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Scleraldehyde as a stabilizing agent for collagen scaffold preparation

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ABSTRACT

Schizophyllan is a natural polysaccharide, produced by fungi of the genus *Schizophyllum*. Periodate oxidation specifically cleaves the vicinal glycols in schizophyllan to form their dialdehyde derivatives. The present study investigates the interaction of scleraldehyde with Type I collagen membrane. The formation of the inter and intra interaction between scleraldehyde and the collagen fibres results in significant increase in viscosity of collagen. Crosslinking efficiency of scleraldehyde was found to increase with concentration of scleraldehyde. Scleraldehyde interacted collagen membrane exhibited an increase in thermal stability by 29 °C at pH 8. The gelling time of collagen fibrils was found to decrease with increase in concentration of scleraldehyde due to shift in nucleation centre. Swelling degree of collagen membrane was also found to decrease with increase in concentration of scleraldehyde. Scleraldehyde treated collagen membrane exhibited 93% resistance to collagenase. The modified collagen membrane exhibited non-toxicity towards the fibroblasts cells. The modified collagen membrane by scleraldehyde finds application as a stabilizing agent in scaffold preparation.

1. Introduction

Schizophyllan is a natural polysaccharide produced by the fungus *Schizophyllum commune* and its repeating unit consists of three β-(1 → 3) glucoses and one β-(1 → 6) glucose side-chain linked at every third main chain of glucose (Maushmi, Shrikant, & Rekha, 2008). Periodate oxidised schizophyllan is prepared under mild aqueous conditions and is characterized by specific cleavage of the C_2–C_3 bond of the glucopyranoside ring that produce two aldehyde groups per unit. Scleraldehyde is thus heteropolymer containing aldehyde groups and if no other bond rupture occurs in oxidation, it should have a molecular weight of 639 Da. In contrast to most polysaccharides, usually poorly soluble in water, modified schizophyllan exhibits a fairly high solubility due to the presence of its lateral residues. Modified schizophyllan (scleraldehyde) is a natural polymer that has established widespread applications (Jayakumar, Swarna, Chandrasekaran, Rao, & Nair, 2010). Scleraldehyde appeared to be an alternative crosslinking agent for stabilizing collagen as it is from natural resources and eco-acceptable biopolymer.

Collagen, the main structural proteins accounting for the structural integrity of vertebrates and many other multicellular organisms, has been extensively applied to the field of tissue engineering (Nimni & Harkness, 1988). This widespread use of collagen emphasizes the need to understand the mechanism of stabilization of collagen against biodegradation and heat, as these studies will have far reaching implications in both industrial and biological applications of collagen (Ramachandran & Chandrasekharan, 1968). Collagen being a protein is amenable to degradation by microbial attack. Therefore, the development of biomaterials based on collagen depends on rendering them resistant to biodegradation. The thermal stability of collagen and the influence of various factors on the denaturation temperature of collagen were studied widely (Madhan, Subramanian, Raghava Rao, Unni Nair, & Ramasami, 2005; Vizarova et al., 1995). It is well known that collagen crosslinked with various crosslinking agents such as plant polyphenols, metal ions and aldehydes is made resistant against the degradation by collagenase (Madhan, Muralidharan, & Jayakumar, 2002). Hence the thermal stability of collagen increases owing to crosslinking.

In recent times, ecological concerns demand alternative crosslinking chemicals (Di & Heath, 2009; Rao, Gayatri, Rajaram, Nair, & Ramasami, 1999). Modified polysaccharides were proved to be biodegradable and toxologically acceptable (Jayakumar, Swarna, Chandrasekaran, Rao, & Nair, 2011; Zhao, Yi, Po, & Jian, 2010). In

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the present work, an attempt is made to understand the effect of scleraldehyde on hydrodynamic property, crosslinking eficiency, thermal stability, swelling ratio, gelling time and enzymatic stability of Type I collagen matrix. The modified collagen membrane is analyzed for the toxicity characteristics for its feasibile application in scaffold preparation.

2. Materials and methods

2.1. Materials

The chemicals used for all the experiments were of analytical grade and purchased from SRL chemicals, India. Type I collagenase purchased from sigma chemical company was used in the study. The Type I collagenase was used after purification by gel filtration on sephadex G-200 according to the method of Keller and Mandl (Keller & Mandl, 1963). Strains of *S. commune* MTCC 1096 were procured from Microbial Type Culture (MTCC), Chandigarh, India.

2.2. Preparation of Type I collagen

Tails were excised and frozen at −20 °C from 6-month-old male albino rats (Wistar strain) that are ideal collagen substrate for crosslinking studies due to its high purity, available lysine residues and collagen content. On removal from the freezer, tails were thawed and tendons were teased out. Teased collagen fibres were washed with 0.9% NaCl at 4 °C, to remove the adhering soluble proteins. RTT were washed extensively in double distilled water at 4 °C and used as collagen fibres. Acid soluble RTT Type I collagen solution was also isolated according to the method described by Chandrakasan, Torchia, and Piez (1976). The procedure included acetic acid extraction and salting out with NaCl. The purity of collagen preparation was confirmed by SDS-polyacrlyamide gel electrophoresis (PAGE). The collagen concentration in the solution was determined from the hydroxyproline content according to the method of Woessner (1961).

