CHAPTER 2

PREPARATION AND CHARACTERIZATION OF CROSSLINKED COLLAGEN SCAFFOLDS WITH D-AMINO ACIDS (COLL-D-AAS)

2.1 INTRODUCTION

The fibrous protein of coiled coil triple helical structure of collagen, one of the most common biomaterials, has found diverse applications in industries like cosmetic, photographic and leather, and biomedical fields such as drug and gene carriers, management of severe burns, and tissue engineering because of its excellent biocompatibility, controllable biodegradability and less immunogenicity (Cen et al 2008; Ramshaw et al 2009; Bachinger et al 2010). Various forms of collagen such as soluble, insoluble and reconstituted collagen, etc, can be obtained, according to the sources, types and the extraction processes. Among these, the insoluble collagen has often been used to construct collagen based biomaterials for tissue engineering and regenerative medicine. Different forms of the collagen matrices such as film, gel, sponge, tube, scaffold and composite have been fabricated and used in practice such as drug carriers and soft and hard tissue augmentation (Madhavan et al 2010). In the past decades, collagen matrix has been used widely in tissue engineering such as cartilage, bone, nerve and skin where they serve as support and template for cell infiltration, proliferation and differentiation (Lee et al 2001).
The poor mechanical strength, thermal instability and fast biodegradation rate of the unmodified collagen matrix cannot match the demand of in vitro and in vivo applications in many cases and have been one of the crucial factors that limit the further use of this biomaterial for biomedical applications. Cross-linking and molecular interaction of the collagen matrix is an effective way to slow down the biodegradation rate and to optimize the mechanical strength and thermal stability (Parenteau-Bareil et al 2010). Currently, there are two kinds of cross-linking methods frequently employed for improving the stability of the collagen matrix: physical and chemical methods. The former includes the use of photooxidation, dehydrothermal treatments (DHT) and UV irradiation which could avoid introducing potential cytotoxic chemical residuals and sustain the excellent biocompatibility of collagen matrix (Charulatha et al 2003). But most of the physical treatments cannot yield enough cross-linking degree to meet the demands of tissue engineering. Therefore, the treatments by chemical methods are still necessary in most cases. In chemical treatment, inorganic and organic agents such as chromium complexes and aldehydes such formaldehyde and glutaraldehyde are the most convenient and traditional agents. These can enhance the stability of the collagen scaffold by bridging amine groups between two adjacent polypeptide chains. This cross-linking can also suppress the immunogenicity of the artificial implant. However, accompanying the increase in stability of the matrix, there are also some other problems arising, such as over cross-linking and the potential cytotoxicity (Eybl et al 1989). Therefore, several alternative cross-linking agents such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) have been reported (Yuanyuan et al 2009). Among them, the EDC/NHS cross-linking is frequently used in the treatment of collagen matrix, which has shown better biocompatibility than glutaraldehyde and other aldehydes (Rault et al 1996). The cross linking method using EDC/NHS induces the formation of an amide bond by the activation of the side chain –COOH group of aspartic
and glutamic acid followed by aminolysis of the O-iso acylurea intermediates by the NH₂ groups of (hydroxy)lysine, which presents in the formation of intra- and interhelical cross links (Pieper et al 2000). There are also some drawbacks in using EDC/NHS as a cross linking agent. Though this zero length cross linker produces collagen matrices with increased stability, this also leads to undesired stiffening of the matrix. The increased stiffness of the collagen matrices may result in a deterioration of function and even in long-term failure of the implant. In order to improve the efficiency of crosslinking, mechanical, thermal and biostability of the collagen matrix with unnatural D-Amino acids (D-AAs) were incorporated during the crosslinking process with EDC/NHS.

![Figure 2.1 Schematic representation of stereochemistry of chiral L and D-AAs](image)

Unnatural AAs are non-genetically coded AAs that either occur naturally or are chemically synthesized. Unnatural D-AAs are mirror images of L-AAs known as left-handed proteins; whereas D-AAs are produce right-handed proteins. D-AAs are stereoisomers or optical isomers of naturally occurring L-AAs. D-AAs and L-AAs have almost the same physical and chemical properties. D-AAs are becoming very important tools for chiral building block, molecular scaffolds and also modern drug discovery. The chiral compounds of minor image of L and D-AAs are represented
(Figure 2.1). The use of unnatural AA for design of novel organomodified clays as components of nanocomposite biomaterials has been studied (Katti et al 2010). Natural AA based biomaterials find limited application as drug carriers and tissue engineering applications due to rapid metabolism and by proteolysis and interaction with multiple receptors (Tsai et al 2007). Partial D-AAs substitution improves enzymatic stability and preserved the peptide (Tugyi et al 2005). Furthermore, D-AAs containing peptides are especially important as pharmaceuticals due to their strong anti-microbial activity and also protease inhibitor (Annedi et al 2006).

**Figure 2.2 Industrial and biomedical applications of unnatural AAs**

The D-AAs may be an advantage in terms of specificity and efficacy, the latter because of longer persistence in an undigested form because they resist enzymatic degradation. And then D-AAs are increasingly growing in research stature for pharmaceuticals, food and feed additives, agrochemicals and other industrial applications (Friedman et al 1999; Friedman et al 2010; Martinez-Rodriguez et al 2010). The industrial and
biomedical applications of Unnatural AAs are presented (Figure 2.2). In order to improve the mechanical strength, thermal stability and resistance to collagenolytic activity, while preserving the biocompatibility, herein the EDC/NHS method was used to cross-link the collagen matrix in the presence of unnatural AAs such as D-form or mirror image of L-AAs of acidic, basic or neutral AAs.

2.1.1 Scope of the Work

This work involved incorporation of unnatural AAs with biomaterials for possible uses in biomedical and industrial applications.

- In this chapter, we focused on the stabilization of the collagen matrix by D-AAs in order to improve the mechanical strength and thermal stability, and resistance to collagenolytic activity.

- The stability of collagen matrices were compared by comparing the mechanical strength, thermal stability and degree of biodegradation before and after adding the D-AAs.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All reagents and chemicals used of were analytical grade. Clostridium histolyticum collagenase (ChC) (Type IA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxy succinimide (NHS) and 2-(N-morpholino) ethanesulfonic acid [MES] were sourced from Fluka and Sigma Aldrich Chemicals Co., USA. D-amino acids (D-AAs) and all other reagents analytical grade and chemicals used for the study were sourced from Hi-Media and SRL Pvt. Ltd, India.
2.2.2 Preparation of Bovine Achilles Tendon (BAT) Collagen

The Achilles tendons, collected fresh from local slaughterhouse, were manually dissected out from surrounding fascia, followed by washing in tap water. They were cut into small bits of 3-4 mm each with a sharp knife and were solubilised by a patented procedure developed at CLRI (Sehgal et al 2001).

