Enhancing Amine Terminals in an Amine-Deprived Collagen Matrix

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Collagen, though widely used as a core biomaterial in many clinical applications, is often limited by its rapid degradability which prevents full exploitation of its potential *in vivo*. Polyamidoamine (PAMAM) dendrimer, a highly branched macromolecule, possesses versatile multiterminal amine surface groups that enable them to be tethered to collagen molecules and enhance their potential. In this study, we hypothesized that incorporation of PAMAM dendrimer in a collagen matrix through cross-linking will result in a durable, cross-linked collagen biomaterial with free $-NH_2$ groups available for further multi-biomolecular tethering. The aim of this study was to assess the physicochemical properties of a G1 PAMAM cross-linked collagen matrix and its cellular sustainability *in vitro*. Different amounts of G1 PAMAM dendrimer (5 or 10 mg) were integrated into bovine-derived collagen matrices through a cross-linking process, mediated by 5 or 25 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 5 mM *N*-hydroxysuccinimide (NHS) and 50 mM 2-morpholinoethane sulfonic acid buffer at pH 5.5. The physicochemical properties of resultant matrices were investigated with scanning electron microscopy (SEM), collagenase degradation assay, differential scanning calorimetry (DSC), Fourier transform infrared (FTIR) spectra, and ninhydrin assay. Cellular sustainability of the matrices was assessed with Alamar Blue assay and SEM. There was no significant difference in cellular behavior between the treated and nontreated groups. However, the benefit of incorporating PAMAM in the cross-linking reaction was limited when higher concentrations of either agent were used. These results confirm the hypothesis that PAMAM dendrimer can be incorporated in the collagen cross-linking process in order to modulate the properties of the resulting cross-linked collagen biomaterial with free $-NH_2$ groups available for multi-biomolecular tethering.

Introduction

Collagen constitutes about 30% of the total body protein with the majority representing structural support to dermis, bone, cartilage, tendons, and ligaments.[1] Among the existing collagens, collagen type I is the most abundant and by far the most commonly used natural polymer for use in tissue engineering scaffold applications.[1−11] It is versatile and can be processed to various forms such as gels, sponges, membranes, films, tubes, powders, and microspheres.[9] Its applications range from skin substitutes and cartilage regeneration to corneal repair.[2−8,10,11] Collagen offers a wide range of advantages such as cytocompatibility and known structural, physical, chemical, and immunological properties.[12] Its disadvantages, however, are its poor mechanical properties and rapid degradability *in vivo*.[13]

As a natural material, collagen can be modified to overcome these drawbacks through fabrication by various cross-linking techniques. The formation of intra- and intermolecular cross-links between collagen molecules in macromolecular fibrils with cross-linkers has been shown to be an effective method to improve mechanical integrity and biological stability and reduce the antigenicity of collagen.[14−16] Collagen can be cross-linked by either physical or chemical means. Each of these different methods serves a different purpose for the type of scaffold application in tissue engineering. Physical cross-linking involves exposure to ultraviolet, or gamma irradiation, or dehydrothermal (thermal dehydration) treatment.[16−18] Although avoiding the formation of potential cytotoxic chemical residues, physical cross-linking is often insufficient in generating high degrees of cross-linking for tissue engineering applications.[19] Chemical cross-linking procedures involve bifunctional reagents containing reactive groups such as aldehydes (e.g., glutaraldehyde), diisocyanates (e.g., hexamethylene diisocyanate), and epoxy compounds (e.g., polyglycidyl ethers) or involve activation of carboxylic acid

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groups followed by reaction with amino groups (e.g., cyanamide (carbodiimide (e.g., 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC) or acyl azide (hydrazine or diphenylphosphorylazide)).[15,16,20−25] Recently, an alternative collagen cross-linking method which uses the enzyme microbial transglutaminase has also been described.[26−30]