2.3. Maintenance of culture

S. commune was maintained on solid medium containing 20 g malt extract/L, 1 g peptone/L, 20 g dextrose/L and 20 g agar/L. A liquid culture was started by transferring approximately 7 mm of a 7–10 day old colony into 250 mL of the same medium without agar in a 500 mL fluted Erlenmeyer flask with three 10 mm glass beads. This culture was grown for 4 days at 30 °C and 240 rpm. Suspensions (0.5 mL) were used to inoculate 300 mL flasks containing 50 mL (w/v) corn fibre pretreated as previously described with alkaline H_2O_2 in a basal medium containing 0.67% yeast nitrogen base (Difco), 0.2% asparagine and 0.5% KH_2PO_4 Cultures containing treated corn fibre were grown for 7 days at 30 °C and 240 rpm (Shrikant, Parag, & Rekha, 2006).

2.4. Isolation of schizophyllan

Mycelial growth was removed from cultures by centrifugation in 50 mL conical tubes at 3220 g for 30 min at 4 °C. Polysaccharides were recovered from culture supernatants using 95% ethanol diluted to 50% (v/v) and 67% (v/v). Polysaccharide pellets were resuspended in distilled H_2O, transferred to 15 mL conical tubes and dried under vacuum at 50 °C for analysis.

2.5. Preparation of scleraldehyde

Scleraldehyde was prepared according to the methods reported earlier with modifications as described (Aalmo & Painter, 1981). Scleraldehyde (∼100 g) was hydrolyzed in 5 N hydrochloric acid (10 h, 85 °C). Hydrolyzed schizophyllan was suspended in demineralized water and subsequently cooled in an ice bath. Sodium periodate of 120 g was added to the sample while stirring with a magnetic stirrer. The pH of the solution was maintained at 4 during the reaction. The reaction was performed in the dark at 35 °C and stopped after 48 h to obtain scleraldehyde of 99% oxidation. The product was extracted with centrifugation in t-butyl alcohol (1:3 sample:solvent). The product was resuspended in the same volume of t-butyl alcohol and the centrifugation cycle was repeated several times until all iodic compounds were removed. The product was dried at 35 °C. The degree of oxidation was determined by measuring the concentrations of unconsumed periodate by iodometry; whereas the dialdehyde content was determined using hydroxylamine method (Swarna et al., 2007).

2.6. Preparation of reconstituted collagen membranes

Collagen fibrils were reconstituted from purified collagen solution (0.5%) by mixing with 0.2 M phosphate buffer (pH 7.4) and NaCl (2 M) in an ice bath (Rajaram & Chu, 1990). The solution was then poured on polythene trays at room temperature to form collagen membrane. The collagen membranes were washed extensively with distilled water to remove buffer salts and then air-dried. The thickness of the membranes as measured using a thickness gauge varied from 20 to 30 mm.

2.7. Crosslinking conditions

The dried reconstituted collagen membranes were swollen in PBS (pH 7.4) at room temperature before scleraldehyde interaction. The collagen membranes were crosslinked by treating them with different ratios of scleraldehyde (0.2–1). After interaction for the described period of time, the membranes were washed extensively with distilled water to remove traces of stabilizing agents. Finally, the washed membranes were then air-dried.

2.8. SDS-PAGE of scleraldehyde interacted collagen

The interaction of scleraldehyde was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli (1983) system with a 10% resolving gel. The gel was allowed to set for 45 min. Distilled water was pipetted over the gel to prevent oxygen inhibition of the polymerisation reaction. The 4% stacking gel was then prepared on top of the resolving gel. The comb was placed carefully to prevent formation of air bubbles. The gel was allowed to set for 30 min. Samples were diluted prior to electrophoresis using sample buffer to give a final protein concentration of 1 μg/mL. Samples were boiled at 100 °C for 5 min. Gels were run at 25 mA per gel for 45 min. On completion of electrophoresis, gels were fixed and stained with coomassie Blue R-250 for 1 h. The gel was then transferred to destained solution and subsequently photographed. Type I collagen purified from rat tail tendon and protein marker was used as a standard.

2.9. Viscometry of scleraldehyde crosslinked collagen solution

Viscosity measurements were performed using an Ostwald type viscometer with a flow time for buffer at 25 °C (Calvini & Conio, 2006). Viscosity measurements were carried out from 10 °C to 40 °C in 5 °C intervals. This heat induced aggregation reverses on cooling. The flow times of collagen samples were measured after a thermal equilibrium time of 30 min. The collagen concentration (2 mg/mL) was fixed and the measurements were carried out with different concentrations of scleraldehyde (0.2–1/1 (w/w) of collagen). The mixture was then incubated for 24 h at room temperature. The viscosity measurement was based on the flow rate of collagen solution

through the capillary of an Ostwald viscometer. In these experiments the viscosity contribution (η) due to collagen was measured as a function of the concentration of scleraldehyde. The flow time was measured with a digital stopwatch at least three times and the average was taken. The viscosity was calculated from the relation, $\eta = (t - t^0)/t^0$, where t^0 is the flow time of buffer and t is the flow time for each sample.

2.10. Determination of free amino groups

The free amino groups present in scleraldehyde interacted collagen was determined using ninhydrin and 2,4,6-trinitrobenzenesulfonic acid assay (Bubnis & Ofner, 1992). The resulting colored products were evaluated using UV–VIS spectroscopy. L-Lysine was used as a standard for the calibration curve.