In brief, the bits were minced in a mincer (Model 4612, Hobart, USA). The minced tissues were washed using non-ionic surfactant. The washed tissues were suspended in sodium peroxide solution (0.3%) for swelling. The tissue was washed with distilled water (qs) after the coagulation of the swollen mass. The coagulated collagen was then suspended in phosphate buffer solution of pH 8.5 and treated overnight with trypsin (0.5% w/w). The tissue was again washed in distilled water to deactivate the enzyme and the dissolved salts were removed. The coagulated tissue was swollen again in distilled water after adjusting the pH of water to 2.5 with HCl and treated with Pepsin (0.3% w/w) overnight. After the 2nd enzyme treatment, tissues were washed repeatedly in water to deactivate the enzyme. The coagulated collagen was dissolved in Millipore water (0.06 μs purity) acidified to pH 3.5 using HCl to get pure collagen solution. The undissolved proteins were removed by centrifugation at 10000 rpm for 30 min using standard polypropylene centrifuge tubes. All the above operations were performed at a temperature of 15 ± 2°C. The purity of collagen and concentration of collagen have been confirmed by SDS-PAGE and Hyp assay methods. The schematic representation of preparation of collagen matrix is presented (Figure 2.3).
## Preparation of collagen scaffold

| 1 | Collection of Achilles tendons from the slaughterhouse |
| 2 | Storing in an ice bucket and transportation to the processing center |
| 3 | Removal of adhering non Collagenous tissues from Achilles, salting the material, followed by preservation in a freezer |
| 4 | Chopping of Achilles tendon into small bits and treatment with non-ionic wetting agents solution at 37°C |
| 5 | Treatment with alkali at ambient temperature and solubilisation of collagen |
| 6 | Precipitation of collagen and washing with several changes of water and repeat process |
| 7 | Enzyme (Trypsin) treatment at 20°C pH 8 to 8.5 using phosphate buffer |
| 8 | Deactivation of enzyme by washing with water |
| 9 | Enzyme treatment (Pepsin) at pH 2.5 (HCl) |
| 10 | Enzyme deactivation by bring down the pH to 6.5 using dilute NaOH |
| 11 | Swelling of tissue in acid pH adjustment (pH 2.5 HCl) |
| 12 | Sheet casting in Laminar Flow |
| 13 | Sealing of each sheet in a polypropylene sachet and sterilisation by ETO |

**Figure 2.3 Schematic representation of preparation of collagen matrix**
The % of Hyp was also determined (Woessner 1961). The Hyp is a unique AA for collagen and it offers itself as a useful marker for identifying collagen in the presence of non-collagenous proteins. The method of determining Hyp involves the oxidation of Hyp to pyrrole-2-carboxylic acid, which complexes with p-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm.

\[
\% \text{ Collagen} = \% \text{ Hyp} \times 7.4
\]

2.2.3 Characterization of Coll-D-AAs

2.2.3.1 SDS-PAGE analysis

SDS-PAGE was carried out according to the standard method using the Tris–HCl/Glycine buffer system with 7.5% resolving gel and 4% stacking gel. Isolated collagen samples were dissolved in sample buffer solutions (0.5 M Tris–HCl, pH 6.8, containing 2% SDS, 25% glycerol and 0.01% bromophenol blue) to reach a final collagen concentration of 1mg/1ml. The mixed solutions were boiled for 3 min and subjected to electrophoresis. The gel was stained with 0.25% Coomassie brilliant blue R-250 solution for 40 min and then distained with a solution containing 7.5 % acetic acid and 5% methanol. Prior to all the experiments, purified 2.5mg of collagen solution was treated with 40mM EDC and 20mM NHS (EDC/NHS, 2:1 ratio). Collagen was incubated with 2.5 mM D-AAs for 3 h prior to cross linking with EDC/NHS (40 mM/20 mM).

2.2.3.2 CD spectral analysis

The collagen solutions prepared were diluted with 5 mM acetic acid and treated with EDC/NHS, D-AAs-EDC/NHS and incubated overnight in a refrigerator at 4°C. The collagen concentration was 2×10^{-6} M for the far UV region. Samples were transferred to a 1 mm path length quartz cuvette, and
ellipticity was measured from 185 to 260 nm using a J-715 spectropolarimeter (Jasco) at 20°C. The CD readings were expressed as molar ellipticity. The molar ellipticity at 220 and 197 nm was measured and Rpn ratio (ratio of the positive to negative band) was calculated for EDC/NHS cross linked collagen at various concentrations. CD experiments were also carried out for collagen cross linked with EDC/NHS (40 mM/20 mM) in the absence and presence of 2.5 mM D-AAs.

2.2.3.3 Collagen fibrillogenesis

Prior to fibrillation, purified native collagen solution was treated with various concentrations of EDC/NHS and keeping the collagen concentration same as 1.5mg/ml. The samples were incubated overnight in a refrigerator. Native collagen was also treated with 5mM D-AAs followed by EDC/NHS and incubated in the same way in a refrigerator before fibrillation. Collagen fibril formation was initiated by mixing collagen with phosphate buffer (0.2M) and sodium chloride (2M) in an ice bath. The pH of the solution was adjusted to 7.4 with sodium hydroxide (1.25N). The final composition in the mixture was collagen (1.5mg/ml), phosphate buffer (0.02M), and sodium chloride (0.13M). A control which did not contain D-AAs and EDC/NHS was also prepared. Turbidimetric analysis was made with Shimadzu UV-1700 having thermostatically controlled cuvette holders. A blank was run simultaneously which contained the same mixture but the collagen solution was replaced with equal volume of 0.05 M acetic acid. The turbidity was measured as the optical density at 313 nm at room temperature for collagen treated with various concentrations of EDC/NHS. The experiments were also done for collagen (1.5 mg/ml) treated with 10 mM EDC/NHS in the absence and presence of D-AAs (5mM). The fibril formation rate was represented by \( T_{1/2} \) determined from the fibril formation curve. The time at which the turbidity was half the plateau value at 9 min was taken as \( T_{1/2} \).
2.2.3.4 Viscosity measurement

Viscosity measurements were performed using an Ostwald type viscometer (10 mm diameter) of 1 ml capacity. The viscometer was thermostated at 25±1 °C. The viscometer was calibrated with 10 and 20% sucrose solution. The intrinsic viscosity of the sucrose solution was found to be in close agreement with the reported values. The flow times of collagen samples were measured. The collagen concentration (0.4 x 10^{-6} M) was fixed and the treatment of collagen with D-AAs in acetate buffer (pH 4.0, Ionic strength, I = 1 x 10^{-2} M) for 24 h was carried out and the viscosity for the same measured. 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 (\eta) due to collagen was measured as a function of concentration of added D-AAs. 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°) / t° where t° is the flow time of buffer (pH 4.2, Ionic strength, I = 2 x 10^{-2} M) and t is the flow time for each sample. A plot of relative viscosity was calculated.

