Chemistry behind the Elastic Nature of the Biomaterial Prepared Using Oxidized Form of Glutaraldehyde and Chitosan - An Approach at 2D and 3D Level

Subtitle: Glutaric Acid Cross-Linked Chitosan Biopolymer

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***Abstract*-This is the time to search an alternative cross-linker which will provide a non-toxic and mechanically stable biopolymer material. In order to achieve the requisite property, in the present study, we have chosen glutaric acid and studied its interaction with chitosan. A 3D scaffold biopolymer material prepared using chitosan and glutaric acid, displayed requisite mechanical strength and in addition found biocompatible for NIH 3T3 fibroblast cells. The interaction chemistry and the characteristics of the biopolymer material obtained upon cross-linking suggest non-covalent interactions play the major role in deciding the property of the said materials and its suitability for biomedical applications.**

Keywords- Ionic Cross-Linking; Glutaric Acid; Glutaraldehyde; Chitosan; Bioinformatics

I. INTRODUCTION

Recent research on biomaterial development suggests, biopolymers of natural origin find immense clinical applications. And most of the research publications discussed the significant role of natural polymers in biomedical applications [1-4]. Several researchers have chosen glutaraldehyde [5, 6] for cross-linking with chitosan because of its cross-linking pattern with natural polymer. The free $-NH_2$ group of chitosan can easily react with –C=O group of glutaraldehyde to form compounds with a carbon-nitrogen double bond (C=N) called imines. The chemistry behind the formation of imine type compound has been given in discussion session. However, glutaraldehyde cross-linked chitosan based biopolymer demonstrates less mechanical strength [7] and cell toxicity [8]. Since, these two properties are of high importance for the biopolymers, an alternative cross-linking agent is the need of the hour.

According to the basic chemistry of oxidation/ reduction reaction of aldehydes, acid formation is the final product and which cannot be oxidized further and needs reducing agent for reduction processes as shown below:

$$\text{Alcohol} \underset{\text{Reduction}}{\overset{\text{Oxidation}}{\rightleftharpoons}} \text{Aldehyde} \underset{\text{Reduction}}{\overset{\text{Oxidation}}{\rightleftharpoons}} \text{Acid}$$

With regard to cross-linking of chitosan with traditional cross-linking agent glutaraldehyde at room temperature, formation of glutaric acid is unavoidable. Hence, it is necessary to study the impact of glutaric acid with chitosan. For chitosan based material, de-acetylated chitosan was in use.

Glutaric acid (pentanedioic acid), C-3 organic dicarboxylic acid, is a white crystalline powder with the formula (HOOC $(CH_2)_3$COOH). Glutaric acid mainly occurs in plant and animal tissues and is found in the blood and urine and finally non-toxic [9].

Thus, the present study emphasizes, how much the effectiveness of the cross-linking of glutaric acid with natural polymer chitosan and further demonstrate the cross-linking chemistry between the molecules using suitable bioinformatics tools. In addition, thermal, mechanical properties and the biocompatibility of the resultant biopolymers also explored in detail.

II. EXPERIMENTAL DETAILS

A. Materials

Chitosan from shrimp shells (≥75% deacetylated), glutaric acid, Picrylsulfonic acid [2, 4, 6-Trinitrobenzene sulfonic acid (TNBS)], were obtained from Sigma- Aldrich (USA). 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.

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III. PREPARATION AND CHARACTERIZATION OF 2D AND 3D BIOPOLYMERS

A. Preparation of Two-Dimensional (Sheet) Biopolymer

For the preparation of biopolymers using glutaric acid, the following procedure was employed. In this procedure, the use of acetic acid for dissolution of natural polymer like Chitosan was completely avoided. In brief, the powder form of chitosan (1%) was added to 20 ml of water taken in the glass beakers and stirred vigorously to ensure the uniform distribution. To that dispersed mixture, glutaric acid was added and the stirring was continued for an hour. Concentration of glutaric acid (GA) was varied between 0.05-0.5% (w/v). Followed by stirring, the samples were subjected to centrifuge and a clear solution obtained upon centrifugation at 5,000 rpm for 10 min. The 2D biomaterial was obtained in the form of sheet by transferring the clear solution to polypropylene plate (Tarson, India) and air dried at 37°C for 12 h and designated as GA cross-linked chitosan (GACCH). In addition, a separate chitosan sheet was prepared using 0.05 M acetic acid for comparative analyses.

B. C. Preparation of Three-Dimensional (Sponge) Biopolymer

According to the above procedure the clear solution of GACCH was prepared and poured in Tarson (India) vial of an inner diameter of 4.5 cm and frozen at -4 ℃ for 2 h, -20 ℃ for 12 h and -80 ℃ for another 12 h. The frozen samples were lyophilized for 48 h at vacuum of 7.5 militorr (1 Pa) and a condenser temperature of -70 ℃ (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 GACCH (glutaric acid cross-linked chitosan). In addition, a separate chitosan sponge was prepared using 0.05 M acetic acid for comparative analyses.

For comparative analysis, glutaraldehyde cross-linked chitosan scaffold (GADCCH) was prepared according to the method described in the above said paragraph using 0.2% glutaraldehyde.

1) Texture and Morphology of the Biopolymers:

The physical texture and the morphology of the biopolymers of glutaric acid cross-linked chitosan was assessed using physical touch followed by scanning electron micrograph. SEM micrograph analysis was made using F E I Quanta FEG 200 - High Resolution Scanning Electron Microscope instrument under high voltage at 20 kV.