2.10.1. Ninhydrin assay

The collagen/scleraldehyde mixture at varied ratios (0.2–1 at an interval of 0.2) was placed into 20 mL vial. Ethanol solution (0.2%) of the ninhydrin was added to the reaction mixture. The solution was stirred and simultaneously heated up at 100 °C for 20 min. Subsequently, the solution was three times diluted (9 mL of miliQ water) and then measured by UV–VIS at 570 nm in a 1 cm cell against blank (water). For each ratio, five samples were prepared and measured. Percentage of free amino groups in the sample were calculated using the equation given below,

$$\text{Free NH groups} \quad (\%) = \frac{C_s}{C_c} \times 100$$

where C_s is the concentration of amino groups in the sample and C_c is the concentration of amino groups of lysine in the pure lyophilized collagen.

2.10.2. TNBS assay

Crosslinking efficiency of scleraldehyde in stabilization of collagen was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay that determines the ε-amino groups in collagen. Crosslinking efficiency was determined by measuring the available lysine and comparing lysine content before and after crosslinking using TNBS. The unreacted ε-amino groups of lysine in native and scleraldehyde crosslinked collagen solution reacts with TNBS to form a soluble complex. Acid soluble collagen solution of concentation 5 mg/mL and 1 mL of varying concentrations of scleraldehyde (0.2–1 (w/w) held at 4 °C for 24 h), 1 mL of 4% (w/v) sodium bicarbonate solution and 1 mL of freshly prepared 0.5% (v/v) TNBS solution in deionized water was added at 60 °C for 4 h. The reaction mixture was treated with 3 mL of 6 M HCl at 40 °C for 1.5 h and the absorbance was measured at 334 nm after dilution. The native collagen solution was also treated with TNBS in a similar manner. All the experiments were carried out in triplicate.

2.11. Swelling ratio

Collagen membrane which was interacted with various concentration of scleraldehyde was swollen in water and then equilibrated overnight in phosphate buffer (pH 7.4) at room temperature. The membrane was removed, quickly blotted with filter paper to remove excess surface water and weighed immediately. The membrane was then placed in a large volume of deionized water to remove buffer salt and air dried to constant weight. The swelling ratio was calculated as the ratio of the weight of swollen sample to that of dry sample (Balakrishnan & Jayakrishnan, 2005).

2.12. Gelling time

Scleraldehyde solution of 1 mL with varied concentration in 0.1 M borax (pH 9.4) and in phosphate buffer was reacted with 1 mL aqueous solution of collagen (2 mg/mL) in glass vials of 15 mL capacity (diameter 20 mm) under magnetic stirring using a Teflon-coated stir bar (diameter 5 mm, length 10 mm) at 37 °C. Gelling time was noted as the time required for the stir bar to stop. Values reported are average of 4–5 determinations. Gelling time was studied by varying the concentration of scleraldehyde (Balakrishnan & Jayakrishnan, 2005).

2.13. Thermal resistance of scleraldehyde stabilized collagen membrane

2.13.1. Hydrothermal temperature

The thermal stability of scleraldehyde treated collagen fibres were determined using a micro-shrinkage tester. The temperature at which the collagenous fibre shrinks to one third of its original length was noted as the hydrothermal temperature of the fibre. A small strip of fibre was cut and placed on a grooved microscopic slide along with water. The slide in turn was placed on a heating stage along with a microscope mounted above the heating stage. The rate of heating was maintained at 2 °C/min (Swarna, Abirami, Rao, & Nair, 2009).

2.13.2. Calorimetric measurement – denaturation temperature

The native and scleraldehyde-treated collagen membrane (1:1) were blotted uniformly and hermetically encapsulated in aluminium pans. The samples were fused in a differential scanning calorimetric cell of a Netzsch DSC 200 PC differential scanning calorimeter. The temperature was calibrated effectively using indium as standard. The heating rate was maintained constant at 5 °C/min. The peak temperature T_D (in °C) and the enthalpy changes ΔH (in J/g of wet weight) associated with the phase change for the shrinkage process for native and scleraldehyde treated collagen membranes was studied (Swarna et al., 2009).

2.14. Collagenolytic activity of scleraldehyde stabilized collagen matrix

The enzymatic degradation of native collagen and scleraldehyde stabilized collagen membrane by bacterial collagenase (Type IA) from *Clostridium histolyticum* was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis (French, Bhown, & Van Wart, 1992). Collagenase treatment was carried out in 0.04 M $CaCl_2$ solution buffered at pH 7.2 with 0.05 M tris–HCl. The collagen:enzyme ratio was maintained at 50:1. The % collagen degradation was determined by estimating the release of hydroxyproline. This method of determining hydroxyproline involves the oxidation of hydroxyproline to pyrrole-2-carboxylic acid, which complexes with p-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm.

$$\%\ \text{Collagen degradation} = 100 - \left[\left\{\frac{\text{Initial collagen} - \text{Soluble collagen}}{\text{Initial collagen}}\right\} \times 100\right]$$

2.15. Morphological studies of scleraldehyde stabilized collagen membrane

The structural stabilization of the collagen membrane was assessed by SEM. The control and 1:1 (collagen:scleraldehyde) (w/w) scleraldehyde interacted membrane samples were cut into specimens of uniform thickness. A Quanta 200 series scanning electron microscope was used for the analysis. The micrographs of the

collagen membrane were obtained by operating the SEM at low vacuum with an accelerating voltage of 5 kV in 20× magnification levels (Lammers, Tjabringa, Schalkwijk, Daamen, & Van Kuppevelt, 2009).

