A Molecular Level Investigation of Dialdehyde Starch Interaction with Collagen for Eco-friendly Stabilization

by

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Abstract

The current study investigates Dialdehyde Starch (DAS) as a stabilizing agent for collagen. DAS is a well established crosslinking agent for protein; however, molecular interaction with collagen was not elucidated. Dialdehyde involves in the formation of inter and intra crosslinking with protein which renders higher stability against heat and enzyme. Crosslinking efficiency of DAS with collagen was found to increase with increase in the concentration. DAS interacted collagen membrane exhibited an increase in the thermal stability of about 35°C at pH 8. Swelling degree of collage-DAS membranes were found to decrease with increase in the concentration of DAS owing to the shift in the nucleation behavior in collagen fibrillogenesis. DAS treated collagen membrane shows 90% resistance to collagenase due to the unavailability of cleaving sites in collagen-DAS fibres. Reconstittued collage-DAS collagen membranes showed increase in cell proliferation which signifies its non-toxic characteristics. Therefore, DAS can be a new class of green tanning agent for skin stabilization and also finds applications in scaffold preparation.

Introduction

Collagens are known as triple helical proteins which are widespread throughout the body and are important for a broad range of functions, including tissue scaffolding, cell adhesion, cell migration, cancer treatments, angiogenesis, tissue morphogenesis and tissue repair.[1] Generally, the vertebrate collagens are categorized in to 7 sub-groups according to their structure and function: fibril-forming collagens, which include Type I, II, III, V, XI, XXIV, XXVII, and they are the major protein in the extracellular matrix. Fibril-associated collagen with interrupted triple helix (FACITs) includes Type IX, XII, XIV, XVI, XIX, XX, XXI, XXII and XXVI and are widely spread for different functions. Type IV, VIII, X are network forming collagens which form basement membranes.[2]

The structure of collagen is stabilized by inter and intra-chain hydrogen bonds and by water-mediated hydrogen bonds. Collagen, being a protein, is amenable to degradation by microbial attack.[3] 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 widely studied.[4,5] 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.[6] Hence, the thermal stability of collagen increases owing to crosslinking. In recent times, ecological concerns demand alternative crosslinking chemicals.[7] Starch is a natural polymer that has established widespread applications.[8] Periodate oxidised starch 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.[9,10] Modified polysaccharides are proved to be biodegradable and toxologically acceptable.[11] DAS appeared to be an alternative crosslinking agent for stabilizing collagen as it is from natural resources and eco-acceptable biopolymer.[12] In the present work, an attempt is made to understand the effect of DAS on crosslinking eficiency, thermal stability, swelling ratio and enzymatic stability of type I collagen matrix. The modified collagen membrane is analysed for the toxicity characteristics for its feasibile application as a non-toxic stabilizing agent.

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Materials and Methods

Reagents and Chemicals

The chemicals used for all the experiments were of analytical grade and purchased from Sigma Chemicals, India. Type I collagenase purchased from Sigma Chemical Company was used in the study.

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 their 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 prepared. 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.[13]

DAS was prepared according to the methods reported earlier with modifications as described. Starch (~100 g) was hydrolyzed in 5 N hydrochloric acid (10 h, 85°C). Hydrolyzed starch 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 DAS 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.[14]

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), NaOH (200 mM) and NaCl (2 M) in an ice bath. 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.

Crosslinking Conditions

The collagen membranes were crosslinked by treating them with different ratios of DAS (0.2-1 w/w) at pH 8.0. After interaction for the described period of time (24 h), the membranes were washed extensively with distilled water to remove traces of stabilizing agents. Finally, the washed membranes were then air-dried.[15]

Crosslinking Efficiency

The free amino groups present in DAS interacted collagens were determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay. The resulting colored products were evaluated using UV-VIS spectroscopy. [16] The unreacted ε-amino groups of lysine in native and DAS crosslinked collagen reacts with TNBS to form soluble complex. Acid soluble collagen solution of concentation 5 mg/mL and 1 mL of varying concentrations of DAS (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.[17] All the experiments were carried out in triplicate.