Many of these chemical cross-linkers, including glutaraldehyde, hexamethylene diisocyanate, and acyl azide, are "bridge-linking", meaning that the collagen molecules are linked by integration of chemical intermediaries, bridging the collagen molecules.[12] Unlike these "bridge-linking" cross-linkers, EDC forms "zero-length" cross-links between collagen molecules by mediating direct covalent amide bond formation between its reactive amino acids side groups.[23] This mechanism involves the activation of carboxylic groups (−COOH) of glutamic and aspartic acid residues and the formation of amide bonds (−CONH−) in the presence of free amine groups ($-NH_2$) of lysine or hydroxylysine residues.[23] This reaction is further enhanced by the addition of *N*-hydrosuccinimide (NHS) which prevents the hydrolysis of activated carboxyl groups.[23,31,32]

EDC cross-linking, however, has two noticeable disadvantages: its cytotoxicity at high concentration and dependency on the limited number of $-NH_2$ groups in collagen for cross-linking reaction.[12,13] Although EDC cytotoxicity is less pertinent than "bridge-linking" cross-linkers, its cytotoxic effect has been reported by Powell and Boyce.[13] The cytotoxicity was seen when high concentrations greater than 10 mM EDC were used for cross-linking.[13]

The ratio of $-NH_2$ groups to −COOH groups in collagen varies from 1:4 to 2:3.[12,19] The amounts of $-NH_2$ groups and −COOH groups in 2.5 mg collagen scaffold are 1.3 and 2 μmol, respectively.[19] Because of the dependency of EDC reaction on $-NH_2$ groups in collagen, the addition of functional free $-NH_2$ groups to collagen during cross-linking with water-soluble EDC solution will provide the required number of $-NH_2$ groups necessary to enhance the cross-linking density of collagen. Multifunctional dendrimers with multiterminal free amine groups, such as polypropyleneimine dendrimer, have been successfully used to enhance collagen cross-linking.[12] Molecules such as diamines, triamines, tetraamines, and polylysine have also been used to serve such purpose with variable success.[12,19,33] The reason for the different effects from these amine sources is unclear, with a possible explanation such as the relatively short size and the inadequacy of diamines to supply free amine groups being considered.[12] Other potential factors include the possibility of selective interaction of intrinsic amine groups from amino acids with the activated carboxyl groups, or the accessibility of the amine groups provided by amine source supplements for the cross-linking reaction.[19] In addition, the extra $-NH_2$ groups provided by the dendrimer also enable tethering of bioactive substrates to the cross-linked collagen.[34]

Polyamidoamine (PAMAM) dendrimer is a spheroidal polymer composed of repeating polyamidoamine units in a starlike cascade, and a highly branched, amine terminal surface.[35,36] It is the first complete dendrimer family synthesized, characterized, and commercialized.[35] PAMAM dendrimer has long been used in the biomedical field due to its low cytotoxicity level, perfectly controllable size, and ease of modification.[37−41] The dendrimer is well recognized for functionalization of a variety of polymers through its addition to polymer surfaces or into polymer blocks.[34,42,43] Dendrimer nanotechnology presents an exciting frontier of biofunctionalization of many polymers for biomedical applications such as imaging, peptide and gene transfection strategies, among others.[44−53] With a size of approximately 1.1 nm, generation 1 (G1) PAMAM dendrimer with its eight terminal $-NH_2$ groups should theoretically provide an ideal free $-NH_2$ group supply for cross-linking and surface modification of collagen polypeptides by acting as a flexible nanoscaffold.[12,34,54,55] Its small size and non-overcrowding eight terminal $-NH_2$ groups are more accessible and present less of a sterical issue compared to higher generation species.[55−59] In addition,