2.2.4 Preparation of Coll-D-AAs Matrix

2.2.4.1 Preparation of collagen matrix

Collagen was dissolved in 0.5M acetic acid solution to prepare a 0.5% (w/v) solution. After deaeration under reduced pressure to remove entrapped air-bubbles, the collagen solution was injected into a homemade mold (diameter: 16 mm; depth: 2 mm), frozen in 70% ethanol bath at -20°C for 1 h and then lyophilized for 24 h to obtain a collagen matrix.
2.2.4.2 Cross-linking treatments

The entire collagen matrices were dehydrated at 105°C under vacuum for 24 h prior to any additional chemical crosslinking. In all the cross-linking experiments, the collagen matrix weighing 2.5 mg was incubated in 1ml 50 mM MES (pH 5.5) solution containing 2.5 mM D-AAs (e.g., D-alanine (D-Ala), D-glutamic acid (D-Glu) and D-lysine (D-Lys)) for 1 h at room temperature. Then 1ml MES solution containing 40mM EDC and 20mM NHS was added. After incubation for 24 h at room temperature, the matrix was washed with double distilled water (10 min×six times) and lyophilized.

2.2.5 Characterization of Coll-D-AAs matrices

The mechanical strength, thermal stability, structural elucidation and morphological changes of Coll-D-AAs matrices were analyzed using by Thermal Mechanical Analysis (TMA, Instron series II Automated Materials Testing System), Thermal Gravimetric Analysis (TGA, TA-TGA Q 50), Differential Scanning Calorimetric (DSC, TA-DSC Q 200), Fourier Transform Infrared (FT-IR) spectral, Scanning Electron Microscope (SEM, FEI-Quanta 200) and Atomic Force Microscope (AFM, Park Systems XE-70).

2.2.5.1 TMA analysis

The mechanical strength of Coll-D-AAs matrices were analyzed using by TMA. The matrices were cut into 10 cm length and immersed in distilled water (1-5 min) before testing the samples. A tensile force was applied at an extension rate of 10 mm/min. The ends of the matrices were held by pneumatic grips (40 psi grip pressure). The ability of the matrices to bear loads can be studied by its stress strain behaviour.
2.2.5.2 TGA analysis

The Tg of Coll-D-AAs matrices were analyzed using by TGA. The reference of TGA was aluminium oxide. TGA analysis was done using an open cell at a heating rate of 20°C/min under dry air at a flowing rate of 300 ml/min from 25 to 800°C.

2.2.5.3 DSC analysis

The Td of Coll-D-AAs matrices were analyzed using by DSC. The matrices were weighed (5mg) into a hermetic aluminium pan which then sealed using a sample pan crimper. The thermogram was obtained by heating to 120°C at the rate of 10°C per 1 min. The reference cell used in the analysis was an empty hermetic aluminium pan. The temperature scale was calibrated with standard indium. Td of the matrices were obtained from the peak temperature.

2.2.5.4 FT-IR spectral analysis

The structural elucidations of Coll-D-AAs matrices were analyzed using by FT-IR spectral analysis. The spectra represented the average of 50 scans. All spectra were recorded from 400 to 4000 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\).

2.2.5.5 Water uptake and swelling ratios

The water-uptake and swelling ratios were obtained by incubation of the samples in water at room temperature for 2 hr. Water uptake ratio of Coll-D-AAs matrices were assessed as follows. The coll-D-AAs matrices were weighed and then equilibrated for 2h in PBS (pH 7.4) at room temperature. After removal of additional water, they were weighed again. The water-uptake ratio was calculated by the following formula. The water-uptake
ratio is defined as \((W_d - W_w) / W_d\), where \(W_w\) and \(W_d\) represents the weight of wet and dry scaffold, respectively.

\[
\% \text{ Water uptake} = \frac{(W_d - W_w)}{W_d} \times 100
\]

Swelling ratios of Coll-D-AAs matrices were assessed as follows. After hydrating in water for 1h, the samples were equilibrated for 2h in PBS (pH 7.4) at room temperature, blotted with filter paper to remove excess surface water and then weighed. The collagen matrices were then placed in deionized water to remove the buffer salts and air-dried to constant weight. The swelling ratio is defined \((L_d - L_w) / L_d\), where \(L_w\) and \(L_d\) represents the length of wet and dry scaffold, respectively.

\[
\% \text{ Swelling} = \frac{(L_d - L_w)}{L_d} \times 100
\]

In addition to assessment of weight and calculation of the swelling ratio, the volume changes of the different cylindrical formed collagen matrices before contact with fluid (dry samples) and after removal of fluid (dried samples) were determined.

2.2.5.6 Morphological analysis

The morphological changes of Coll-D-AAs matrices were analyzed using SEM and AFM analyses. The matrices were cut with uniform thickness without any pre-treatment. The micrographs for the matrices surface were obtained by operating the SEM and AFM.

2.2.6 Biodegradation Assay

The % of Coll-D-AAs matrices degradation against ChC activity was studied by the following method. The Coll-D-AAs matrices were further treated with ChC and the treatment was carried out in 0.04 M CaCl\(_2\) solution buffered at pH 7.2 with 0.05 M tris HCl. The collagen matrix: ChC ratio was
maintained at 50:1 (2mg of collagen: 0.04mg ChC). The samples were incubated at 37°C. Samples were collected at 96 h and stored in freezer. The cleavages of native and Coll-D-AAs matrices were monitored by the release of soluble form of Hyp from insoluble collagen (Ryan et al 1971). The % of Hyp was also determined (Woessner 1961). Hyp is a unique AA for collagen and it offers itself as a useful marker for identifying collagen in the presence of non-collagenous proteins. The method of determining Hyp involves the oxidation of Hyp to pyrrole-2-carboxylic acid, which complexes with p-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm.

\[
% \text{ Soluble collagen} = % \text{ Hyp} \times 7.4
\]

Based on the soluble (solubilized due to enzymatic hydrolysis) collagen content in the supernatant solution of the ChC treated matrices the % degradation of collagen for native and Coll-D-AAs matrices are calculated as

