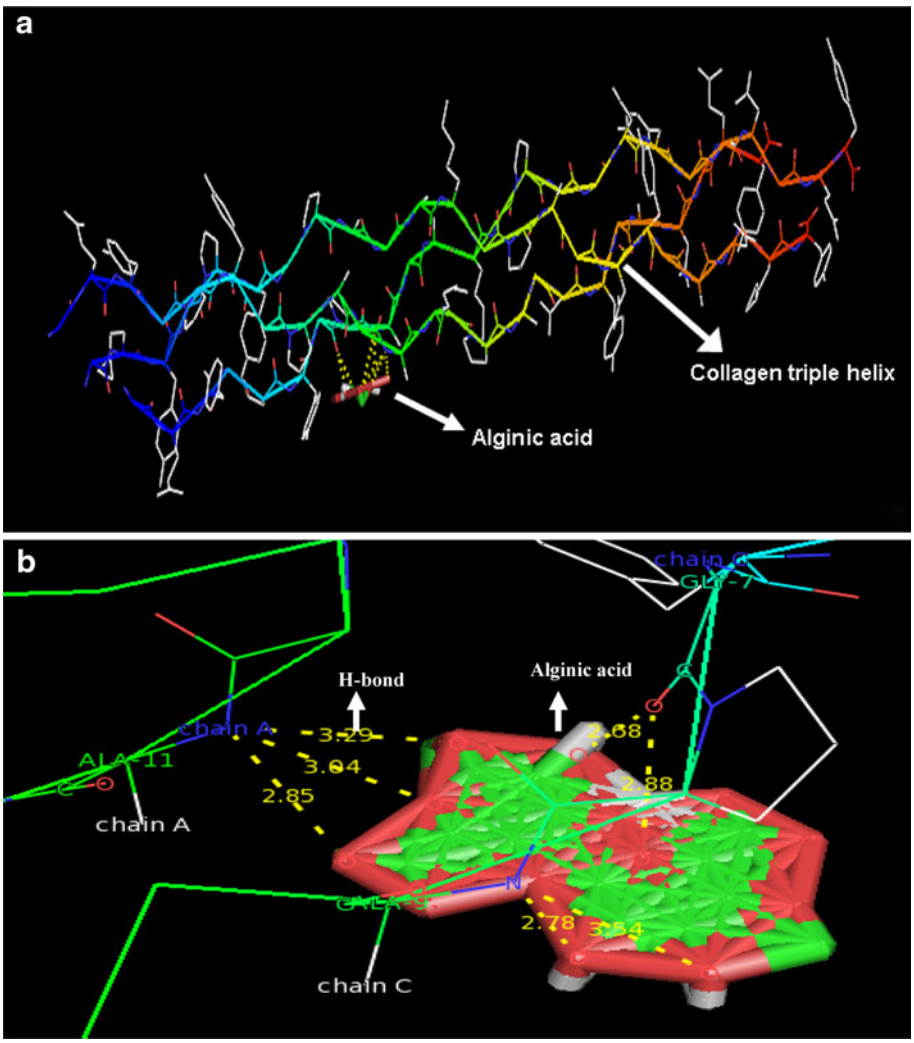

Fig. 7 **a** Bonding pattern of collagen with AA and **b** magnified image of the cross-linking obtained from bioinformatics tools

Table 2 Interaction sites, binding energy, hydrogen bond sites and bond distance, calculated based on the bioinformatics tools for the cross-linking between collagen (type III) and AA

Interaction site	Binding energy (kcal/mol)	H-bond	Bond distance (Å)
Eleventh residue Ala of A-chain (α_1)	−7.14	Ala A 11-(C_α)-N···H–O(AA)	3.29, 3.04, 2.85
Ninth residue Ala of C-chain (α_1)		Ala C 9-(C_α)-N···H–O(AA)	2.78, 3.54
Seventh residue Gly of C-chain (α_1)		Gly C 7-(C_α)-O···H–O(AA)	2.88, 2.68
Seventh residue Gly of C-chain (α_1)	−6.89	Gly C 7-(C_α)-O···H–O(AA)	3.27
Eleventh residue Ala of A-chain (α_1)		Ala A 11-(C_α)-N···H–O(AA)	3.02, 2.97, 3.03
Eleventh residue Ala of A-chain (α_1)	−6.5	Ala A 11-(C_α)-N···H–O(AA)	3.08, 2.98
Ninth residue Ala of C-chain (α_1)		Ala C 9-(C_α)-N···H–O(AA)	2.78
Seventh residue Gly of C-chain (α_1)		Gly C 7-(C_α)-O···H–O(AA)	2.57
Twelfth residue Lys of A-chain (α_1)	−6.07	Lys A 12-(C_α)-N···H–O(AA)	2.88, 3.10
Thirteenth residue Gly of A-chain (α_1)		Gly A 13-(C_α)-O···H–O(AA)	2.89, 3.12
Thirteenth residue Gly of A-chain (α_1)	−5.92	Gly A 13-(C_α)-O···H–O(AA)	3.22, 2.79, 2.59
Fourteen residue Pro of A-chain (α_1)		Pro A 14-(C_α)-N···H–O(AA)	3.53
Twelfth residue Lys of A-chain (α_1)		Lys A 12-(C_α)-N···H–O(AA)	3.25, 3.32

hydroxy proline and –OH group of AA (OH–OH) which ultimately protects the material from the action of collagenase enzyme and the random action of collagenase enzyme releases amino acids mostly leucine compared to hydroxy proline. Thus, the results of the study showed the cross-linked collagen biopolymer material is biodegradable.

5 Conclusion

In the present study the choice for the preparation of thermally stable and biodegradable biopolymer material was type III collagen of avian intestine and a natural anionic polysaccharide AA. Though numbers of studies were available for the different use of AA, its cross-linking potential with collagen has not yet in reports. The present study describes, the cross-linking ability of AA with type-III collagen assessed using FT-IR, TGA, DSC, SEM and degree of cross-linking measurements using standard procedures. The results obtained proves, AA was cross-linked with collagen through (i) multiple intermolecular hydrogen bonding (proved by bioinformatics study) and (ii) by covalent amide linkage (proved by TNBS/percentage of cross-linking degree assay and FT-IR analysis). The biopolymer material (sheet) prepared upon cross-linking of AA was the green method of preparation. No toxic compounds were involved in this preparation and the resultant material found application as wound dressing sheet in clinical applications.

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