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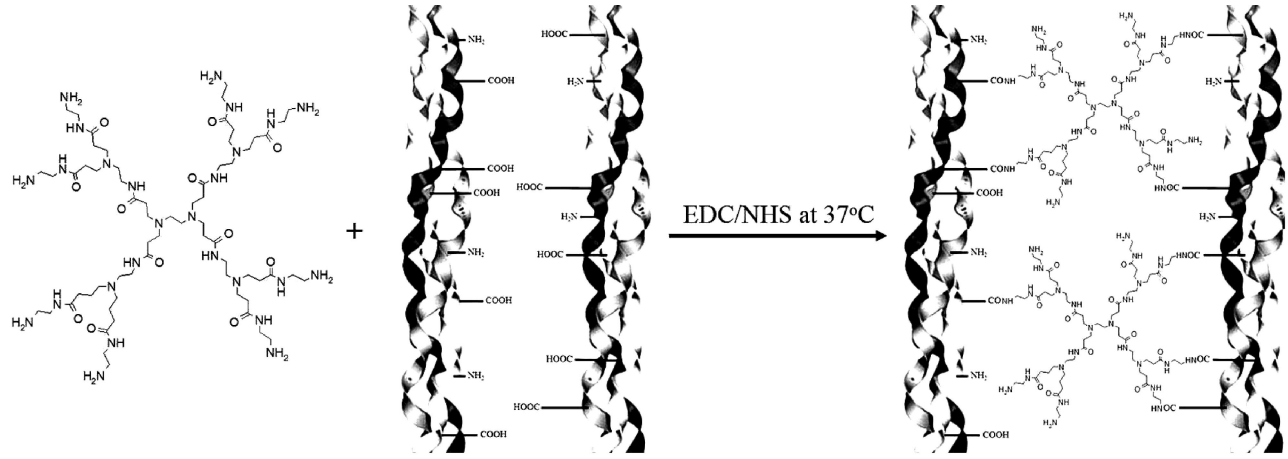
Scheme 1. Generation 1 (G1) PAMAM Dendrimer Incorporation in Carbodiimide Cross-linking of Collagen



PAMAM dendrimer of equivalent surface functionality has also been shown to be less toxic than polypropyleneimine dendrimer with the same number of surface groups.[36,40,56] For the above reasons, incorporation of G1 PAMAM dendrimer in cross-linking collagen, its physicochemical properties, and its *in vitro* cellular sustainability have been examined in this study. The cross-linking reaction was mediated by conventional EDC methods, through the activation of −COOH groups followed by −CONH− bond formation with the free $-NH_2$ groups (Scheme 1). It is hypothesized that the successful incorporation of G1 PAMAM dendrimer will result in a cross-linked collagen biomaterial with free $-NH_2$ groups available for further multi-biomolecular tethering.

Materials and Experimental Techniques

All the materials and reagents used in these experiments were supplied by Sigma-Aldrich (Tallaght, Dublin) unless otherwise stated. The source of all water used was Millipore deionized water (Elix S, Automatic Sanitization Module, AGB Scientific Ltd.), and sterilization was carried out using an autoclave at 121 °C for 1 h. The PAMAM dendrimer used in this study was G1 PAMAM solution in methanol from Sigma-Aldrich (Tallaght, Dublin).

Collagen Isolation. Insoluble type I collagen was isolated from bovine Achilles tendon using neutral salt and 0.5 M acetic acid extraction methods as previously described.[60] Collagen purity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Sircol collagen assay methods. The collagen preparation was essentially free of other proteins.

Preparation of Collagen Gels. Collagen solutions (0.3 (wt/vol) %) were prepared by dissolving 3 mg/mL collagen in 0.5 M acetic acid at pH 2.5 and then incubated at 4 °C for 16 h. This solution was then homogenized using a BarBoss blender and filtrated with a nylon 90 μm gauze filter (TG 100, Schleicher and Schuell, Dassel, Germany). The filtered collagen solution was deaerated under vacuum to remove the entrapped air content. Using a 24-well tissue culture plate, 1 mL of solubilized collagen was added to each well, and the pH was adjusted to 7.5 by using phosphate buffered saline and 1 M sodium hydroxide. The pH-adjusted collagen solution was then incubated at 37 °C overnight to complete the gelling process.