2) Analysis of Functional Groups:

Functional group analysis (FT-IR) for GA, chitosan and GACCH biopolymers were made by spectrum one (Perkin-Elmer Co., USA model) FT- IR instrument. All spectra were recorded with the resolution of 4 cm^{-1} in the range of 400-4000 cm^{-1}

3) Estimation of Percentage of cross-Linking Degree (TNBS Assay):

Degree of cross-linking was quantified using TNBS assay according to the procedure summarized by Bubnis et al [10]. In brief, native (chitosan alone) and cross-linked (GACCH) biopolymer materials were cut into small pieces of size 4.5 mm. Six mg of cut pieces were 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 ℃ for 2 h. Glutaric acid alone at respective concentration was also treated with 2 ml solution in separate test tubes. Termination of reaction was by the addition of 3 ml of 6 M (V/V) HCl and the incubation was continued at 60 ℃ for 90 min. The absorbance of the resulting solution was measured at 345 nm using UV-Visible spectrophotometer (Shimadzu, UV-2450, Japan) and the percentage of cross-linking was calculated from the difference in the absorbance divided by the absorbance of the native (chitosan alone) material and then multiplied by 100.

4) Analysis of Mechanical Properties of Chitosan, GACCH and GADCCH 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 cross head speed of 5 mm min^{-1} at 25 ℃ and 65% relative humidity. Length and width of the dumbbell shaped test sample maintained as 20 and 5 mm respectively. All the mechanical tests were performed with dried samples and were examined in triplicate way.

5) Thermo Gravimetric Analysis (TGA):

Thermal decomposition analysis of GA, native and cross-linked biopolymers (chitosan, GACCH and GADCCH) were carried out under nitrogen flow (40 & 60 ml min^{-1}) with ramp 20 ℃ min^{-1} using TGA Q 50(V20.6 build 31) instrument.

6) Differential Scanning Calorimetry (DSC):

Thermal properties of GA, native and cross-linked biopolymers (chitosan, GACCH and GADCCH) were analyzed using differential scanning calorimeter, model -DSC Q 200(V 23.10 Build 79) with standard mode at nitrogen (50 ml min^{-1}) atmosphere with ramp 10 ℃ min^{-1}.

7) Binding Energy Calculations Using Bioinformatics Tools:

For the docking study, chemical structures of chitosan and GA were generated using ACD/ChemSketch [11]. Docking

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technique is useful to find out the binding efficiency with ligand and a chemical compound. To find out the interaction between chitosan and GA, AUTODOCK has been used and AutoDock 4.2 used to calculate [12] the free energy of binding of GA with chitosan. It's faster than other versions and force field includes an updated charge-based desolvation term, improvements in the directionality of hydrogen bonds, and several improved models of the unbound state. Current procedure categorized under semi-flexible docking protocol in that chitosan was kept as rigid and ligands being docked was kept flexible; Kollman united atom charges, salvation parameters and polar hydrogens were added to chitosan PDB file for the polysaccharide, to ligands Gasteiger charges were assigned and then non polar hydrogen was merged before docking simulation. Total number of rigid roots was defined using automatically with amide bond kept as non-rotatable. The possible dihedrals in the ligand are allowed to rotate freely using Auto-Tors. Pre-calculated grid maps for each atom in the ligand were generated using Auto-grid. The 5A º grid was built surrounding the binding pocket. Three-dimensional grids of interaction energy for all possible atom types that were already present in the Auto Dock default parameter set were calculated. This grid maps were of dimension $60 \times 60 \times 60$ points with the spacing of 0.375 A º yielding a receptor model that included atoms within 0.5 A º of the grid center. The Graphical User Interface program "Auto-Dock Tools" was used to prepare, run and analyze the docking simulations. The Lamarckian Genetic Algorithm (LGA) was chosen to search for the best conformers.

8) In Vitro Assessment on Cell Compatibility of the Biopolymers:

Biocompatibility in terms of cytotoxicity, cell proliferation, live cell detection and cell attachment on the prepared scaffold biopolymer were analyzed using NIH 3T3 fibroblast cell line. According to Trentani et al [13] this cell line is a robust and durable platform for investigating common cellular functions: attachment, viability, proliferation and cellular properties, etc.

9) Cell Proliferation Study (MTT Assay):

Cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal bovine serum and 1% antibiotic and were incubated at 37 ℃ in 5% CO_2 humidified atmosphere. Polystyrene 24 well culture plates (Tarson, India) were coated with native chitosan and GA cross-linked chitosan (GACCH) biopolymers. The plates were dried under laminar air flow hood followed by UV sterilization. The cells were seeded at the density of $0.5 \text{ X } 10^6$ per well and incubated at 37 ℃ 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 ℃ 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 a few minutes to ensure all crystals are dissolved. Finally, absorbance was measured at 570 nm using UV spectrophotometer [14]. Each experiment was performed at least three times. The sets of three wells for the MTT assay were used for each experimental variable.

10) Cell Tracker Assay to Detect Live Cells:

Cell viability was measured using 5-chloromethylfluorescein diacetate probe (CMFDA) (Invitrogen). 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 [15].