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

### 2.2.7 Anti-microbial Activity

The zone of inhibition assay on solid media was used for determination of the antimicrobial effects of Coll-D-AAs matrices against Escherichia coli and Bacillus subtilis. 10 ml of Molten Nutrient agar was inoculated by 20 ml of bacterial cultures. Test discs were placed on the bacterial lawns. The plates were incubated at 37°C for 24 h in the appropriate incubation chamber. The plates were examined for zone of inhibition of the Coll-D-AAs matrices discs and the diameter of the zone was measured with a caliper. The area of the whole zone was calculated and then subtracted from the Coll-D-AAs matrices disc area and this difference in area was reported as the zone of inhibition.
2.2.8 Biocompatibility Analysis

2.2.8.1 Cell viability assay

Monolayers of fibroblast cell line NIH 3T3 sourced from National Centre for Cell Science (NCCS), Pune, India were grown on dispersion of Coll-D-AAs matrices on 96 Well Culture plate (Corning, NY) and maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% Fetal Calf Serum (FCS) supplemented with antibiotics (Sigma) such as penicillin (120 units/mL), streptomycin (75 mg/mL), gentamycin (160 mg/mL), and amphotericin B (3 mg/mL) at 37°C humidified with 5% CO₂. The culture medium was changed every other day.

2.2.8.2 Cell adhesion

For study of the cell adhesion on Coll-D-AAs matrices, cells were attached themselves to the matrices placed on the bottom of the 96-well tissue culture plates and cultured for 6, 12, 18, 24, 30, 36, 42 and 48 hrs, respectively. The cells were seeded with the density of 5×10³/ml. At each time interval, three specimens of each matrices were rinsed with PBS and the average cell numbers of each matrix were counted.

\[ \text{Na (attached)} = \text{Ns (seeded)} + \text{Nr (rinsed)} \]

2.2.9 Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). The significant level was set as *P<0.05, **p<0.001 and ***p<0.0001.
2.3 RESULTS

Incorporating variable unnatural AAs of D-configuration stereochemistry in molecular design has the potential to improve existing protein stability and create new topologies inaccessible to homo chiral molecules (Annavarapu et al 2009). From a functional point of view, the unnatural stereochemistry often means that compounds containing D-residues are much more resistant to enzyme-catalyzed breakdown than L-residues, a property of considerable pharmaceutical and microbiological importance (Friedman et al 1999; Friedman et al 2010; Martinez-Rodriguez et al 2010). It is of profound interest to establish how the D-AAs exhibit better crosslinking of the collagen matrix in order to improve the mechanical strength and thermal stability and resistance to collagenolytic activity.

2.3.1 SDS-PAGE Analysis

Acid soluble bovine Achilles tendon type I collagen was electrophoresed by SDS-PAGE which gave typical patterns in which alpha1 (I), alpha2 (I) chains, beta dimers and gamma trimers could be clearly distinguished (Figure 2.4a). Figure 2.4b shows the appearance of banding patterns of Coll (control), Coll-EDC/NHS (Lane 1), Coll-D-Ala-EDC/NHS (Lane 2), Coll-D-Glu-EDC/NHS (Lane 3) and Coll-D-Lys-EDC/NHS (Lane 4). Typical banding pattern disappear on D-Lys assisted EDC/NHS initiated crosslinking compared to D-Ala and D-Glu. This is covalent crosslinking of collagen with D-Lys-EDC/NHS.
Figure 2.4 (a) SDS-PAGE analysis of purified type I BAT collagen

Figure 2.4 (b) SDS-PAGE analysis of Coll-D-AAs
2.3.2 Turbidity Measurements

The fibril formation of collagen involves the aggregation and alignment of collagen molecules. Fibril formation can occur in vitro and it follows a nucleation–propagation mechanism. Fibril formation was monitored using a turbidity change at 313 nm (Figure 2.5) for collagen treated with EDC/NHS, D-Ala-EDC/NHS, D-Glu-EDC/NHS and D-Lys-EDC/NHS. The turbidities at the plateaus decreased with D-AAs-EDC/NHS.

![Figure 2.5 Turbidity measurement of Coll-D-AAs](image)

The introduction of D-Lys along with the EDC/NHS among collagen fibres during fibril formation also result in a further increase in the stability of collagen. The experimental results suggest that D-Lys plays a pivotal role in the self assembly process of EDC/NHS cross linked collagen fibrils.
2.2.3  CD Spectral Analysis

The triple-helical structure of type 1 collagen molecules cross linked with D-AAs-EDC/NHS was demonstrated by CD spectra. Collagen and collagen-like triple helical structures exhibit CD spectra in solution which are characterized by a large negative peak at 197 nm and a small positive peak around 220 nm. These features provide information about the presence of triple helical structures of collagen. Figure 2.6 represents the CD spectra of collagen samples treated with D-AAs-EDC/NHS and exhibit a positive peak at 220 and a negative peak at 197 nm. There are no significant changes in the CD spectrum of Coll-EDC/NHS, Coll-D-Ala-EDC/NHS, Coll-D-Glu-EDC/NHS and Coll-D-Lys-EDC/NHS compared to that of native collagen.

![CD spectra of native and Coll-D-AAs](image)

Figure 2.6  CD spectra of native and Coll-D-AAs
This may be due to the formation of aggregates. The cross linking of collagen solution with EDC/NHS seemed to be self terminating because the cross linking reaction did not proceed to precipitations. The effect of D-AAs on the CD spectra of collagen treated with EDC/NHS was also studied and is given in Figure 2.6 and Table 2.1. The CD spectra show an increase in peak intensity at 220 nm in the presence of D-Lys which could be due to the increase in tripe helical propensity of the structure.

**Table 2.1 Rpn values of native and Coll-D-AAs**

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>Rpn (characteristic ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coll</td>
<td>0.1006</td>
</tr>
<tr>
<td>Coll-EDC/NHS</td>
<td>0.1158</td>
</tr>
<tr>
<td>Coll-D-Ala-EDC/NHS</td>
<td>0.10377</td>
</tr>
<tr>
<td>Coll-D-Glu-EDC/NHS</td>
<td>0.9237</td>
</tr>
<tr>
<td>Coll-D-Lys-EDC/NHS</td>
<td>0.9822</td>
</tr>
</tbody>
</table>

However, these CD spectral shapes and peak position cannot be used conclusively to establish the presence of triple helical conformation. Rpn value denoting the ratio of positive peak intensity to negative peak intensity (absolute value) is also found to be useful in establishing the triple helical conformation. There is no significant change in the Rpn ratio (~ 0.1) between non-cross linked and cross linked collagen indicating that the triple helical structure is conserved after cross linking with EDC/NHS in the absence and presence of D-Lys.