Table 1. Terminology of Collagen Matrices Used in This Study

terminology	collagen matrix cross-linking specifics
NC	non-cross-linked
EC-L	5 mM EDC alone/5 mM NHS
PC-L5	5 mg of G1 PAMAM with 5 mM EDC/5 mM NHS
PC-L10	10 mg of G1 PAMAM with 5 mM EDC/5 mM NHS
EC-H	25 mM EDC/5 mM NHS
PC-H5	5 mg of G1 PAMAM with 25 mM EDC/5 mM NHS
PC-H10	10 mg of G1 PAMAM with 25 mM EDC/5 mM NHS

Cross-Linking of Collagen Gels. Collagen gels were cross-linked with PAMAM dendrimer in the presence of EDC/NHS. Non-cross-linked collagen gels and collagen gels cross-linked with EDC alone in the presence of NHS were used as controls. For preparation of the EDC cross-linked collagen, the gels were added into precooled glass vials with either 5 or 25 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 5 mM *N*-hydroxysuccinimide (NHS) in 50 mM 2-morpholinoethane sulfonic acid (MES) buffer solution (pH 5.5). The pH was adjusted to 5.5 with 0.1 N NaOH and/or 0.1 N HCl, and the resultant solution was incubated in an oven at 37 °C overnight.

For preparation of the dendrimeric cross-linked collagen, the gels were added into precooled 24-well tissue culture plates containing G1 PAMAM (5 or 10 mg) with either 5 or 25 mM solution of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 5 mM *N*-hydroxysuccinimide (NHS) in 50 mM MES buffer solution (pH 5.5). The pH was adjusted to 5.5 with 0.1 N NaOH and/or 0.1 N HCl, and the resultant solution was incubated in an oven at 37 °C overnight.

Finally, the gels were washed with distilled water for 1 h with three changes of distilled water. All collagen gels were lyophilized using a freeze-dryer (VirTis Advantage, Wizard 20 freeze-dryer, SP Industries, New York, NY) prior to characterization. Table 1 denotes the terminology of resultant collagen matrices used in this study. Table 2 indicates the molar amount of $-NH_2$ and −COOH groups in each collagen matrix with their PAMAM dendrimer and EDC, and the NH_2/COOH reactive ratio.

Characterization of Matrices. *Structural Morphology of Matrices.* Collagen matrices were observed under scanning electron microscopy (SEM) to evaluate structural changes before and after cross-linking. All samples were mounted on carbon pads attached to aluminum stubs and gold coated (Emitech K550 sputter coater, Emitech Limited, Ashford, Kent, U.K.) before analysis with the scanning electron microscope (Hitachi scanning electron microscope S-4700, Hitachi-Hisco Europe GmbH, Berkshire, U.K.).

Enzymatic Degradative Resistance. Collagenase assay was performed to examine the extent of the cross-linking in the matrices to provide indication of their biological stability. Degradation of all

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Table 2. Molar Amount of $-NH_2$ and $-COOH$ Groups in Collagen Matrices, PAMAM and EDC, and Their NH_2/COOH Reactive Ratio

collagen matrix	$-NH_2$ (μmol)		$-NH_{2\,(total)}$ (μmol)	$-COOH$ (μmol)	EDC (μmol)	$NH_{2\,(total)}$/COOH ratio
	collagen	PAMAM				
NC	1.6		1.6	2.4		0.66
EC-L	1.6		1.6	2.4	5.0	0.66
PC-L5	1.6	28.0	29.6	2.4	5.0	12.3
PC-L10	1.6	56.0	57.6	2.4	5.0	24.0
EC-H	1.6		1.6	2.4	25.0	0.66
PC-H5	1.6	28.0	29.6	2.4	25.0	12.3
PC-H10	1.6	56.0	57.6	2.4	25.0	24.0

matrices was carried out with collagenase IA from *Clostridium histolyticum* with collagenase activity of 10 units/mg. Collagen matrices of 3 mg were incubated for 24 h to characterize the degradation profile among different matrices. Each matrix was immersed and incubated in 1 mL of collagenase solution containing 0.1 M Trizma hydrochloride (Tris-HCl) and 50 mM calcium chloride at 37 °C. The reaction was stopped by addition of 0.25 M ethylenediaminetetraacetic acid followed by cooling on ice. The undigested samples were weighed to ascertain their remaining weight.