11) Cell Growth and Morphology of NIH 3T3 Cells in GACCH Biopolymer:

GACCH scaffold ($2 \times 2 \times 1$ cm) was placed in 6 well culture plates (Tarson, India) and ETO (Ethylene Oxide) sterilized. Culture media added to the scaffolds for overnight. NIH 3T3 fibroblast cells were seeded onto the scaffolds at a density of 5×10^4 cells and incubated in an atmosphere of 5% CO_2 at 37 ℃. The medium was changed every 24 h. Morphology of the cells examined after 12 days according to the following procedure. The cells-scaffold constructs were fixed in 2.5% glutaraldehyde and dehydrated through graded ethanol series [16]. The dried cells-scaffold were coated with gold (E-1010 Ion sputter, HITACHI) and examined under SEM (S-3400 N Scanning Electron Microscope. HITACHI).

IV. RESULTS AND DISCUSSION

As described in the Introduction, cross-linkers are used to stabilize natural polymer materials for biomedical applications. Glutaraldehyde is the maximum choice compared to other cross-linkers. However, the recent realization of the toxicity and the mechanical properties of glutaraldehyde cross-linked biopolymers necessitates the alternative cross-linkers. In the present study, suitability of glutaric acid as cross-linker was evaluated using natural polymer and in addition understanding the chemistry behind the interactions.

Biopolymer preparation using glutaric acid: Understanding the cross-linking chemistry

For the preparation of any biopolymer materials, the solution form of parent compound/polymer is required to proceed further. However, the natural polymer chosen for the present study were insoluble in water and acetic and formic acids were used for dissolution [17, 18]. The "proton exchange" between –COOH groups of acid molecule and free $–NH_2$ groups of chitosan shown in the following scheme could be reasoned for the dissolution in the said acids.

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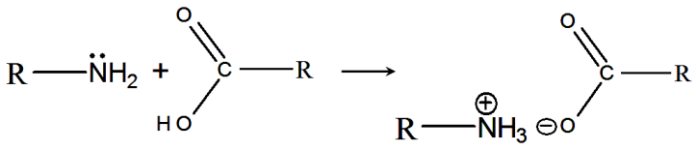
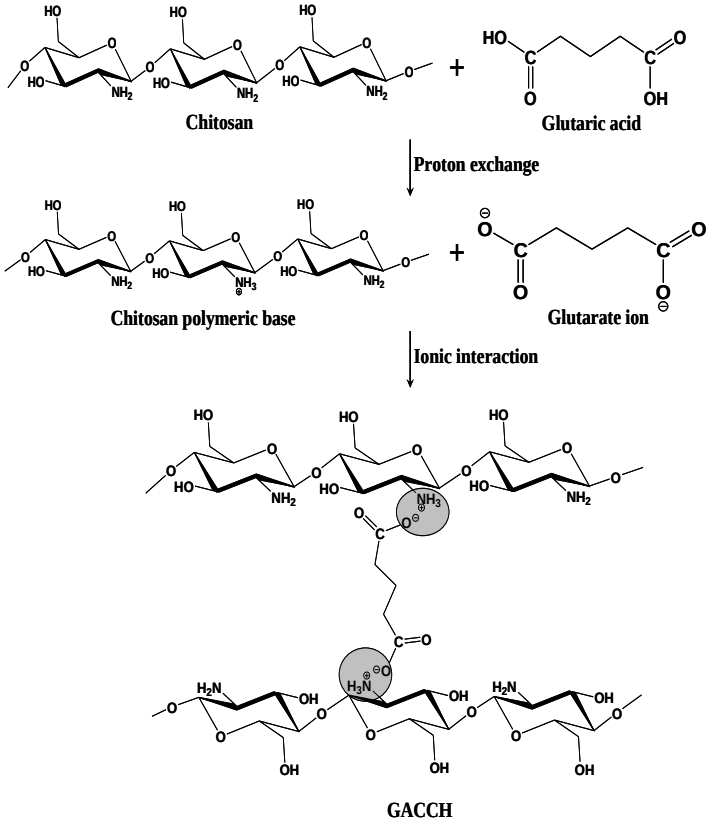


Therefore, it has been expected that like acetic acid, glutaric acid is also possible to provide protons to dissolve chitosan. Further, alike interaction of TPP (tripolyphosphate) [19] with chitosan, glutaric acid also interacts with the natural polymer through ionic interaction.

Because of the said proton exchange, chitosan get dissolved in the presence of glutaric acid in water and the following schematic representations (Scheme 1A and 1B) illustrates the nature of proton exchange between glutaric acid and chitosan for better understanding. The ionic interaction and the hydrogen bonding between –COOH group of cross-linker and $–NH_2$ group of natural polymers (chitosan/collagen) was already in reports [20-23].

Scheme: Possible reaction mechanism between chitosan and glutaric acid



Because of the said interactions, the natural polymer chitosan were completely dissolved in water in the presence of glutaric acid. With the resulting solution, scaffolds were prepared and subjected to characterization studies. Fig. 1 and Fig. 2 demonstrate the morphological features of the cross-linked biopolymer (GACCH). 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.

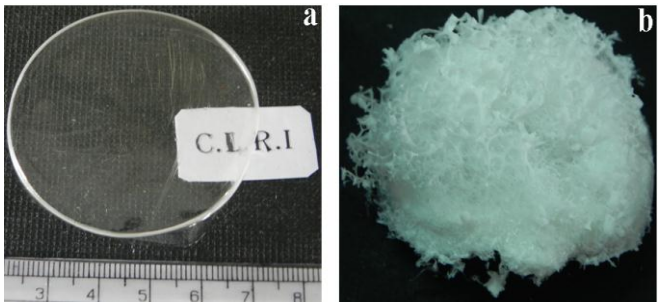


Fig. 1 (a) Digital image of two dimensional (2D) scaffold (sheet) of glutaric acid cross-linked chitosan (GACCH) biopolymer and (b) Digital image of three dimensional (3D) scaffold (sponge) of glutaric acid cross-linked chitosan (GACCH) biopolymer.