**2.3.4 Viscosity Measurement**

Relative viscosity of collagen in the presence of D-AAs-EDC/NHS is shown as a plot in Figure 2.7. From the plot, it is clear that there is change
only a slight increase in the relative viscosity of collagen with D-Lys has been observed. But D-Ala and D-Glu decrease the relative viscosity of collagen.

Figure 2.7  A Plot of relative viscosity ($\eta/\eta_0$) against 1/R ($\eta$ and $\eta_0$ are the viscosity of collagen in the presence and absence of D-AAs). R = [collagen]/[D-AAs]

2.3.5 Mechanical Strength

The TS, % E and E of Coll-D-AAs matrices are given in Table 2.2. TS of the Coll-D-AAs matrices increased with the presence of different kinds of D-AAs with EDC/NHS. The % E too showed the same trend. Furthermore, Coll-D-Lys matrix leads to improved TS compared to native matrix. The E of the Coll-D-AAs matrices increases highly in contrast with native matrix. The Coll-D-Lys 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. The ratio of carboxyl groups to amino groups significantly affects the TS of matrix. According to the molecular structure, the amino and carboxyl groups of D-AAs and the collagen all provide possibilities for crosslinking. The statistical tests showed that there was significant difference in the TS between native and Coll-D-AAs matrices. The Coll-D-Lys matrix exhibited high TS and low % E where as
Coll-D-Ala and Coll-D-Glu matrix exhibited low Ts with high % E. And this is due to the fact that the fibre bundles are well separated in the case of D-Ala and D-Glu, while Coll-D-Lys matrix shows cemented fibres bundles as revealed by AFM studies. By D-AA's with EDC/NHS crosslinking, the biomechanical properties of Coll-D-AA's matrices are improved.

### Table 2.2  TMA, DSC and TGA properties

<table>
<thead>
<tr>
<th>Process</th>
<th>TS (MPa)</th>
<th>%E</th>
<th>E</th>
<th>T_d (C)</th>
<th>% Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coll</td>
<td>59±4*</td>
<td>48±3</td>
<td>87±8*</td>
<td>69±3</td>
<td>79±7</td>
</tr>
<tr>
<td>Coll-EDC/NHS</td>
<td>79±3*</td>
<td>48±4</td>
<td>131±5</td>
<td>73±5</td>
<td>79±5</td>
</tr>
<tr>
<td>Coll-D-Ala-EDC/NHS</td>
<td>120±2*</td>
<td>65±6</td>
<td>132±7*</td>
<td>74±4</td>
<td>74±4</td>
</tr>
<tr>
<td>Coll-D-Glu-EDC/NHS</td>
<td>160±6</td>
<td>75±4</td>
<td>160±7</td>
<td>84±3*</td>
<td>71±5</td>
</tr>
<tr>
<td>Coll-D-Lys-EDC/NHS</td>
<td>180±3*</td>
<td>80±9</td>
<td>170±4***</td>
<td>108±4*</td>
<td>64±6</td>
</tr>
</tbody>
</table>

### 2.3.6  DSC Analysis

The Td of Coll-D-AA's matrices were computed from DSC data (Figure 2.8) and also presented in Table 2.2. The Coll-D-AA's matrices exhibit an increase in the T_d values when compared to native matrix value. DSC curves for Coll-D-AA's matrices show characteristic endothermic transitions of structural changes of the collagen triple helix i.e., the protein denaturation. This may be due to a net increase in the number of inter and intra molecular crosslinks and interactions between the D-AA's with 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. The hydrophobic interactions determined predominantly by glycine residues and H-bonds formed between hyp residues play an important role in stabilization of collagen molecules. 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 matrix structure.
This could be possibly due to the decrease in the availability of the active sites in the matrices. The Td of matrices depends on stable intermolecular cross-links formed through the D-Lys than D-Ala and D-Glu.

### 2.3.7 TGA Analysis

The thermal behavior of Coll-D-AAs matrices were determined by TGA data and characterized by three stages of mass loss (Figure 2.9). The first one between 25 and 200°C is associated with release of water content of samples, the second around 200–400°C is due to the degradation of collagen molecule and the third that occurs in the range 400–650°C is related to the combustion of the residual organic components. The sample material weight of native and Coll-D-AAs matrices in the TG curves decreased with increasing temperature and reached a stationary value at about 185°C and then drastically decreased above 185°C.
Figure 2.9 TGA analyses of Coll-D-AAs matrices

The water contents of all, which lie in the region from 12 to 15%, is estimated from the weight loss at this stationary value. From this data, it can be observed that an initial thermal transitional change occurs between the temperature range of 180-500°C for all the collagen matrices, with maximum % transitional weight loss at 79.75, 79.59, 74.18, 71.56 and 64.79% (Table 2.2) for all the Coll-D-AAs matrices respectively. After the initial transition, a broad equilibrium peak occurs, even with an increase in temperature (200–300°C). The corresponding weight losses for all collagen matrices are no significantly different. This stage is associated with slow decomposition of matrices over a temperature range of 150–500°C. D-Lys decreases the weight loss of collagen matrix.

2.3.8 Water Uptake and Swelling Studies

Water-uptake and swelling ratio of Coll-D-AAs matrices are presented in Table 2.3. Water-uptake and swelling ratio of matrices were 98±03, 98±02, 99±09 and 98±08% and 80±2, 82±3, 85±3 and 75±4% at pH 7.4 respectively.
Table 2.3 Water uptake and swelling ratios

<table>
<thead>
<tr>
<th>Process</th>
<th>% Water uptake</th>
<th>% Swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coll-EDC/NHS</td>
<td>98±03**</td>
<td>80±2</td>
</tr>
<tr>
<td>Coll-D-Ala-EDC/NHS</td>
<td>98±02**</td>
<td>82±3</td>
</tr>
<tr>
<td>Coll-D-Glu-EDC/NHS</td>
<td>99±09**</td>
<td>85±3</td>
</tr>
<tr>
<td>Cool-D-Lys-EDC/NHS</td>
<td>98±08**</td>
<td>75±4</td>
</tr>
</tbody>
</table>

Although formation of Coll-D-Lys matrix can decrease more or less the amount of hydrophilic groups and also presented lower swelling ratio, this decrease should be mainly attributed to the partial diminishing of the hydrophilic groups of the scaffold after crosslinking. The initially existing hydrophilic groups, such as amino groups and carboxyl groups, were partially transformed into hydrophobic amide groups under the EDC/NHS treatment. Nevertheless, the swelling ratios of the Coll-D-AAs matrices are still big enough to meet the demand of the matrices used in tissue engineering. Consequently, both water uptake and swelling time can be controlled by characteristics of D-AAs.