Thermal Stability. The shrink temperature (T_s) of the collagen matrices was determined by using a differential scanning calorimetry (DSC; DSC-60, Shimadzu, Japan) instrument. The shrink temperature provides information about the cross-linking density of collagen matrices. Collagen matrices (3 mg) were heated in hermetic pans at 5 °C/min from 0 to 105 °C while the endothermic peak of the thermogram were monitored and recorded.

Collagen IR Spectra. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was used to determine the structural and conformational changes in collagen matrices after cross-linking. Each spectrum was obtained with FTIR spectroscopy (Shimadzu FTIR-8600, Shimadzu, Japan). An average of 40 scans between 4000 and 500 cm^{-1} wavenumber was recorded for each spectrum.

Amine Group Content. During cross-linking, amine groups react with carboxylic groups. Thus, the higher the cross-link density, the lower the free amine group content remaining after cross-linking. To assess the degree of collagen cross-linking and the availability of unreacted $-NH_2$ groups, the free $-NH_2$ group content of collagen matrices was determined spectrophotometrically using the ninhydrin assay.[61] All matrices were washed thoroughly and incubated with water in order to remove unreacted dendrimer. The absorbance measurement was taken at wavelength 570 nm with a spectrophotometer (Nanodrop ND-1000 spectrophotometer, Labtech International, U.K.).

Cell Viability. Cell viability on collagen matrices was evaluated based on their morphology and metabolic activity. Human fetal foreskin fibroblasts (HFFFs; CAMR, Porton Down, U.K.) were seeded at a density of 4×10^4 cells on matrices in a 24-well plate using Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin−streptomycin. The cells were maintained at 37 °C in a 5% CO_2 humidified incubator.

Cell Morphology. Cells seeded on collagen matrices were visualized using SEM at 500× magnification after a 7 day incubation period. The samples were fixed with 2.5% glutaraldehyde (Agar Scientific Ltd., Cambridge) in 0.1 M sodium cacodylate (sodium cacodylate trihydrate) for 2 h at room temperature and were subsequently immersed in increasing concentrations of ethanol/water (50%, 75%, 80%, 90%, and 100%) for 5 min periods, followed by their immersion in hexamethyldisilazane for 30 min. All samples were mounted on carbon pads attached to aluminum stubs and gold-coated (Emitech K550 Sputter Coater, Emitech Limited, Ashford, Kent, U.K.) before visualization via SEM (Hitachi scanning electron microscope S-4700, Hitachi-Hisco Europe GmbH, Berkshire, U.K.).

Cell Metabolic Activity. Alamar Blue (Biosource, U.K.) assay was used in the evaluation of cellular metabolic activity. The culture media was changed daily, and the assays were taken on day 3, 5, and 7. Fluorescence emission was measured using a microplate fluorescence reader (FLx800 microplate fluorescence reader, BIOTEK Instruments, Inc.) with excitation and emission wavelengths of 530 and 590 nm, respectively.

Statistical Analysis. Numerical data are expressed as mean ± standard deviation (SD). Analysis was performed using statistical software (MINITAB version 13.32, Minitab, Inc.) with one-way analysis of variance (ANOVA) for evaluation of data and posthoc differences between groups identified using the Tukey pairwise comparison test. A p-value of less than 0.05 was considered statistically significant.

Results

Characterization of Matrices. *Structural Morphology of Matrices.* Scanning electron microscopy images of the surfaces and cross sections of the collagen matrices at 1000× and 500× magnifications, respectively, showed more collagen fibers to be fused with less defined fibrillar architecture on the cross-linked collagen matrices (Figure 1). In the case of cross-linked collagen matrices, collagen fibers fused and formed a sheet of collagen on the surface but this transformation was not uniform throughout the whole depth of the matrices. All matrices remained porous as seen in the cross section (Figure 1). There was no obvious structural difference between collagen matrices cross-linked with EDC alone or with the addition of PAMAM. The difference in concentration used for cross-linking between each matrix also did not appear to affect their physical morphology. This implies that the incorporation of PAMAM with its bridge-linking effect did not physically interfere with the EDC cross-linking mechanism.