Swelling Ratio

Reconstituted collagen membranes, which were interacted with various concentrations of DAS, were swollen in water and then equilibrated overnight in phosphate buffer (pH 7.4) at room temperature. The membranes were removed, quickly blotted with filter paper to remove excess surface water and weighed immediately. The membranes were 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 dried sample.[18,19]

Thermal Resistance of DAS Stabilized Collagen Membrane Hydrothermal Temperature

Thermal stability of DAS 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 microscope mounted above the heating stage. The rate of heating was maintained at 2°C/min. [20]

Calorimetric measurement – Denaturation Temperature

The native and DAS-treated collagen membrane (1:1 w/w) 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)) associated with the phase change for the shrinkage process for native and DAS treated collagen membranes was studied.[21]

Collagenolytic Activity of DAS Stabilized Collagen Matrix

The enzymatic degradation of native collagen and DAS 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. 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.[22]

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

Scanning Electron Microscopy Studies of DAS Stabilized Collagen Membrane

The structurally stabilization of the collagen membrane was assessed by SEM. The control and 1:1 (Collagen: DAS) (w/w) DAS 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 at low vacuum with an accelerating voltage of 15 KV in 10x magnification levels.

***In Vitro* Cytotoxicity Analysis**

Fibroblasts used in all the experiments were isolated by the method as described by Purna and Mary 2001.[23] Native collagen and DAS crosslinked collagen samples were reconstituted in 24 well plates 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 $4x10^4$ cells/well were directly seeded into culture plates into which a collagen sample were placed and then cultured for 48 h. 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 was 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.

Results and Discussion

DAS with 65% 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 distinguished feature of DAS 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.

Efficiency of DAS Interaction with Biomembrane – TNBS Assay

The crosslinking efficiency brought about by DAS was estimated by TNBS assay. The crosslinking efficiency of DAS at varied concentrations is shown in Figure 1. It is observed that DAS results in interaction with collagen and an increase in concentration enhances the crosslinking efficiency by formation of stable crosslinks with ε-amino groups of collagen. Collagen: DAS at 1:0.2 w/w ratio results in the interaction efficiency of 15% as compared to 76% at 1:1 w/w DAS ratio. The increase in crosslinking efficiency of DAS with collagen was observed due to the decrease in ε-amino groups of lysine in collagen. At a ratio of 1:1 w/w (collagen:DAS) maximum quantity of aldehyde groups are available for interaction with ε-amino groups of collagen. The high interaction ability of DAS with collagen can be attributed to strong binding as DAS can have both covalent and non-covalent interactions with collagen. Hence, aldehydic functionality in DAS 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.

Water Absorption in Collagen-DAS Biomembrane

Swelling ratios of the modified collagen membrane for various ratios of DAS are shown in Figure 2. From the figure, it is observed that interaction of DAS had reduced the swelling behavior of collagen. The swelling ratio for native collagen was found to be 6.8. In the presence of DAS, there was significant decrease in swelling ratio from 6.8 to 3.2 (1:1, collagen: DAS), which substantiates the fact that the water accessibility reduced with increased interaction. At increased concentration, the modified collagen membrane found to be suitable for cell proliferation.

Thermal Stability of Collagen-Das Biomembrane

The thermal stability of DAS interacted collagen is a characteristic of the effectiveness for 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 differential scanning calorimetry thermograms for native and DAS treated collagen membrane (1:1) is shown in Figure 3. The denaturation temperature for control collagen membrane was 59°C. DAS treated collagen shown an increase of 35°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. It could be seen from Figure 4, the denaturation temperature measured using DSC follows the same trend as the hydrothermal shrinkage temperature of DAS stabilized collagen fibres. Crosslinking results in a change in the size of the co-operative 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. Hence, DAS 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. [24]