2.3.9 FT-IR Analysis

The FT-IR spectral analysis determining the structural changes of Coll-D-AAs amide I, amide II and amide III band were shown (Figure 2.10). The spectra were scaled to equal absorption at 3350 cm\(^{-1}\), assigned to the \(-\text{CH}_2\) group, which remains unchanged during the crosslinking reaction. Crosslinking of matrices by EDC/NHS with presence of D-AAs results in an increased absorption at 1175 and 1050 cm\(^{-1}\) as compared to the native matrix. The FT-IR spectrum showed the retention of all the major three amide bands viz. I, II, and III in the region of 1600–1670 cm\(^{-1}\), 1510–1560 cm\(^{-1}\) and 1220–1450 cm\(^{-1}\) symmetric stretching of carboxylate salts region 1403 cm\(^{-1}\) and ester bond 1082 cm\(^{-1}\), which validated the integrity in the conformation of the collagen molecule.
Figure 2.10 FT-IR spectral analysis of native, EDC/NHS, D-Ala-EDC/NHS, D-Glu-EDC/NHS and D-Lys-EDC/NHS matrices

The absorbance at the region of collagen bend (1640-1690 cm$^{-1}$) decreased, characteristic region (1620-1630 cm$^{-1}$) was increased, as well as the increase of ester bond (1080 cm$^{-1}$), while the symmetric stretching of carboxylate salts (1403 cm$^{-1}$) was decreased compared to C-H bond (2958 cm$^{-1}$), indicating that the amidation reaction has occurred between carboxyl groups and available amino groups of Lys and Hyl residues.

2.3.10 SEM Analysis

The SEM images of Coll, Coll-EDC/NHS, Coll-D-Ala-EDC/NHS, Coll-D-Glu-EDC/NHS and Coll-D-Lys-EDC/NHS are shown in Figure 2.11. SEM image of control collagen matrix shows fibrous nature, even though pores are observed on surface and the pores are very less. The SEM image of Coll-EDC/NHS matrix shows excellent fibrous nature and multilayered matrix observed, porous nature is clearly visible. SEM image of Coll-D-Ala-EDC/NHS matrix shows fibrous nature alongwith pores. Some of the fibres
are aggregated and multilared matrix is also observed. The swollen fibrous nature is observed in the Coll-D-Glu-EDC/NHS matrix. The sample exhibited less number of pores at the swollen fibres and aggregated nature. The multilayered fibrous matrix is observed with porous nature. Most of the fibres are aggregated. The entire sample except control matrix exhibited multilayered fibrous matrix with pores. However, the control matrix shows fibrous nature with less number of pores.

Figure 2.11 (Continued)
(c) Coll-D-Ala-EDC/NHS

(d) Coll-D-Glu-EDC/NHS

Figure 2.11 (Continued)
Figure 2.11 Morphological characterization of SEM analysis of Coll-D-AAs matrices

2.3.11 AFM Analysis

Figure 2.12 shows the plane and three-dimensional profiles of the Coll-D-AAs matrices surfaces. Lighter areas of the images correspond to higher topography and darker areas correspond to lower topography. EDC/NHS crosslinked Coll-D-AAs matrices have been attempted for the first time in this present study. EDC/NHS initiated crosslinked Coll-D-AAs matrices show a rougher texture with higher peaks than native matrix. Crosslinking by D-AAs is thought to be the reason for the increased stability of the matrix that is manifested by higher TS. The Coll-D-AAs matrices clearly formed fibrils in the presence of D-AAs as shown by the AFM images where the typical collagen banding pattern of grooves and ridges.
(a) Coll

Figure 2.12 (Continued)
(b) Coll-EDC/NHS

Figure 2.12 (Continued)
Figure 2.12 (Continued)

(c) Coll-D-Ala+EDC/NHS
(e) Coll-D-Glu+EDC/NHS

Figure 2.12 (Continued)
(e) Coll-D-Lys-EDC/NHS

Figure 2.12 Morphological characterization of AFM analysis of Coll-D-AAs matrices
The ability of the collagen monomers to align in a quarter-stagger arrangement in the presence of D-Ala shows that the size of these molecules does not hinder the formation of collagen fibrils. Although it has been shown that D-Glu results in the formation of reactive adducts of some AA residues found in collagen, which in turn may result in the formation of inter and intramolecular cross-links between collagen molecules, it does not appear to affect the structure or the arrangement of the collagen fibrils.

A similar study performed with D-Lys showed that though D-Lys helped form the quarter-stagger structure and induced a fibrous network of aggregated collagen molecules that lacked the D-periodicity, possibly because of size constraints. The presence of D-Lys during lateral assembly of collagen decreased the rate of lateral disassembly thus helping a faster net rate of stable fibril formation. In the present study with D-Lys, no increase in collagen molecules lacking D-periodicity or a fibrous aggregated network was observed. On the contrary, AFM analysis of collagen fibrils cross-linked with D-Lys revealed a well-ordered structure with the fibrils properly oriented and well aligned compared to collagen fibrils in the absence of D-Lys.

### 2.3.12 Biodegradation Against ChC

% Biodegradation (based on hyp released) of the Coll-D-AAs matrices by ChC at different D-AAs and various concentration has been determined (Figure 2.13 (a) and (b)). The EDC/NHS, Coll-D-Ala, Coll-D-Glu and Coll-D-Lys matrices exhibited 23±3, 17±2, 80±6 and 9±3% degradation of the matrices as against 93% degradation in the case of native matrix at 96 h period of incubation. Addition of neutral AA, D-Ala, into the matrix, biodegradation degree was slightly reduced. The lowest biodegradation degree, 9±3%, that corresponds the best biostability was achieved for the Coll-D-Lys matrix. A surprising result was got with the collagen matrix treated with D-Glu. The addition of D-Glu has deteriorated the stability
comparing with EDC/NHS treated matrix. D-Glu, D-Ala and D-Lys, that contain 1/2, 1/1 and 2/1 of NH₂/COOH groups respectively were selected as bridging agents to enhance the stability to collagen matrix. The stability of Coll-D-AAs matrices against ChC would have been brought about by protecting the active sites in collagen through interaction with D-AAs recognized by ChC. It has to be noted that a higher biostability was observed for D-Lys than D-Ala and D-Glu. The D-Lys can interact with collagen through multipoint peptide, H-bonding and hydrophobic interactions. The di-amino groups of the D-Lys molecules, likely crosslink with collagen may establish multiple peptide and H-bonds with neighboring collagen molecules, resulting in improved stability of matrix and prevent the free access of ChC to reactive sites on the collagen chains. The stability of Coll-D-Lys matrix against ChC would have been brought about by protecting the active sites in collagen. The significant differences in the enzymatic stability offered by D-Lys could be due to the effectiveness of the latter in exhibiting better crosslinking with collagen through multiple peptide bond and H-bonded inter and intramolecular crosslinks.