2.16. In vitro cytotoxicity analysis

Fibroblasts used in all the experiments were isolated by the method described by Kumar, Sai, and Babu (2002). The cytotoxicity of collagenous matrices was evaluated by the MTT assay and cell morphology in contact with the tested sample. Collagen samples were cut into suitable sizes and sterilized by submersion in 75% alcohol. After being washed with PBS buffer to remove any residual alcohol, samples were soaked in dulbecco's modified eagle's medium until they reached equilibrium before use. Fibroblasts at a concentration of 4×10^4 cells/well were directly seeded into 12-well culture plates into which a collagen sample were placed and then cultured for 48 h. Cell morphology was observed by optical microscopy. The reductions in cell viability under conditions of co-culture with the tested samples were measured using the MTT assay. The well into which no tested matrix was placed were used as the control. At the end of culture, the yellow tetrazolium MTT solution was added and incubated for 3 h until a purple precipitate was visible. The absorbance of each well was recorded at 550 nm.

3. Results and discussion

Scleraldehyde with 45% dialdehyde content and 100% solubility was acomplished with 99% periodate oxidation. Periodate oxidation specifically cleaves the vicinal glycols in polysaccharides to form their dialdehyde derivatives. This reaction is generally used for the elucidation of polysaccharide structure. The advantage of periodic acid lies in the specificity of its oxidation. The degree of oxidation can be readily controlled, and a complete range of aldehyde derivatives of scleraldehyde was made available as the oxidation level varies between 0 and 100% depending on the quantity of periodate employed. Each α-glycol group consumes one mole of periodate under given conditions. The rate of oxidation is dependent principally on the stereochemistry of the α-glycol group. Even though, the oxidation reaction was carried out in aqueous medium scleraldehyde was precipitated out in heterogeneous medium (3:1 t-butyl alcohol:water) as a dispersion. The distinguished feature of scleraldehyde is that it is soluble in water after complete oxidation. As water being the main medium through which the chemicals can be transported into the collagen matrix, water solubility for the stabilizing agent is vital.

3.1. Interaction of scleraldehyde with collagen

The amino groups present in the collagen are involved in the interaction of dialdehydes. The most probable reaction of aldehydes is the formation of Schiff's base and the methylene linkages resulting from mannich reaction with the amino functional groups in collagen (Bowes & Cater, 1968). For effective interaction, the molecule used as a stabilizing agent should posses difunctionality to exhibit reactivity between two polypeptide chains. Hence, dialdehydes find application as stabilizing agents for collagen. Interaction of scleraldehyde results due to interaction with amino groups of lysine, hydroxylysine or arginine side groups of collagen with scleraldehyde. The amino groups are generally protonated at lower pH and hence, aldehyde interaction with proteins is preferable at pH above isoelectric point (IEP) of the protein. The IEP of collagen is 6.7 and above this pH, collagen will remain negatively charged and amino sites are available for the interaction of aldehydes. Earlier studies indicate pH 8 seems to be conducive for interaction of aldehydes with collagen. There is also an increase in uncharged amino groups (NH_2) of side chain functional groups of amino acids like lysine and arginine with increased pH, which enables the fixation of scleraldehyde through aldehyde groups as it is well known that aldehydic groups covalently crosslink with collagen by amino functional groups of the protein.

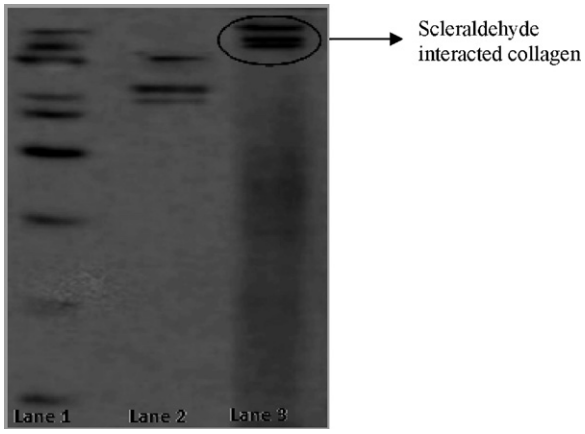

Fig. 1. SDS PAGE of native collagen and scleraldehdye interacted collagen (lane 1 – protein marker, lane 2 – Collagen, lane 3 – scleraldehyde interacted collagen).

3.2. Electrophoretic studies of scleraldehyde interacted collagen

The pure collagen and scleraldehyde crosslinked collagen samples were analyzed in SDS PAGE (10%, w/v) and stained with 1× staining solution. The electrophoresis patterns of the samples are shown in Fig. 1 collagen on lane-2 displayed one 'β' band (200 kDa) and two 'α' bands (100 kDa for α1 and α2), which are the unfolding polypeptide chains of the triple helix ([α1(I)]2[α2(I)]). The molecular weight of Type I collagen is 300 kDa with reference to protein marker on lane-1. The scleraldehyde crosslinked collagen on lane-3 showed higher molecular weight than pure collagen one 'β' band (300 kDa) and two 'α' bands (200 kDa for α1 and α2), which were the unfolding polypeptide chains of the triple helix ([α1(I)]2[α2(I)]). SDS PAGE analysis indeed confirms the increasing molecular weight of collagen due to inter and intra crosslinking by scleraldehyde.