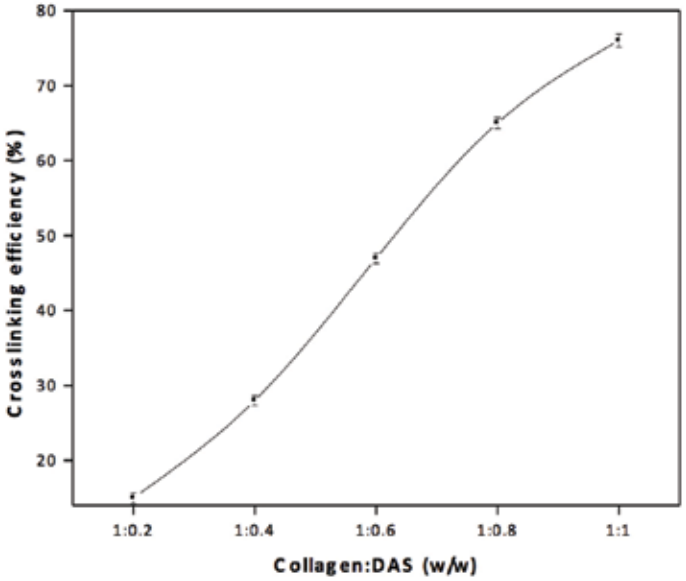

Figure 1. Crosslinking efficiency of collagen and DAS crosslinked collagen.

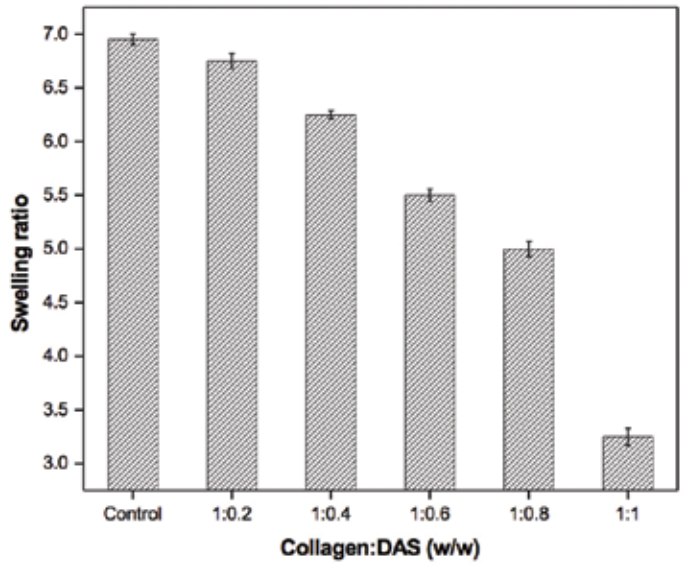

Figure 2. Swelling ratio of control collagen and DAS crosslinked collagen.

DAS-Collagen Biomembrane Resistance Against Collagenase

The stability of the DAS treated collagen membrane against enzymatic degradation was studied by analyzing the rate of hydrolysis of collagen on treatment with bacterial collagenase. Degradation of collagen (based on hydroxyproline released) for native and DAS treated collagen by collagenase at various concentration of DAS was determined. Significant reduction in the degradation of collagen was observed for the collagen membrane treated with DAS compared to native collagen. DAS treated collagen membrane exhibited 10% degradation of collagen as against 99% degradation in the case of native

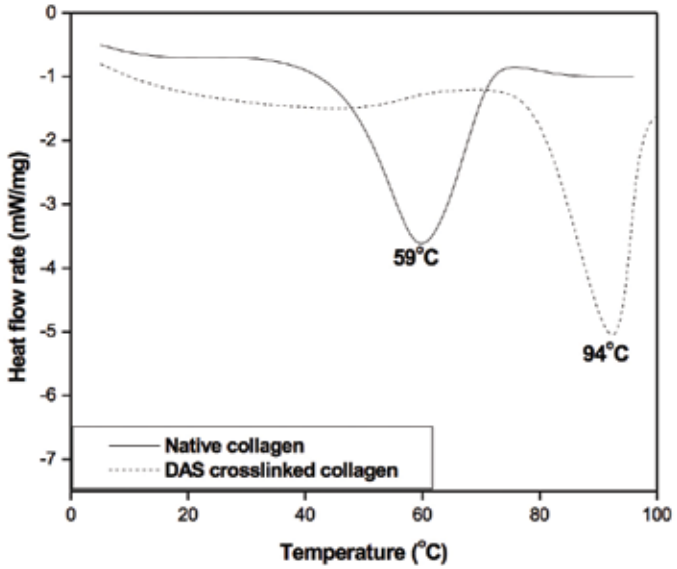

Figure 3. Differential Scanning Calorimetry (DSC) thermograms of collagen and DAS interacted collagen membrane.