2.3.13 Anti-microbial Activity

The antimicrobial activity of Coll, Coll-EDC/NHS, Coll-D-Ala-EDC/NHS, Coll-D-Glu-EDC/NHS, Coll-D-Lys-EDC/NHS matrices determined by disc diffusion assay showed a zone of inhibition for E. coli (6±2, 14±3, 17±2 mm) and B. Subtilis (7±3, 13±3, 16±5 mm) after 24 h. These D-AAs are very useful for biomaterials stabilization. The matrices that have been crosslinked with D-Lys demonstrate resistance to microbes than D-Ala and D-Glu. In vitro antimicrobial study showing the activity of Coll-D-AAs matrices against bacterial species such as E. coli and B. Subtilis are presented in Table 2.4.
Figure 2.13 Biodegradation degree of (a) Coll-AAs matrices and (b) various concentrations of D-AAs

2.3.14 Cell Viability

The fibroblasts in direct contact with Coll-D-AAs matrices showed typical morphology compatible with their surroundings, as presented (Figure 2.14). No abnormal morphology or cellular lysis was detected.
The structural appearance of cultured fibroblasts on Coll-D-AAs matrices showed linear patterns of arrangement with the cells oriented towards the edges of the membrane. Further, the cultured fibroblasts on the Coll-D-AAs matrices showed a regular arrangement with a tightly packed striated and spindle shape appearance. On Coll-D-AAs matrices, cells exhibited better proliferation than those on the native collagen matrix. This indicates that the Coll-D-lys matrix has potential for tissue engineering applications.

2.3.15 Cell Adhesion

The D-AAs could improve the cell adhesion to the surface of collagen matrix. After D-AAs are introduced into the matrix, the cell-adhesion ratio on Coll-D-Lys matrix can be observed to increase significantly (Figure 2.15 and Table 2.4).
Table 2.4 Cell viability and antimicrobial activity of Coll-D-AAs matrices

<table>
<thead>
<tr>
<th>Process</th>
<th>% Cell viability 24h</th>
<th>% Cell viability 48h</th>
<th>Zone of inhibition (mm) E. coli</th>
<th>Zone of inhibition (mm) B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coll</td>
<td>98±4*</td>
<td>100±4*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coll-EDC/NHS</td>
<td>78±6*</td>
<td>99±6*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coll-D-Ala-EDC/NHS</td>
<td>75±2</td>
<td>87±6</td>
<td>6±2</td>
<td>7±3</td>
</tr>
<tr>
<td>Coll-D-Glu-EDC/NHS</td>
<td>79±4</td>
<td>87±4</td>
<td>14±3</td>
<td>13±3</td>
</tr>
<tr>
<td>Coll-D-Lys-EDC/NHS</td>
<td>95±9</td>
<td>99±5</td>
<td>17±2</td>
<td>16±5</td>
</tr>
</tbody>
</table>

D-AAs exhibited more fibroblast adhesion than EDC/NHS crosslinked collagen matrix. It is interesting to note that fibroblast adhesion to Coll-D-AAs matrices demonstrated similar trend to native matrix. It suggests that D-Lys may induce the adhesion and spreading of fibroblasts than D-Ala and D-Glu.

Figure 2.15 Biocompatibility analysis of adhesion % of Coll-D-AAs matrices

2.4 DISCUSSION

Unnatural AAs, utilized as building blocks, molecular scaffolds, composite matrix, represent a nearly infinite array of diverse structural components for the development of new leads in peptidic and non-peptidic compounds (Oh et al 1999). The D-AAs are used to design and obtain new
peptidomimetics by their incorporation in place of the natural L-AAs, either at a specific position, or throughout the whole peptide (Latacz et al 2006). The potential of poly-α-AAs as biomaterials have been investigated for possible use in a wide variety of biomedical applications such as tissue engineering and drug carriers (Anderson et al 1974; Dickinson et al 1981; Anderson et al 1985). Though synthetic peptides formed of natural AAs dominate applications in a wide variety of fields, it is largely felt that sooner the trend will favor peptides formed of unnatural AAs. This is because peptides with novel characteristics are possible only by incorporating unnatural AAs. The D-AAs can be incorporated into proteins in order to study protein structure and function (Haack et al 1997). They have also been used to create drugs or other therapeutic agents that will not be degraded as quickly as natural AAs by proteolytic activity. Structure based designs of non-natural amino-acid inhibitors of amyloid fibril formation have been studied (Sievers et al 2011). Non-canonical AAs in protein polymer design has been reviewed for development of biosensors, novel surfaces and materials (So et al 1998; Magliery et al 2005; Connor et al 2007). Fabrication of self-assembling D-form peptide nanofiber scaffold has been studied for rapid hemostasis and clinical applications (Luo et al 2011). Improvement on the structural consequences of D-AAs in collagen triple-helical peptides have been studied (Shah et al 1999; Horng et al 2007). Pharmaceutical application of D-AAs dipeptides has been studied for improving the peptide stability and controlled drug release (Liang et al 2009; Luca et al 2010). The effect of the D-AAs used as a novel crosslinking bridge on enhancing the ability of collagen matrix to mechanical strength and thermal stability, and resist the collagenases biodegradation and biocompatibility well documented.
AAs

<table>
<thead>
<tr>
<th></th>
<th>L-Configuration</th>
<th>D-Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td><img src="image" alt="Ala L-Configuration" /></td>
<td><img src="image" alt="Ala D-Configuration" /></td>
</tr>
<tr>
<td>Glu</td>
<td><img src="image" alt="Glu L-Configuration" /></td>
<td><img src="image" alt="Glu D-Configuration" /></td>
</tr>
<tr>
<td>Lys</td>
<td><img src="image" alt="Lys L-Configuration" /></td>
<td><img src="image" alt="Lys D-Configuration" /></td>
</tr>
</tbody>
</table>

**Figure 2.16 Schematic representation of stereochemistry of D-AAs**

In this study, collagen matrix was stabilized by EDC/NHS in the presence of D-AAs. The D-AAs, i.e. D-Asp/Glu, D-Ala and D-Lys, that contain 1/2, 1/1 and 2/1 of NH₂/COOH groups respectively were selected as bridging agents to enhance the stability to collagen matrix (Figure 2.16). Schematic representations of collagen side chain crosslinking with D-AAs leading to formation of peptide bond are presented (Figure 2.17). As expected, enhanced mechanical, thermal stability and resistance to ChC activity for the matrix can be surely gained when the cross-linking is conducted in the presence of basic D-Lys. However, the surprising results were got in the presence of D-Ala or D-Asp/Glu. The former has less efficiency to improve the stability of the matrix and the latter even results in a worse stability. The possible reason of the different results is the different self-condensation ability of the D-AAs. Then the cross-linking degree between the collagen molecules shall be greatly affected. To clarify if the self-condensation is the main reason causing the worse stability in case of D-
Glu, the concentrations of D-Glu were varied and their corresponding model compounds, D-Asp, were employed.