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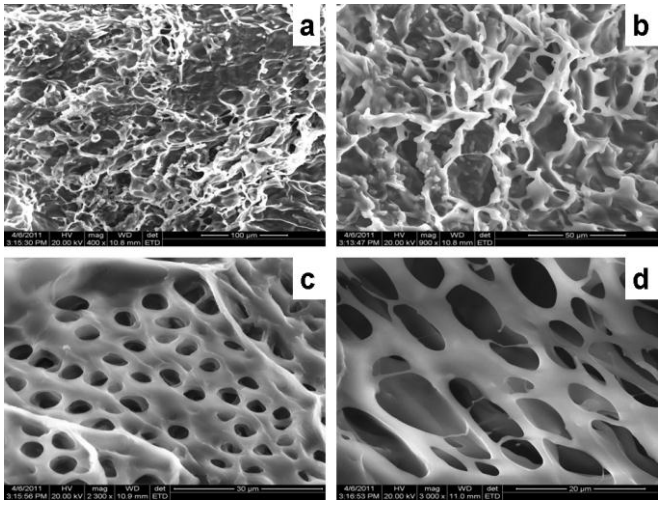

Fig. 2 SEM Micrographs of three dimensional (3D) scaffold of glutaric acid cross-linked chitosan (GACCH) biopolymer at different magnifications (100, 50, 30 and 20 μm)

FT-IR studies were conducted to monitor the chemical modifications in chitosan structure due to cross-linking with GA. Fig. 3 illustrates the FT-IR spectral details of GA, chitosan and GACCH. Table I demonstrates the FT-IR peak assignments of GA and chitosan. In GACCH spectrum few significant changes were observed. A broad, strong absorption in the region of 3446-2860 cm^{-1} was resulting from superimposed –OH and $–NH_3^+$ stretching band. Absorption at 1640 and 1572 cm^{-1} correspond to the presence of asymmetric N–H ($–NH_3^+$) bend and asymmetric $–COO^-$ stretching respectively, Peak observed at 1534 and 1409 cm^{-1} was due to symmetric N–H ($–NH_3^+$) bend and symmetric $–COO^-$ stretching respectively. Other absorption peaks around 1257, 1157 and 899 cm^{-1} observed in GACCH spectrum were similar to the native chitosan spectrum which exhibits that there was no change in main backbone of chitosan structure. Results from FT-IR analysis reflected that GA was ionically cross-linked with chitosan [24].

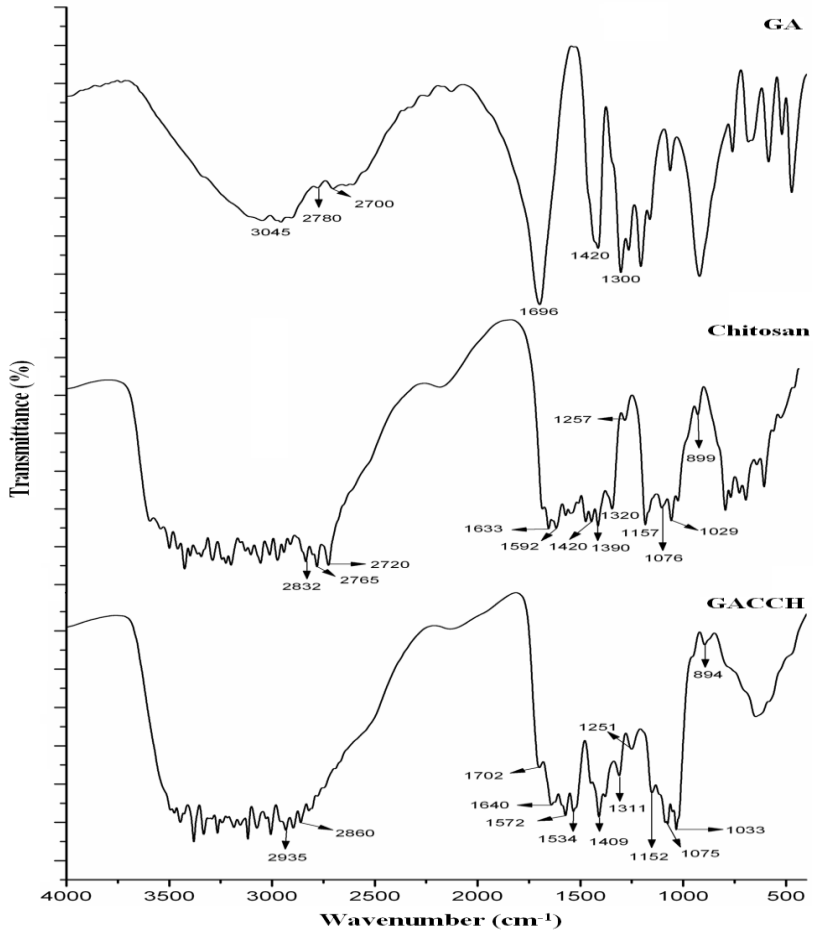

Fig. 3 FT-IR spectrum of Glutaric acid (GA), Chitosan and glutaric acid cross-linked chitosan (GACCH) biopolymer

