# DEVELOPMENT OF GALLIC ACID ASSISTED EDC/NHS CROSSLINKING OF COLLAGEN MATRIX FOR DESIGN OF SCAFFOLD

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The use of collagen matrix in the field of tissue engineering applications is increasing because of its excellent biocompatibility with low antigenicity. To solve undesired stiffness of the collagen matrix, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) / N-hydroxysuccinimide (NHS) initiated crosslinking of collagen is adapted with Gallic acid (GA). The crosslinked matrix was investigated using Thermo Differential scanning calorimetric (DSC) analysis, water uptake and swelling properties and resistance to collagenase activity and biocompatibility. The results indicate an improved tensile strength (TS, 180±3), % elongation (%E, 32±4) and denaturation temperature (Td, 80.03), structural and biostability (15% biodegradation), and swelling and water uptake properties, resulting in high biocompatibility with greater flexibility. The crosslinked matrix has been produced in highly stable and biocompatible forms that can be further manipulated into functionalized scaffold suitable for biomedical applications.

Keywords: tissue engineering, collagen scaffold, gallic acid.

## INTRODUCTION

To devise strategies for using collagen in the development of advanced biomaterials for biomedical engineering, it is necessary to confer mechanical strength, thermal stability and resistance to proteolytic degradation with chemical or physical crosslinking strategies. There are several strategies for crosslinking collagen-based biomaterials. The chemical crosslinking, aldehydes, diphosphorylazide, acyl azides etc, are the most widely used for collagen-based biomaterials (Anderson et al., 2008). However, the above crosslinking agents and the reaction products are associated with cytotoxicity and poor biocompatibility in vivo, because of the presence of crosslinking byproducts and the release of aldehyde-crosslinked collagen peptides during enzymatic degradation. To avoid in vivo cytotoxicity, poor biocompatibility and subsequent calcification of aldehyde treated collagen, several alternative agents have been examined as potential crosslinking such as, carbodiimides and succinimide etc, and physical methods, photo oxidation and gamma or UV-irradiation, etc. While physical methods have no toxicity problems and can provide sufficient stability. Although non-covalent and covalent crosslinks do afford molecular stability, the long-term preservation of collagen matrix has required (Madhavan et al., 2010; Lee et al., 2001; Friess, 1998 and 2003).

A number of polyphenols (PPs) compounds derived from plants have been reported to be interactive or reactive with proteins and resulted in improved properties for collagen-based materials. There are several molecular species of phenolics, which appear to be a promising choice for the stabilization of collagen matrix. These PPs are two types, hydrolysable (polyester of GA and polysaccharides) and condensed (polymerized products of flavan-3-ols and flavan-3, 4-diols, or a mixture of the two), although other PPs occur which are combinations of these two basic structures. The process for crosslinking of Type I collagen fibers with PPs significantly improved the properties. In addition, it has been shown that PPs crosslinked collagen fibers neither elicit a foreign body response nor did they stimulate an immune reaction (Ahmed et al., 2010; Haslam et al., 2007; Tang et al., 2003; Covington et al., 2005).

Gallic acid (GA) is a 3,4,5-trihydroxybenzoic acid, a type of organic phenolic acid, found in gallnuts, sumac, tea leaves, oak bark, and other plants, found both free and as part of tannins, and chemical formula is $C_6H_2(OH)_3COOH$. GA and its derivative are known to form multiple H-bonds with proteins, particularly those rich in proline such as elastin and collagen and used in the manufacture of gelatin product and leather adhesives. Its derivative has been suggested as a potential cross-linking agent and improves the elastin and collagen stability in cardiovascular implants as evidenced by an increased resistance to proteolytic degradation against collagenase activity. The pharmacological properties such as anti-oxidant, anti-inflammatory and antimicrobial activities of GA derivative have been studied (Shahrzad et al., 2001; Punithavathi et al., 2011; Zhang et al., 2008; Jackson et al., 2010). Therefore, it has been considered that GA has a potential possibility as an abundant and natural resource for stabilization of collagen scaffold (Isenburg et al., 2005). An attempt has been made to the process of prepare stable collagen scaffold by GA with presence of EDC/NHS. Achilles tendon which predominantly contains type I collagen has been used in this study.

This study was to develop a collagen scaffold in order to improve the thermal stability, swelling and water uptake properties, resistance to collagenolytic activity, biocompatibility and cell adhesion properties. The molecular level understanding of interaction of collagen with GA was carried out by docking studies.

## MATERIALS AND METHODS

### Materials

All reagents and chemicals used were analytical grade. Clostridium histolyticum collagenases (Type IA), Gallic acid (GA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxy succinimide (NHS) and 2-(N-morpholino) ethanesulfonic acid [MES] were sourced from Fluka and Sigma Chemicals Co., USA. All other reagents and chemicals used for the study were sourced from SRL Ltd., India.

### Preparation of Collagen Scaffolds

The collagen scaffolds viz. Coll, Coll-GA, Coll-EDC/NHS and Coll-GA-EDC/NHS were prepared by the described in our earlier method (Krishnamoorthy et al., 2012).