Enzymatic Degradative Resistance. The collagenase degradation study gave an indication of the biological stability of the matrices from enzymatic degradation. The extent of cross-linking, as reflected by resistance to enzymatic degradation, was quantified by the percentage of weight loss after 24 h of degradation. The results were expressed as percentages of degraded collagen relative to non-cross-linked matrices (Figure 2). The non-cross-linked collagen matrices (NC) were completely degraded by 24 h. After cross-linking, the degradation of matrices varied, depending on the combination and concentrations of the cross-linkers used.

Collagenase adsorbed into the collagen fibers cleaves the helical segment. Cross-linking maintains this helical structure and, hence, extends the stability of the collagen. For EC-L matrices (cross-linked with a low concentration (5 mM) of EDC), approximately 76.4% of the samples were degraded after 24 h. At 25 mM concentration of EDC, EC-H matrices were degraded by 59.7%, which is significantly less in comparison to EC-L. With the addition of 5 mg of PAMAM, PC-L5 matrices achieved enzymatic resistance comparable to EC-H matrices. At 5 mM EDC and 10 mg of PAMAM cross-linking, PC-L10 matrices seemed to be more susceptible to enzymatic digestion, as more than 80% of the matrix was degraded after 24 h. The beneficial effect of incorporating PAMAM to improve the enzymatic resistance of collagen matrices was not evident in all the matrices cross-linked with 25 mM EDC concentration, as EC-H, PC-H5, and PC-H10 matrices had similar weight loss. This suggests that low concentration such as 5 mg of PAMAM contributed to cross-linking when mediated by 5 mM EDC concentration, giving rise to more resistant collagen matrices against enzymatic digestion. However, at 25 mM EDC concentration, the advantage of incorporating PAMAM cross-linking was not seen.

Thermal Stability. The shrink temperature (T_s) of collagen matrices provides an indication of the efficiency and extent of cross-linking.[12] Overall, the cross-linked collagen matrices had