3.3. Rheological property of scleraldehyde-collagen

Hydrodynamic feature of collagen is one of the inevitable properties for its biomedical applications. Ostwald viscometer was used to study the effect of scleraldehyde on the fluid dynamics of collagen. The studies were carried out using varying the collagen:scleraldehyde ratio. The effect of temperature on the viscosity of collagen before and after treatment with scleraldehyde is given in Fig. 2. The temperature range between 10 and 40 °C was chosen because above 37 °C the thermal denaturation of collagen and the proteins show a highly pronounced tendency to aggregate.

3.4. Determination of the free amino groups – ninhydrin method

The interaction of scleraldehyde with collagen was estimated by free amino groups present in collagen before and after treatment of scleraldehyde. Ninhydrin assay was used to estimate the amino groups in native and scleraldehyde interacted collagen. The percentage available amino groups in collagen are given in Table 1. The native collagen has 0.3% free amino groups. Scleraldehyde interacted collagen showed a steady decrease in free amino groups from 0.363 to 0.064%. The decrease in free amino groups in collagen confirms that scleraldehyde interacts with collagen. Moreover the

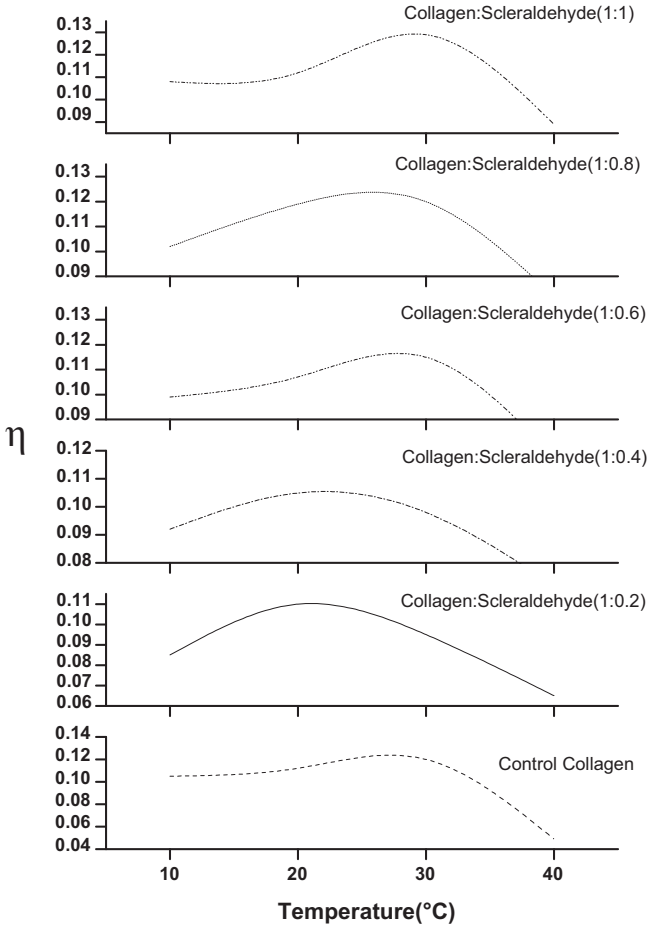

Fig. 2. Viscosity η with various temperatures ranging from 10 to 40 °C at different concentrations.

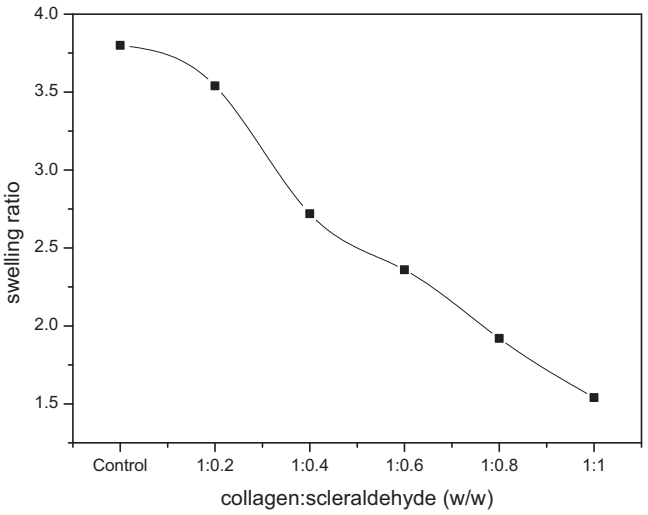

Fig. 3. Swelling ratios and Gelling time of control collagen and scleraldehyde corsslinked collagen.

reaction mixture of scleraldehyde and ninhydrin shows no color formation. It is evident from Table 1, the stabilizing effect to collagen by scleraldehyde up to ratio of 1:1. It is confirmed by the decreasing amount of free amino groups of about 0.06%.