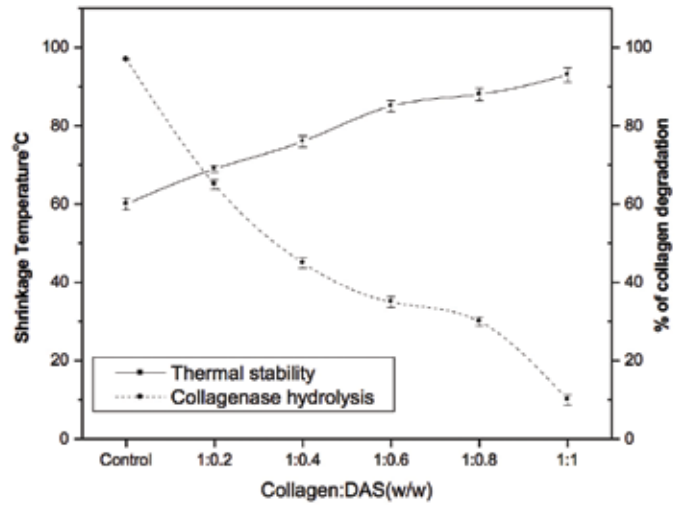

Figure 4. Thermal stability and collagenase hydrolysis of collagen and DAS crosslinked collagen.

collagen at 96 h period of incubation. As seen from Figure 4, the enzymatic stability of the collagen matrix increases with increase in concentration of DAS. The stability of DAS treated collagen fibres against collagenase could be brought about by protecting the active sites in collagen (through interaction with DAS) which are recognized by collagenase.[25]

Morphological Study of Collagen-DAS Biomembrane

The scanning electron micrographs of control (native collagen) and experimental membrane (1:1, collagen: DAS) are shown in Figure 5A and B respectively. From the Figure 5B it can be observed that there was crosslinking of DAS as a there was aggregation between fibrils due to strong interactions. The morphology study confirms that DAS actively participates different mode of interaction with collagen.

Evaluation of Cell Proliferation on Collagen – DAS Biomembrane Substrate

The cytotoxicity of DAS treated collagen membranes were evaluated by MTT method. From the Figure 6, it is observed that the absorbance of the resultant formazan crystals among variously treated collagen matrices showed no significant differences. The modified collagen membranes provide 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 DAS, showed no signs of reduced cell viability and thereby it can be termed as eco-friendly and non-toxic stabilizing agent.

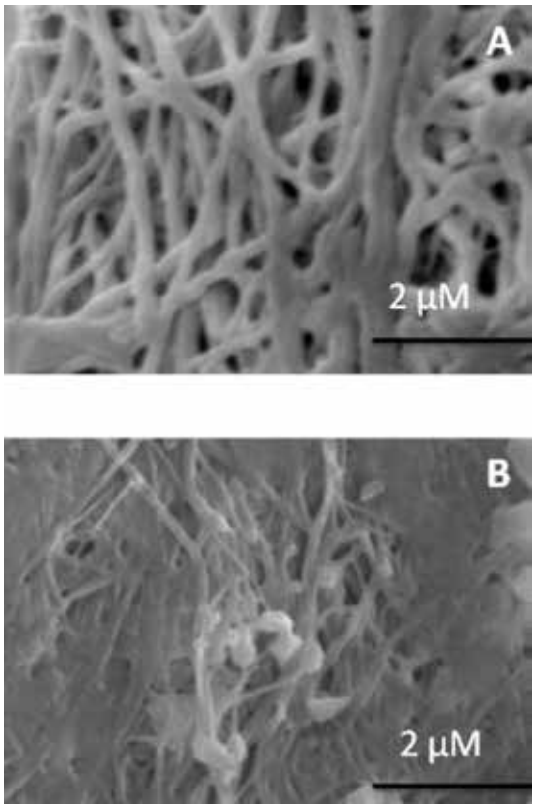

Figure 5.Scanning Electron Micrograph of A) control collagen and B) DAS interacted collagen membrane (collagen: DAS::1:1 w/w).