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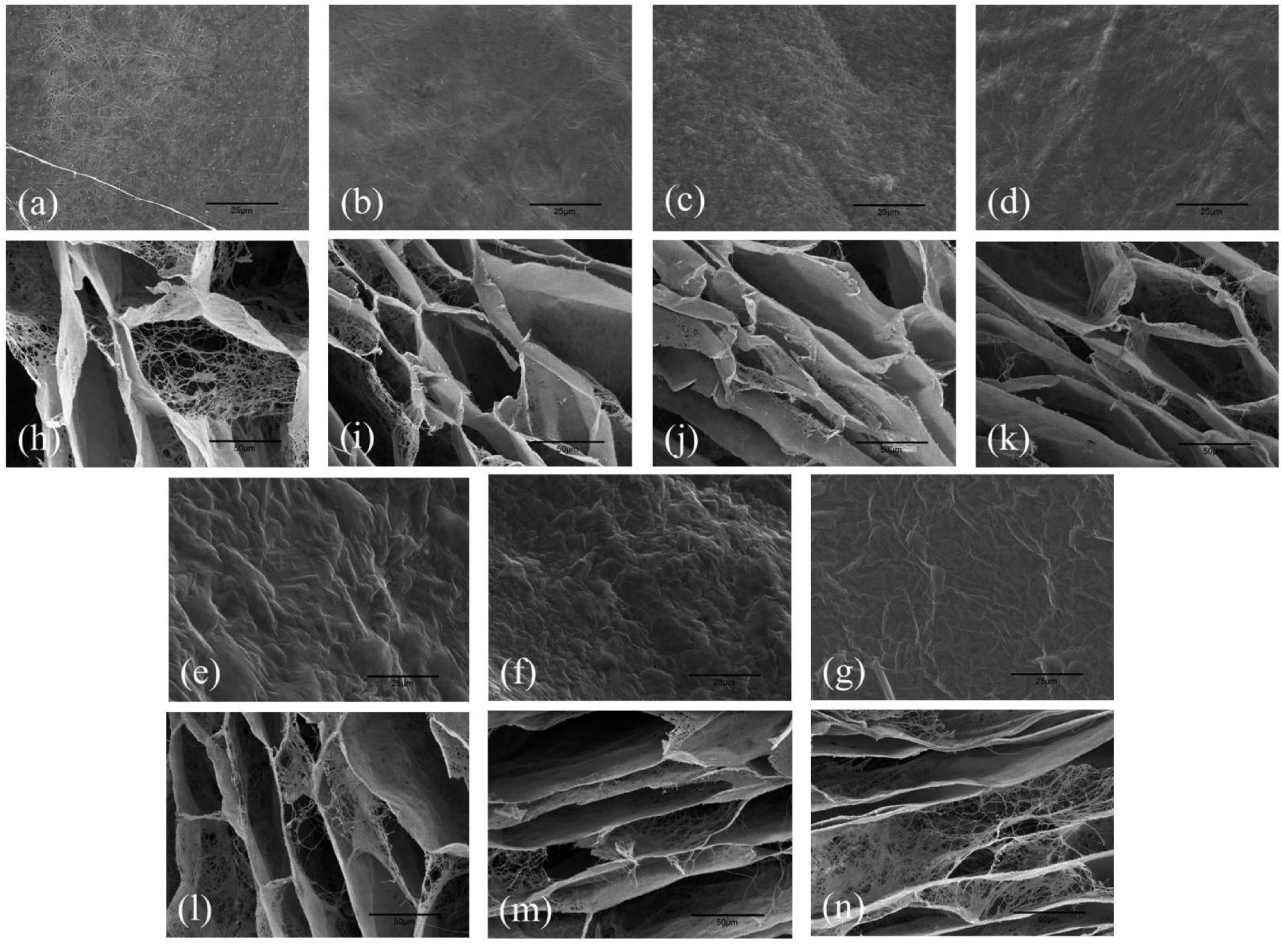

Figure 1. Scanning electron microscopy views of the surfaces (a−g) and cross sections (h−n) of collagen matrices. Collagen matrices were non-cross-linked (a,h), cross-linked with 5 mM EDC/5 mM NHS (b,i) with the addition of 5 mg of PAMAM (c,j) or 10 mg of PAMAM (d,k), or cross-linked with 25 mM EDC/5 mM NHS (e,l) with addition of 5 mg of PAMAM (f,m) or 10 mg of PAMAM (g,n).