3.5. Crosslinking efficiency – TNBS assay

In order to study the crosslinking efficiency brought about by scleraldehyde was estimated by TNBS assay. The crosslinking efficiency of scleraldehyde at varied concentrations is given in Table 1. It is observed that scleraldehyde results in interaction with collagen and an increase in concentration enhances the crosslinking efficiency by formation of stable crosslinks with amino groups of collagen. Scleraldehyde at 1:0.2 ratio results in interaction efficiency of 33% as compared to 79% at 1:1 scleraldehyde ratio. The increase in crosslinking efficiency of scleraldehyde with collagen was observed due to decrease in the amino groups of lysine in collagen. At a ratio of 1:1 (collagen:scleraldehyde) maximum quantity of aldehyde groups are available for interaction with amino groups of collagen. Increase in the ratio above 1:1, crosslinking eficiency remains constant. Higher concentration of scleraldehyde facilitate higher crosslinking due to the presence of more availability of aldehyde groups that crosslink with amino groups in collagen. The high interaction ability of scleraldehyde with collagen can be attributed to strong binding between the two, as scleraldehyde can have both covalent and non-covalent interactions with collagen. Hence, aldehydic functionality in scleraldehyde covalently crosslinks with amino groups of collagen and the hydroxyl groups can involve in hydrogen bonding interaction that brings about significant increase in thermal and enzymatic stability.

3.6. Swelling properties of scleraldehyde interacted collagen membrane

Swelling ratios of the modified collagen membrane for various ratios of scleraldehyde are shown in Fig. 3. From the figure, it is observed that interaction of scleraldehyde had reduced the swelling behavior of collagen. The swelling ratio for native collagen is found to be 3.8. In the presence of scleraldehyde, there is significant decrease in swelling ratio from 3.8 to 1.6 (1:1, collagen:scleraldehyde). The interaction of scleraldehyde to collagen sites has reduced the access of solvent (water) which resulted in lower uptake of the solvent and hence lowers the degree of swelling. There was significant reduction in the swelling ratio of collagen with an increase in the amount of scleraldehyde offer, which substantiates the fact that the solvent accessibility reduced with increased interaction. Moreover, the present study

Table 1
Free amino groups, crosslinking eficiency, hydrothermal temperature and collagenase hydrolysis of scleraldehyde crosslinked collagen membrane.

Process	Ratio (collagen/scleraldehyde) (w/w)	Free amino groups (%)	Crosslinking efficiency (TNBS) (%)	Hydrothermal temperature (°C)	Collagenase hydrolysis (96 h) (%)
Native collagen	1:0	0.363	–	62 ± 0.5	99.3 ± 0.59
Scleraldehyde	1:0.2	0.334	33.45 ± 0.38	65 ± 1.0	67.38 ± 0.47
stabilized collagen	1:0.4	0.284	49.27 ± 0.41	67 ± 0.5	47.92 ± 0.12
at pH 8 for 24 h at	1:0.6	0.201	59.87 ± 0.59	74 ± 0.5	38.74 ± 0.11
varying	1:0.8	0.154	68.64 ± 0.43	82 ± 0.5	20.14 ± 0.24
concentration of	1:1	0.064	79.24 ± 1.04	89 ± 0.5	6.96 ± 0.12
scleraldehyde	0:1	–	–	–	–

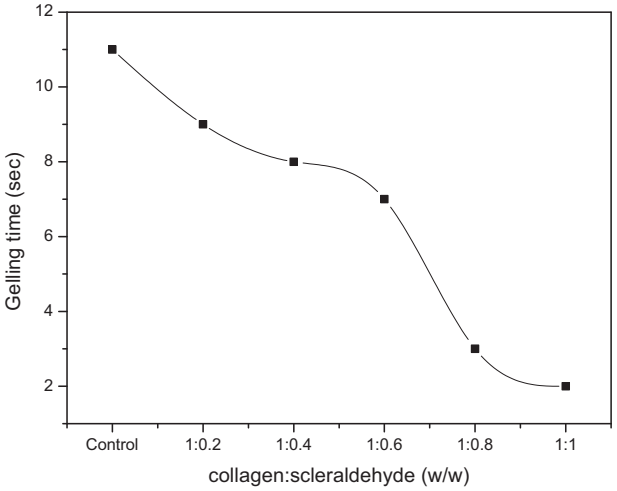

Fig. 4. Gelling time of control collagen and scleraldehyde corsslinked collagen.

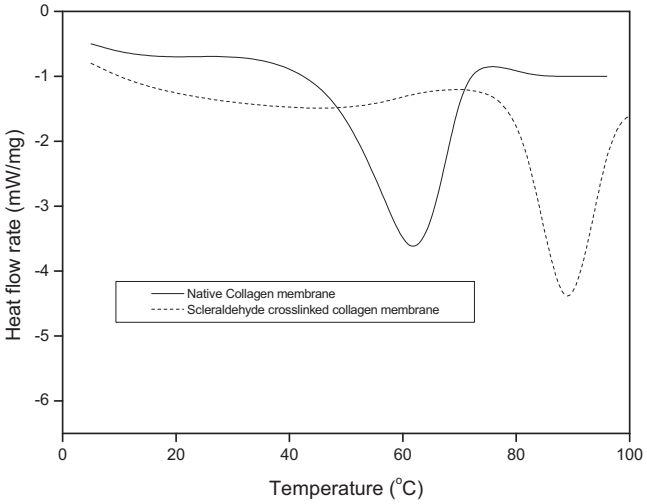

Fig. 5. Differential Scanning Calorimetry (DSC) thermograms of collagen and scleraldehyde interacted collagen membrane.

that crosslinking with scleraldehyde, which reduces the equilibrium swelling of collagen matrix. The hydration of collagen membrane causes the penetration of the cell into the membrane, thereby hindering the proliferation and growth although viability is achieved. At increased concentration, the modified collagen membrane is found to be suitable as the cell substratum.