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TABLE I FT-IR ANALYSIS OF GLUTARIC ACID AND CHITOSAN

Wavenumber (cm^{-1})	Peak assignment
Glutaric acid	
3300-2500	Broad O–H stretch (ν_{O-H})
2957	$-CH_2$ stretch (ν_{C-H}) superimposed upon O–H stretch
1698	Carboxylic –C=O group stretch ($\nu_{C=O}$)
1414	C–O–H in-plane bending (δ_{C-O-H})
1304	C–O stretching vibration (ν_{C-O})
921	Out- of- plane bending of the bonded O–H (δ_{O-H})
Chitosan	
3200	$-NH_2$ stretching vibration (ν_{NH})
2832, 2765, 2720	Symmetric or asymmetric $-CH_2$ stretching vibration attributed to pyranose ring (ν_{C-H})
1633	–C=O in acetamide group (amide I band)
1592	$-NH_2$ bending vibration in amino group (δ_{NH})
1420, 1320	Vibrations of OH, CH in the ring
1257	C–O group
1157	–C–O–C in glycosidic linkage
1076, 1029	C–O stretching in acetamide (ν_{C-O})
899	Corresponds to saccharide structure

ν- stretching, δ- bending

Though FT-IR analysis displayed the ionic interaction between the cross-linker and chitosan, results on the percentage of cross-linking degree suggests that increasing the concentration of glutaric acid increases the degree of cross-linking upto 0.4% and confirmed the interaction. Table II depicts the percentage of cross-linking degree for chitosan in the presence of increasing concentration of glutaric acid. About 60-66% cross linking was observed with 0.2% glutaric acid with chitosan. However, in the case of experiments with glutaraldehyde, about 88-93% of cross-linking was observed with 0.2% concentration of glutaraldehyde.

TABLE II MEASUREMENT OF CROSS-LINKING DEGREE OF GLUTARIC ACID CROSS-LINKED CHITOSAN (GACCH) PREPARED USING DIFFERENT CONCENTRATIONS OF GLUTARIC ACID (0.05-0.5%)

of Glutaric acid (%, concentration w/v)	**Percentage of cross-linking degree of GACCH (%)***
0.05	53.6 ±1.2
0.1	54.4 ±2.1
0.2	64.2 ±1.8
0.3	70.5 ±1.2
0.4	72.4 ±1.8
0.5	72.5 ±1.2

*mean ±SD values

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 glutaric acid concentration up to 0.2%. Further increase in GA concentration leads to the decrease in mechanical strength (results not shown). Table III illustrates tensile strength, young's modulus, stiffness of native and glutaric acid (0.2%) cross-linked biopolymers. Compared to native (chitosan alone), GACCH displayed six fold increase in percentage elongation at break as well as extension at maximum load (mm) in 2D materials and suggested, interaction with GA increases the elastic nature of chitosan. Similarly, 3D materials also demonstrated more than a six fold increase in elongation at break (%) and more than nine fold increases in extension at maximum load. Correspondingly, the Young's modulus value was observed as 1.38 and 0.772 MPa for 2D and 3D materials of GACCH respectively, whereas it was 135.89 and 4.43 MPa for native 2D and 3D forms. With respect to stiffness, GA interaction reduces the stiffness (κ) of the native chitosan material.

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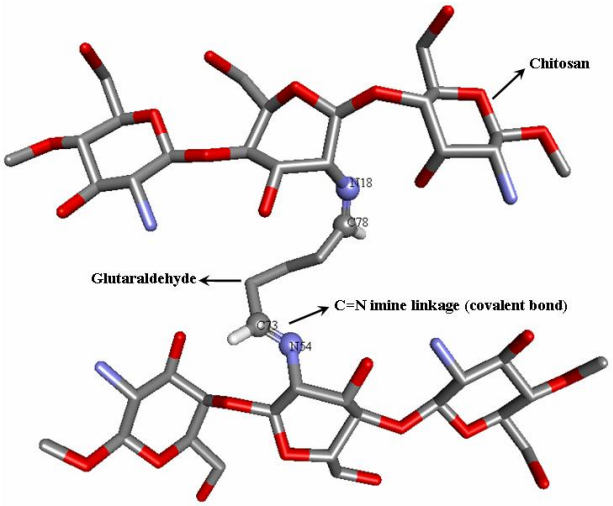
TABLE III ASSESSMENT OF MECHANICAL PROPERTIES OF CHITOSAN AND GLUTARIC ACID CROSS-LINKED CHITOSAN (GACCH) IN TERMS OF TENSILE STRENGTH, ELONGATION AT BREAK, YOUNG'S MODULUS AND STIFFNESS

Samples	Tensile strength (MPa)*	Elongation at break (%)*	Extension at maximum load(mm)*	Young's Modulus (MPa)*	Stiffness (κ) (N/mm)*
Chitosan 2D scaffold (sheet)	28.74 ±1.12	21.13 ±1.85	4.23 ±0.47	135.89 ±10.24	2.04 ±0.87
GACCH 2D scaffold (sheet)	1.67 ±0.39	120.75 ±9.64	24.15 ±2.15	1.38 ±0.24	0.066 ±0.008
Chitosan 3D scaffold (sponge)	0.37 ±0.08	8.33 ±1.28	1.67 ±0.87	4.43 ±1.03	0.79 ±0.06
GACCH 3D scaffold (sponge)	0.42 ±0.13	54.38 ±2.4	10.88 ±2.1	0.772 ±0.07	0.22 ±0.05

*mean ±SD values

All these observations on mechanical properties suggest, glutaric acid cross-linked biopolymer materials demonstrated appreciable mechanical strength compared to glutaraldehyde, where, we observed brittleness. Schiffman et al [7] reported brittle nature of the biomaterial upon cross-linking with glutaraldehyde. Further, when the concentration of GA was increased > 0.2%, a decrease in mechanical strength was observed and this could be reasoned to high degree of cross-linking of GA with the biopolymers which is clearly proved from the results of TNBS assay [25].