Figure 2.17 Schematic representation of side chain crosslinking of collagen with D-AAs

Both the similar effect of Asp on resulting in a worse stability and the greatly excessive quantity of EDC/NHS indicate that the self-condensation of AA is not the main reason to weaken the cross-linking efficiency. Another possible reason is that the adding D-AAs changed the ratio of NH$_2$/COOH in the crosslinking. There are two competing reactions in the cross-linking system: the bridging linking between collagen molecules and the blocking of the functional groups of the collagen matrix by under the assistance of the added D-AAs. The ratio of NH$_2$/COOH will determine which kind of reaction is dominant.
In general, the ratio of NH$_2$/COOH is about 2:3 in the BAT collagen. This is understandable that over existence of one kind of group shall decrease the general cross-linking efficiency, leading to poor stability. For example, addition of Asp/Glu shall introduce more COOH groups than NH$_2$ groups, yielding far excess of COOH groups, which shall cause a tendency that one Glu molecule can just react with one amino group, so that the Glu not only loses the function of cross-linking bridge but also blocks a large amount of functional groups in the collagen molecules. As a result, the cross-linking efficiency is decreased. In contrast to D-Glu, the addition of D-Lys can amend this unbalance of NH$_2$/COOH ratio closing to a constant, for which the biodegradation degree tends to be a constant with increasing D-Lys concentration, resulting in higher cross-linking efficiency. It is easy to understand that the biodegradation degree will decrease with increasing concentration of D-Lys. This can explain that D-Lys can enhance the mechanical strength and thermal stability and resistance to collagenolytic activity in a wide range of concentrations. Representative products isopeptide formed from reactions between collagen Lys and Glu side chain with L-AAs and D-AAs such Ala, Glu and Lys are shown (Figure 2.18). The side chain crosslinking of D-AAs into polypeptide imposes local conformational constraints. To devise strategies for using collagen in the development of advanced biomaterials for biomedical engineering, it is necessary to confer resistance to proteolytic (collagenase) degradation with chemical or physical crosslinking strategies. The MMPs are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for remodeling and degradation of the ECM and also MMPs-1, 8, 13, 18 (collagenase I, II, III and IV) are capable of degrading native interstitial collagens I, II and III and at the cellular surface producing changes in cellular behavior and subsequent pathological responses (Jones et al 2008; Anderson et al 2008).
Figure 2.18 Proposed structure of side chain crosslinking of collagen with D-Ala, D-Glu and D-Lys. The result of crosslinking via amino and carboxyl of two collagen like peptide are directly joined via an isopeptide bond playing the major role in stabilization of collagen
The D-AAs can crosslink with collagen through peptide and multipoint H-bond interactions resulting in improved stability of collagen scaffold could prevent the free access of MMPs to reactive sites on the collagen chains. Based on the reported molecular docking evidence, D-Lys efficiently crosslink with collagen. Most importantly, multipoint H-bond and hydrophobic interactions of D-Lys greatly interact the collagen than D-Ala and D-Glu. The D-AAs along a peptide chain may be less digestible than the L-forms. D-D, D-L and L-D form of peptide bonds partly or fully inaccessible to proteolytic activities has been studied (Luca et al 2010; Hamamoto et al 2002). The proteolytic machinery is not well equipped to deal with D-AAs polypeptides, however, and it is this finding above all else that has spurned research into stereochemical and directional manipulation of peptide chains. The expectation has been that systematic inversion of the stereochemistry at the peptide backbone α-carbon atoms, if accompanied by chain reversal, should yield proteolytically stable retro-inverso peptide isomers. These D-AAs derivatives form the basis of important families of enzyme inhibitors (Hong et al 1999; Fischer et al 2003). The study and design of the collagen matrix largely relies on D-AAs possessing non-natural structures or non-natural absolute configurations. The crosslinked matrix could have increased application as in vivo stability in tissue and increased selectivity in biological response.

Furthermore, the good biocompatibility of D-AAs, accompanied by the improvement of the mechanical, thermal and biological stability, a better preservation of the original biocompatibility of collagen matrix can be expected. SEM was used for revealing the influence of EDC/NHS treatment on the microstructure. The results indicate that the collagen matrix could preserve the structure during the cross-linking treatment in the presence of D-AAs. It is a crucial aspect to evaluate a cross-linking method to be used to modify a tissue engineering scaffold. Cell culture results demonstrate that the
ability to induce cell infiltration and growth of the original collagen matrix have been preserved after cross-linking treatment in the presence of D-Lys. The addition of D-Lys in the collagen matrix would not decrease the cytocompatibility of the collagen matrix. Hyaluronic acid-poly-D-Lys-based three-dimensional hydrogel for traumatic brain injury have been studied (Tian et al 2005). The synthesis of D-trihydroxyllysine-based oligopeptides as a hydrophilic scaffold and its application to the synthesis of bi-functional chelating agents for use as bone tracers has been studied (Tanaka et al 2008). D-Lys is an unnatural AA that is positively charged and widely could be used as a coating to enhance cell attachment and adhesion, growth and differentiation of many cell types. This matrix is resistant to enzymatic degradation and has been used to culture a wide variety of cell types. This matrix demonstrates a higher resistance to microbial activities than conventional method. Using this D-configuration of unnatural AA tools for crosslinking of biomaterial and collagen based biomaterial is an effective method for design of scaffold for tissue engineering and regenerative medical applications that are inaccessible to proteolytic activity.

2.5 CONCLUSIONS

The Coll-D-AA matrices were shown here that improved TS, T_D, structural integrity, swelling and water uptake properties, biocompatibility and effective in the prevention of matrices degradation by collagenolytic activity. The D-AAs stabilize the collagen matrix through covalent and non–covalent bonds such as carboxamide and H-bonding interaction, this might have lead to deactivation of ChC. The collagen functional groups, to which D-AAs are bound via multipoint H-bonds and play the main role during collagen stabilization. Also, Coll-D-AA matrices in the blended networks promote a reduction in both swelling and biodegradation. The Coll-D-AA matrices are more biostable and, hence may find use in the preparation of collagen scaffolds for tissue engineering and regenerative medical applications.