3.7. Gelling time of scleraldehyde interacted collagen fibrils

Gelling time of the crosslinked collagen for the various ratios of scleraldehyde is shown in Fig. 4. The effect of concentration of scleraldehyde, collagen and borax on gelling time is systematically examined in the present study. The gelling time decreased to 2 s for 1:1 ratio of scleraldehyde as compared to 11 s for native collagen. Particularly striking is the influence of borax on the gelling time. This might be due to the alkaline pH of the medium which facilitates the formation of the Schiff's base as well as the ability of borax to complex with hydroxyl groups of polysaccharides. Since the gelling time decreased rapidly with increase in the concentration of scleraldehyde, it supports the fact that not only the alkaline pH of the medium, but the ability of scleraldehyde to complex with hydroxyl groups of collagen is also responsible for the rapid gelation. When scleraldehyde is employed at lower concentration, gelation took place slowly, demonstrating that minimum aldehyde content is essential for rapid gelation. Gelation of scleraldehyde with collagen also occurred in phosphate buffer, although reaction was less rapid in this medium. The gelling time was found to decreases with an increase in the concentration of scleraldehyde. Gelling time follows the same pattern as swelling ratio. Higher degree of crosslinking reduces the gelling time, which in turn facilitates the aggregation of collagen, as the concentration of the scleraldehyde was increased.

3.8. Thermal stability investigation of scleraldehyde stabilized collagen membrane

The thermal stability of scleraldehyde interacted collagen is a characteristic of the effectiveness of stabilization. Thermally induced structural transitions in the fibrous collagenous network lead to denaturation. Increase in resistance against hydrothermal stress is one of the important aspects in the stabilization of collagen matrix. The characteristic temperature at which collagen shrinks to one-third of its original length was 62 °C. The temperature at which the shrinkage of collagen matrix occurs is highly specific and occurs due to destabilization of the protein. Crosslinking results in a change in the size of the cooperative units of collagen and tight packing of the molecules leading to dehydration of the fibre. The basic forces, which are responsible for the high denaturation temperature of collagen, could be attributed to long range interactions. Scleraldehyde interacted collagen has resulted in increase in thermal stability of 89 °C. As seen from Table 1, the thermal stability of the collagen matrix increases with increase in scleraldehyde concentration. Hence, scleraldehyde modified collagen increases the long range ordering by forming a large number of crosslinks involving covalent linkages with the amino groups and also hydrogen bonding, which could be responsible for the increased thermal stability of the collagen fibres.

The differential scanning calorimetry thermograms for native and scleraldehyde treated collagen membrane (1:1) is shown in Fig. 5. It is an endothermic process in which a phase transition takes place involving changes in the lattice and long-range order. The denaturation temperature for control collagen membrane is 60 °C. Scleraldehyde treated collagen shown an increase of 29 °C as compared to that of native collagen. An increase in thermal stability could be related to the increase in the number of crosslinks because they decrease the entropy of transition (Pineri, Escoubes, & Roche, 1978). The collagen fibres were kept in the presence of water during the hydrothermal shrinkage measurements and in the absence of water during DSC measurements. And it is known that the thermal helix-coil transition depends on the degree of hydration (Covington, Hancock, & Ioannidis, 1989). It was observed that the scleraldehyde treated collagen membrane exhibits a marked increase in the denaturation peak temperature of 89 °C as well as in the enthalpy changes when compared to native collagen.

3.9. Collagenase resistance of scleraldehyde treated collagen membrane

The stability of the scleraldehyde treated collagen membrane against enzymatic degradation was studied by analyzing the rate of hydrolysis of collagen on treatment with bacterial collagenase. Bacterial collagenase preferentially cleaves X-Gly (X is most frequently a neutral amino acid) bond of the –Gly-Pro-X-Gly-Pro-X– sequence in the non polar regions of the collagen molecule (Galardy & Grobelny, 1983). Degradation of collagen (based on hydroxyproline released) for native and scleraldehyde treated collagen by collagenase at various concentration of scleraldehyde was determined. Significant reduction in the degradation of collagen was observed for the collagen membrane treated with scleraldehyde compared to native collagen. Scleraldehyde treated collagen

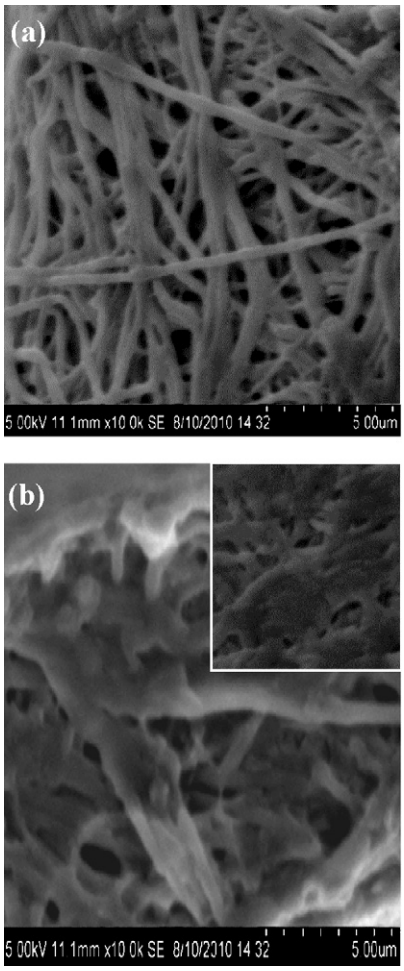

Fig. 6. Scanning electron micrograph of (a) control collagen and (b) scleraldehyde interacted collagen membrane (collage:scleraldehyde 1:1).