The following schematic illustrations emphasize the cross-linking chemistry of glutaraldehyde with the chosen natural polymer and reasons out the brittle nature.



The above illustrations suggested that glutaraldehyde could covalently cross-linked with chitosan through the formation of double bond (C=N, imine bond) between –CHO group of glutaraldehyde and $–NH_2$ group of chitosan, result 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.

Thermo gravimetric analysis for the experimental samples GA, chitosan, GACCH and GADCCH were illustrated in Fig. 4 and the corresponding thermal degradation values were displayed in Table IV. From the results we observed that incorporation of glutaric acid with chitosan tends to shift the thermal region to higher temperature and such a shift is attributed to an increase in thermal stability.

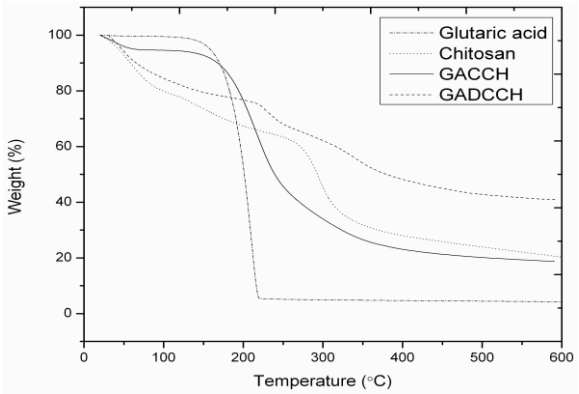


Fig. 4 Thermo gravimetric analysis of Glutaric acid (GA), Chitosan, glutaric acid cross-linked chitosan (GACCH) and glutaraldehyde cross-linked chitosan (GADCCH) biopolymers

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TABLE IV THERMAL ANALYSIS OF GLUTARIC ACID (GA), CHITOSAN, GLUTARIC ACID CROSS-LINKED CHITOSAN (GACCH), GLUTARALDEHYDE CROSS-LINKED CHITOSAN (GADCCH) UNDER N_2 AIR ATMOSPHERE

Temperature (℃)	% of weight loss (heating rate 20 ℃/min)			
	Glutaric acid	Chitosan	GACCH	GADCCH
100	0	21	5	16
200	48	33	24	24
300	96	56	66	38
400	96	73	77	52
500	96	77	80	58
600	96	81	81	60

Differential scanning calorimetry (DSC) studies were performed to understand the behavior of GACCH on application of thermal energy. The thermo grams of GA,

Chitosan, GACCH and GADCCH were shown in Fig. 5. DSC studies recorded melting temperature differences among GA (96 ℃), chitosan (107 ℃) and GACCH (159 ℃) whereas for GADCCH it was observed at 149 ℃ respectively. The higher transition temperature suggests, GACCH had higher stability at high temperature environment. The thermal stability also influences on the durability of the biopolymers. Similar kind of observation was reported by Bhumkar et al [19].

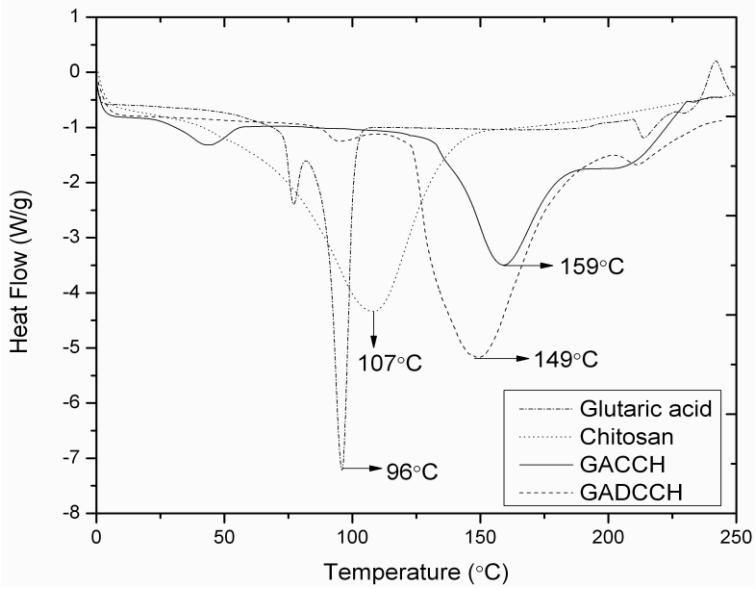

Fig. 5 Differential scanning calorimetry analysis of Glutaric acid (GA), Chitosan, glutaric acid cross-linked chitosan (GACCH) and glutaraldehyde cross-linked chitosan (GADCCH) biopolymers.

Results on binding energy calculations based on bioinformatics tool for the cross-linking of GA with chitosan using Auto Dock software proved that chitosan can cross-link with glutaric acid not only with ionic interaction but also through multiple intermolecular hydrogen bonding. Autodock is an automated procedure for predicting the interaction of ligands with bio-macromolecular targets. Hundred runs were given for docking GA with chitosan. The best binding energy values and their corresponding rank and run numbers were depicted in Table V.