### Characterization

The thermal properties of collagen scaffolds were analyzed using by DSC (TA-DSC Q 200) analysis. The water-uptake and swelling ratio were obtained by incubation of the samples in water at room temperature for 2 hrs. The percentage (%) of all scaffolds degradation compared with native scaffold against ChC activity was calculated by the method described in our earlier method (Krishnamoorthy et al., 2012). The MTT assays were performed to assess the % of cell viability and cell adhesion.

### Molecular Docking of Collagen-GA and ChC-GA Interaction

The molecular docking studies comprise the determination of the three-dimensional structure of collagen by energy minimization methods and the simulation of the interaction between collagen, GA and ChC active site (Autodock 4.2, Accelrys Discovery Studio 2.5). The collagen like peptide (CLP), ChC and GA structure were

obtained from Protein Data Bank (collagen PDB: 3A1H, ChC PDB: 1NQD) and PubChem Database (CID: 370) for this study.

## RESULTS AND DISCUSSION

### Tensile Strength

The tensile strength (TS) and % elongation (% E) are given in Table 1. TS of the collagen matrix increased with the presence of GA with EDC/NHS. Furthermore, GA-EDC/NHS treated matrix leads to improved TS compared to native, GA and EDC/NHS. The GA-EDC/NHS treated matrix resulted in maximum TS, this is due to the fact that inter and intra molecular crosslinking with hardness lower than that of matrix may well increase thermo mechanical stability.

Table 1. TS, % E, TD, % weight loss of collagen scaffold

| Concentration | TS (MPa) | % E | Td (°C) | % Weight Loss |
|---|---|---|---|---|
| Native scaffold | 59±4 | 8±3 | 64.11 | 68.98 |
| EDC/NHS | 79±3 | 19±4 | 69.09 | 53.31 |
| GA | 120±2 | 25±6 | 69.50 | 52.45 |
| GA-EDC/NHS | 180±3 | 32±4 | 80.03 | 31.76 |

Our data imply that the ratio of -COOH groups to $NH_2$ groups significantly affects the TS of matrix. According to the molecular structure, the OH and COOH groups of GA and the collagen all provide possibilities for crosslinking. GA-EDC/NHS treated matrix exhibited high TS and high % E where as GA and EDC/NHS treated matrix exhibited low Ts with low % E. By GA-EDC/NHS treatment, the mechanical properties of collagen matrix are improved.

### DSC Analysis

The $T_D$ of scaffolds were computed from DSC data and presented in Table 1. The GA-EDC/NHS treated scaffold exhibits an increase in the $T_D$ when compared to native matrix. This may be due to a net increase in the number of inter and intra molecular crosslinks and interactions between the GA-EDC/NHS and collagen. It is known that $T_D$ of matrix is paralleled by destruction of H-bonds and hydrophobic interactions between the protein subunits and helix-coil transformation. Presumably, the combination of high hydrophobicity and capacity to form peptide bonds permits these molecules incorporate in certain areas of collagen fibrils and promote stabilization of their structure. The Ts of matrix depends on stable intermolecular cross-links formed through the GA-EDC/NHS than GA and EDC/NHS.

### Water Uptake and Swelling Studies

Water-uptake and swelling ratio of matrix were increased with presence of GA (Figure 1). Although formation of GA-EDC/NHS treated matrix can increase more amount of hydrophilic groups and also presented higher swelling ratio than EDC/NHS. This decrease should be mainly attributed to the partial diminishing of the hydrophilic groups of the scaffold after crosslinking.

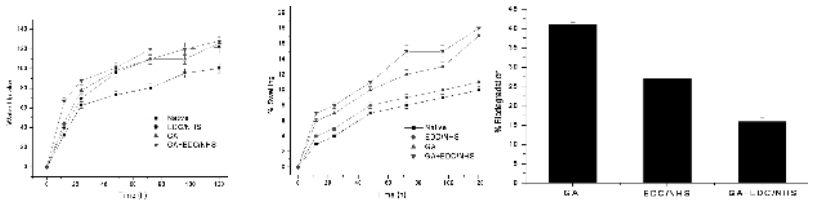


Figure 1. The % of water uptake and swelling ratio and % biodegradation of scaffolds

The swelling ratios of the GA-EDC/NHS treated scaffolds are still big enough to meet the demand of the matrix used in tissue engineering. Consequently, both water uptake and swelling time can be increased by characteristics of GA. Thus, we concluded that the addition of GA-EDC/NHS to collagen scaffold significantly increased the water uptake ability and made them more hydrophilic. Also, it is seen that % swelling of GA-EDC/NHS treated scaffold increased with increase of concentration of GA.