membrane exhibited 7% degradation of collagen as against 99% degradation in the case of native collagen at 96 h period of incubation. As seen from Table 1, the enzymatic stability of the collagen matrix increases with increase in concentration of scleraldehyde. Scleraldehyde interacts with collagen through covalent and hydrogen bonding. The stability of scleraldehyde treated collagen fibres against collagenase could brought about by protecting the active sites in collagen (through interaction with scleraldehyde) recognized by collagenase.

3.10. Scanning electron microscopic analysis of scleraldehyde interacted collagen membrane

The scanning electron micrographs of control (native collagen) and experimental membrane with (1:1, collagen:scleraldehyde) are shown in Fig. 6a and b, respectively. From Fig. 6b it can be observed that there is crosslinking of scleraldehyde as a deposition on collagen membrane. The morphology study confirms the interaction of scleraldehyde with collagen.

3.11. Evaluation of cytotoxicity: biocompatibility study of scleraldehyde

The cytotoxicity of scleraldehyde treated collagen membranes were evaluated by MTT method. According to observations under light microscopy, human dermal fibroblasts in direct contact with scleraldehyde interacted collagen matrices showed typical

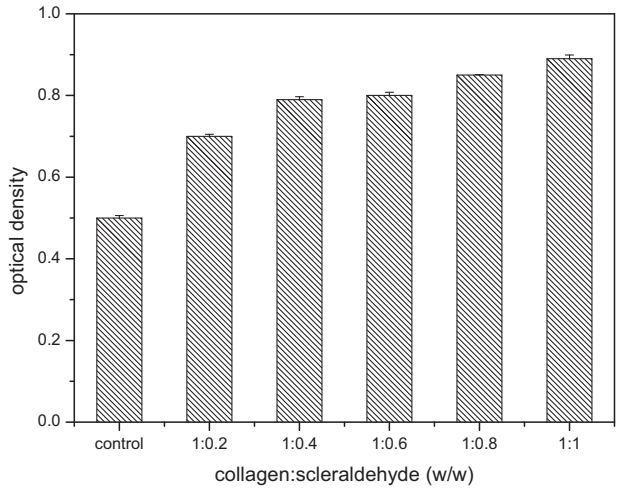

Fig. 7. *In vitro* assay of control collagen and scleraldehyde interacted collagen.

shuttle-like morphology compatible with their surroundings. No abnormal morphology or cellular lysis was detected. From Fig. 7, the absorbance of the resultant formazan crystals among variously treated collagen matrices showed no significant differences. The modified collagen membrane provides an adhesion point for cells, which enhance the rate of cell proliferation. Viability of cells was sustained on the collagen matrix due to its non toxic behavior. The study confirms that modified collagen matrices by scleraldehyde, showed no signs of reduced cell viability and thereby it can be used for biomedical applications.

4. Conclusions

The current investigation establishes scleraldehyde as a stabilizing agent for collagen. The characteristic feature of scleraldehyde like aldehyde content and molecular weight found suitable for protein interaction. The electrophoretic study visually confirms the crosslinking of scleraldehyde with collagen. There was significant increase in molecular weight of collagen owing to inter and intra crosslinking. The hydrodynamic property of collagen was found to increase in the presence of scleraldehyde. Shift in formation of nuclei centre and aggregation of monomeric collagen occurred by the influence of scleraldehyde. The interaction of scleraldehyde with collagen was validated by estimating the involvement of aminogroups in collagen by ninhydrin and TNBS assay. The maximum crosslinking efficiency of about 79% was achieved at a ratio of 1:1 (collagen:scleraldehyde). Higher concentration of scleraldehyde increases the gelling behavior of collagen due to rapid aggregation of collagen.

The degree of swelling and the hydrophilicity nature of the collagen matrices being reduced in the presence of scleraldehyde, which is an inevitable property required for biomedeical applications. The thermal stability and the enzymatic stability of scleraldehyde treated collagen increased with increase in concentration of stabilizing agent. The higher crosslinking of scleraldehyde with collagen can be attributed to strong binding between the two, as scleraldehyde can have both covalent and non-covalent interactions with collagen. Scleraldehyde significantly brings about an increase in thermal temperature of 29 °C as compared to native collagen. The SEM analysis clearly indicates the structural changes in the morphology of scleraldehyde interacted collagen matrices with that of native collagen matrix. The scleraldehyde treated collagen was found to be resistant to degradation by collagenase and the resistance to degradation was found to be high at higher concentration of scleraldehyde. The modified collagen matrices exhibited

non-toxicity towards fibroblast cells. Therefore, scleraldehyde treated collagen membrane found suitable as a substratum for cell proliferation. Thus, the scleraldehyde finds a versatile applications as a stabilizing agent in the preparation of scaffolds.

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