TABLE V BINDING ENERGY VALUES OF GLUTARIC ACID CROSS-LINKED CHITOSAN (GACCH)

The binding energy calculation between chitosan and Glutaric acid based on autodock tool software.		
Rank	Binding energy (Kcal/mol)	No. of runs
1	-4.48	54
2	-4.25	13
3	-4.18	55
4	-3.99	67
5	-3.84	57
6	-3.75	58

The binding energy of -4.48 (kcal/mol) was observed when GA were interacted with chitosan. These interactions were made by multiple intermolecular hydrogen bonds between –COOH group of GA and $–NH_2$ group of chitosan (Fig. 6). In addition to ionic cross-linking, hydrogen bonding interaction can also improve the mechanical property of the biopolymer. The details of these intermolecular hydrogen bonding sites were given below.

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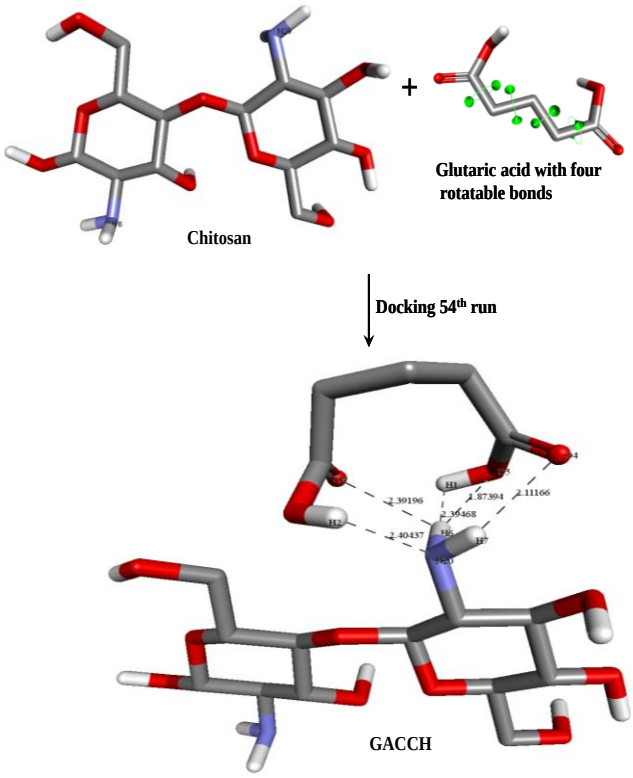

Fig. 6 Multiple hydrogen bonding interaction between chitosan and glutaric acid (GA)
(The black dotted line indicates the hydrogen bond. In figure white colour is for hydrogen atom (H), red colour indicates oxygen atom (O), grey colour for carbon atom (C) and blue corresponds to nitrogen atom (N)).

Intermolecular hydrogen bond details between GA and chitosan

(i) H (2) of GA is linked with N (20) of chitosan with the bond distance of 2.40437,

(ii) H (6) of chitosan is linked with O (8) of GA with the bond distance of 2.39196,

(iii) H (1) of GA is linked with N (20) of chitosan with the bond distance of 2.39468,

(iv) H (6) of chitosan is linked with O (5) of GA with the bond distance of 1.87394,

(v) H (7) of chitosan is linked with O (4) of GA with the bond distance of 2.11166.

(H- Hydrogen, O- Oxygen, N- Nitrogen, GA- Glutaric 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 biopolymer (GACCH). Only cells that are metabolically normal can turn the tetrazolium salts into purple crystals. Compared with the native chitosan, GACCH showed no significant differences in absorbance (Fig. 7), 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 GA cross-linked biopolymer (GACCH).

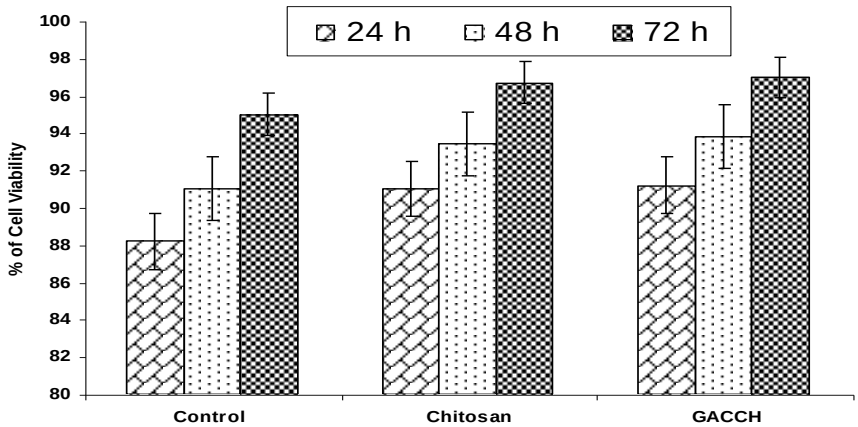

Fig. 7 MTT analysis of control, chitosan and glutaric acid cross-linked chitosan (GACCH) biopolymer at 24, 48 and 72h time interval

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In cell viability assay we observed intense fluorescence of the cells on the surface of the native and cross-linked biopolymers and suggests the viability of the cells as illustrated in Fig. 8.