Plausible Mechanism of Dialdehyde Starch Interaction with Collagen

In the present study, an attempt was made to understand the mechanism of DAS interaction with collagen. For effective interaction, the molecule used as a stabilizing agent should posses difunctionality to exhibit reactivity between two polypeptide chains. The fixation rate of DAS with collagen can be attributed to strong binding between the two, as DAS can have both covalent and non-covalent interactions with collagen. The aldehyde functionality in the DAS can covalently bind with amino groups of the collagen and the hydroxyl groups of the DAS can also involve in electrostatic interaction with side chain functional groups available in collagen matrix. The stability influenced was much higher when compared to conventional aldehydes. The pragmatic investigation of DAS interaction with collagen unequivocally reveals the stability factor. The interatomic forces between DAS and collagen can be induced and transient dipoles. Crosslinking condition can also participate in the efficiency of the conjugation between collagen and DAS. Though, covalent and hydrogen bond well pronounced in protein and protein interaction, it is necessary to appreciate the interaction by diatomic forces. Weak forces between DAS and peptide bond might result in hydrophobic interactions through non-polar amino acids. The intricate process of interaction in the quarter-staggered polypeptides might show marginally repulsion and attractive force. Influence of weak force at fibrillar level plays a vital role in shifting the nucleation centers in monomeric collagen. These forces play a crucial role in stabilization of collagen against heat and enzyme.

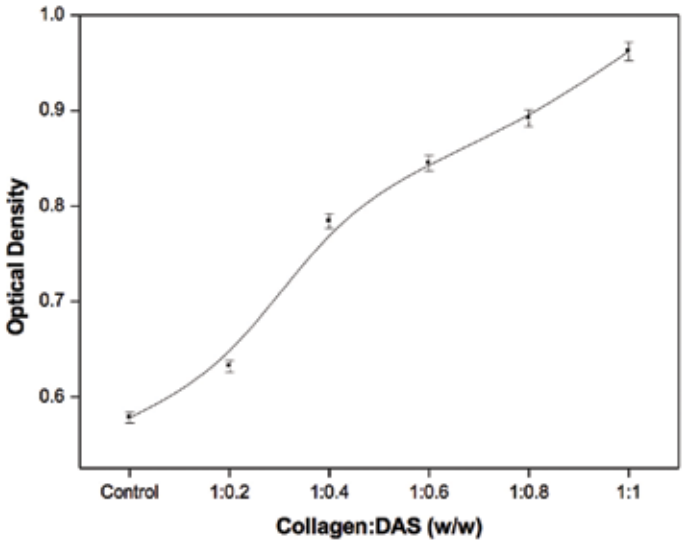

Figure 6. *In vitro* assay of control collagen and DAS interacted collagen.

Conclusions

The present investigation confirms DAS as a stabilizing agent for collagen. The interaction of DAS with collagen was validated by estimating the involvement of amino groups in collagen by TNBS assay. The maximum crosslinking efficiency of about 76% was achieved at a ratio of 1:1 w/w (collagen:DAS). The degree of swelling and the hydrophilicity nature of collagen matrices being reduced in the presence of DAS. The thermal and enzymatic stability of DAS treated collagen increased with increase in concentration of stabilizing agent. DAS significantly brings about an increase in thermal stability of 35°C as compared to native collagen. The SEM analysis indicates the structural changes in the morphology of DAS interacted collagen membrane as compared to native collagen. The DAS treated collagen was found to be resistant against collagen degradation and the resistance was found to be high at higher concentration of DAS. The modified collagen matrices exhibited non-toxicity towards fibroblast cells.

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