**Stability of GA Treated Scaffold Against ChC Activity**

% Collagen degradation (based on hydroxyproline released) scaffolds by ChC at various time periods were determined (Figure 1). Significant reduction in the degradation of collagen was observed for the scaffold treated with the GA-EDC/NHS compared to Coll, GA and EDC/NHS. The GA, EDC/NHS and GA-EDC/NHS treated scaffold exhibited 44, 27 and 15 % degradation of collagen as against 93% degradation in the case of Coll at 96 h period of incubation. The GA can interact with collagen through multipoint H-bonding, carboxyamide and hydrophobic interactions. The hydrophobic core of the GA molecule, likely incorporates itself into hydrophobic areas, while OH moieties of GA may establish multiple H-bonds with neighboring collagen molecules, resulting in improved stability of scaffold and prevent the free access of ChC to reactive sites on the collagen chains (Krishnamoorthy et al., 2008, 2011 and 2012).

**Molecular Docking of Collagen-GA and ChC-GA Interaction**

Figure 2A, B, C and D shows the closest structure to the collagen interlayer region with GA, H-bonding, hydrophobic and closest binding site respectively, where the contact surfaces are colored according to their distance. The best geometries obtained through the docking represent the low interaction energy -5.08 Kcal/mol between collagen-GA, resulting in stronger H-bonds. The interaction of CLP with GA and binding at multiple sites and the predicted H-bonds (dashed yellow lines) are shown (Figure 2B). The light yellow dots represent the possible H-bonds and are involved in hydrophobic interactions. These representations clearly show the possible anchoring points of GA moieties with the collagen interlayer region.

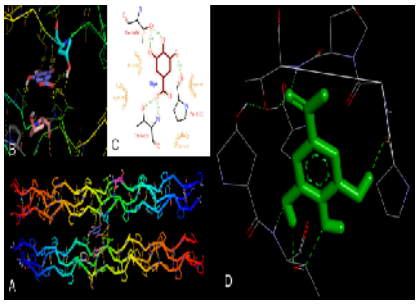

Figure 2. A) GA binding to interlayer region of collagen, B) H-bonding, C) Hydrophobic interaction and D) binding site

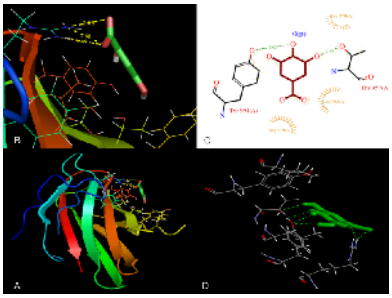

Figure 3. A) GA binding to ChC active site, B) H-bonding, C) hydrophobic interaction and D) binding site

Molecular docking of interaction of GA with ChC active site was carried out. Figure 3A, B, C and D shows the closest structure to the ChC with GA, H-bonding, hydrophobic and closest binding site respectively, where the contact surfaces are colored according to their distance. The best geometries obtained through the docking represent the low interaction energy -4.27 Kcal/mol between ChC-GA, resulting in stronger H-bonds. ChC is represented by its solvent-accessible surface. The yellow dots represent the possible H-bonds and are involved in hydrophobic interactions. This clearly show the possible anchoring points of GA with the ChC active site.

**Cell Viability**

Fibroblast incubated in the scaffold showed no cytotoxic effect. MTT assay showed more than 85% fibroblast viability (NIH 3T3) after 72 h of culture on scaffold when compared with native scaffold and EDC/NHS (Figure 4). GA-EDC/NHS could improve the cell adhesion to the surface of matrix are shown (Figure 4). After GA-EDC/NHS is introduced into the matrix, the cell-adhesion ratio can be observed to increase significantly.

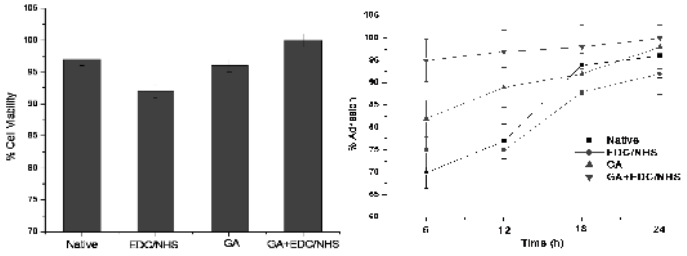


Figure 4. % Cell viability and % Cell adhesion assay of scaffolds

GA-EDC/NHS exhibited more fibroblast adhesion than GA and EDC/NHS treated matrix. It is interesting to note that fibroblast adhesion to EDC/NHS treated matrix demonstrated similar trend to native matrix. It suggests that GA-EDC/NHS may induce the adhesion and spreading of fibroblasts than GA and EDC/NHS.

## CONCLUSIONS

The GA with presence of EDC/NHS was shown to improve mechanical strength and thermal stability, swelling and water uptake properties, biocompatibility and cell adhesive properties, effective in the prevention of collagen scaffold degradation. We have shown here that GA interaction with collagen imparts a less binding energy and also shown the possible anchoring points of GA galloyl moieties with the ChC active site. This matrix is more stable and can be effective in the prevention of degradation by collagenase activity and, hence may find use in the preparation of collagenous biomaterials.

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