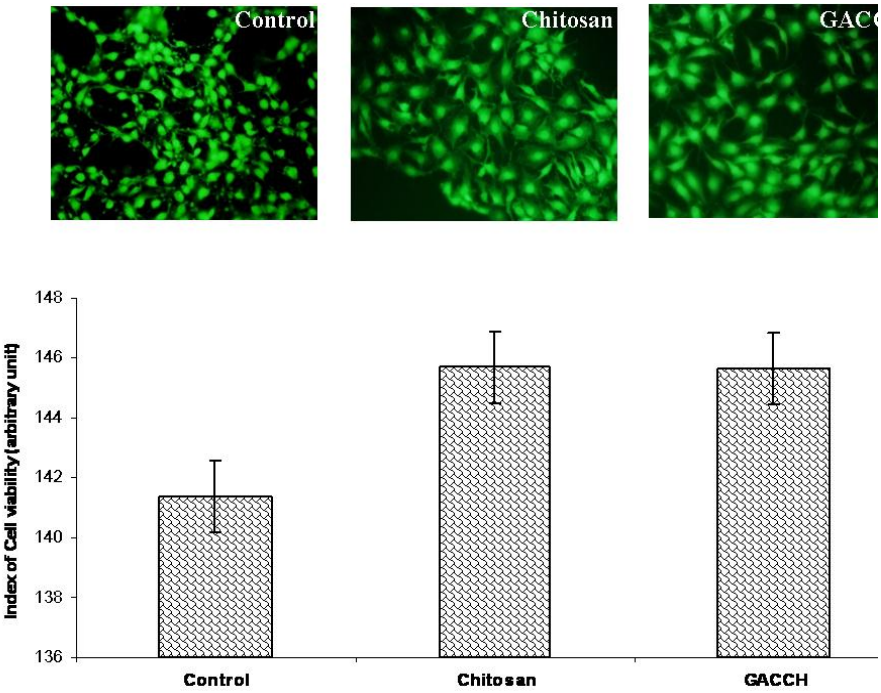

Fig. 8 Index of cell viability (AU) assessed in GACCH (glutaric acid cross-linked chitosan) in comparison with the parent molecule (chitosan) and control

(The assay was carried out using cell tracker kit. NIH3T3 Cells were treated on the surface of native and cross-linked biopolymers for 6h followed by incubation with cell viable dye cell tracker for 30mins. Fluorescence images of the cells were acquired by DP71 camera adapted to an Olympus IX71 microscope. Intensity of green positive cells were counted and plotted. Next, fluorescence intensities of the images were calculated using Adobe Photoshop version 7.0).

The SEM images of the cell seeded GACCH scaffold displayed in (Fig. 9) demonstrated that after being cultured for prolonged time (12 days) fibroblasts were detected in the scaffold (GACCH) with typical spindle shaped morphology and suggested that the cells were infiltrated into the scaffolds and further proliferated.

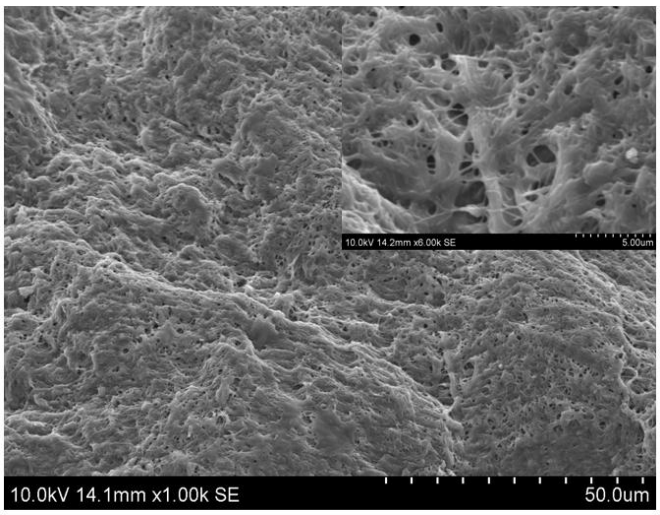

Fig. 9 Attachment of fibroblast cells on the GACCH (glutaric acid cross-linked chitosan) scaffold biopolymer (Porous GACCH scaffold was completely covered by fibroblast cells).

Cytocombatility studies for GADCCH have not been performed in the present study. Though glutaraldehyde (GAD) has been widely used as chemical cross-linking agent [26] because of stabilizing the collagen efficiently and the cross-linking is thought to involve the formation of Schiff bases [27] However, GAD-cross-linked biomaterials are poorly biocompatible with some cell lines including human fibroblasts, osteoblasts, Chang cells and endothelial cells [28-30]. The side effects of GAD treatment were attributed to the degradation of the GAD-derived cross-links and the continuous release of aldehydes contributing to prolonged toxic effects [26, 31].

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V. CONCLUSIONS

The present study explicitly demonstrated that glutaric acid acts as suitable cross-linker for the preparation of biocompatible biopolymer from natural polymer (Chitosan) with appreciable mechanical properties. The interaction between glutaric acid and chitosan was identified as non-covalent interactions, i.e., both ionic and multiple intermolecular hydrogen bonding interactions. These non-covalent interactions provided the resulting biopolymer with high mechanical strength. All the instrumental analysis and bioinformatics tool authenticated the non-covalent interactions. The biopolymer material (scaffold) prepared upon cross-linking of glutaric acid with chitosan was the green method of preparation. No toxic compounds were involved in this preparation and the resultant material found application as wound dressing material or as implant in clinical applications.

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