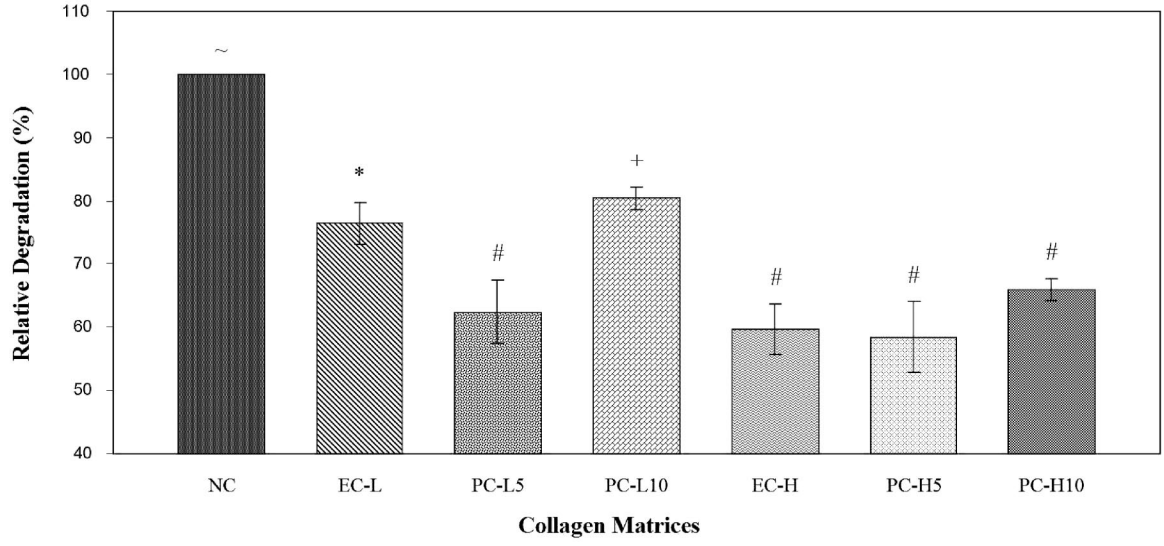

Figure 2. Percentage degradation of collagen matrices after 24 h exposure to collagenase solution at 37 °C. Values are mean ± SD ($n = 3$). (∼) $p < 0.05$ vs all; (*) $p < 0.05$ vs all except PC-L10; (#) $p < 0.05$ vs NC, EC-L, PC-L10; (+) $p < 0.05$ vs all except EC-L.

significantly higher T_s values compared tothose of non-cross-linked (NC) groups (Figure 3). At 5 mM EDC concentration, EC-L matrices showed a modest increase of T_s from 70.4 to 75.9 °C. Addition of 5 mg of PAMAM in PC-L5 resulted in a matrix that had a significantly higher T_s, 86.8 °C. However, further increase of PAMAM to 10 mg in PC-L10 only achieved a T_s of 74.5 °C with no significant difference in thermal stability compared to the case of EC-L. This trend was consistent with the pattern seen in the respective different groups in the collagenase assay.

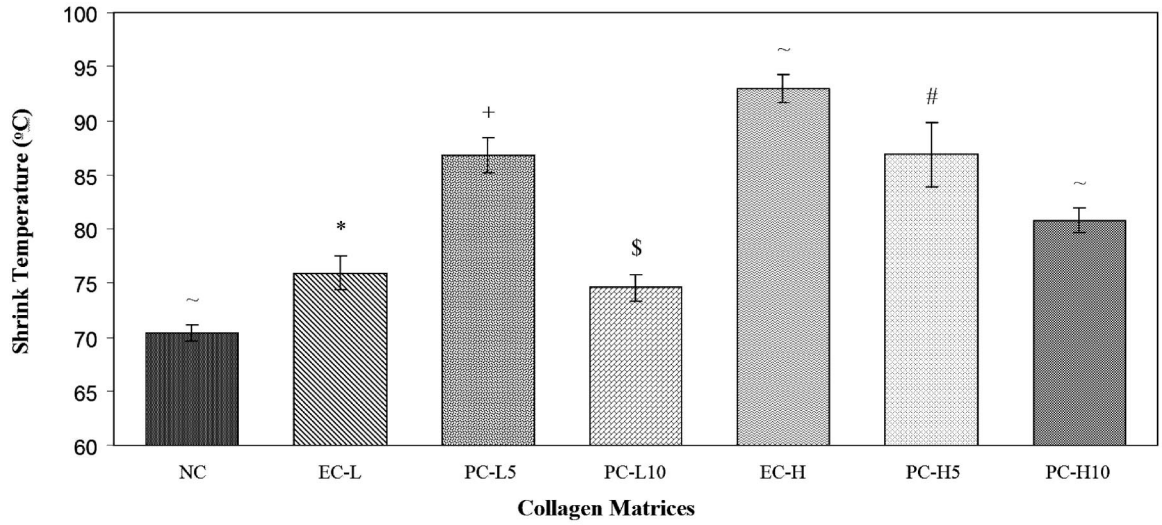

Figure 3. Shrink temperatures of collagen matrices. Values are mean ± SD ($n = 3$). (~) $p < 0.05$ vs all matrices; (*) $p < 0.05$ vs all except PC-L10; (+) $p < 0.05$ vs all except PC-H5; ($) $p < 0.05$ vs all except EC-L; (#) $p < 0.05$ vs all except PC-L5.

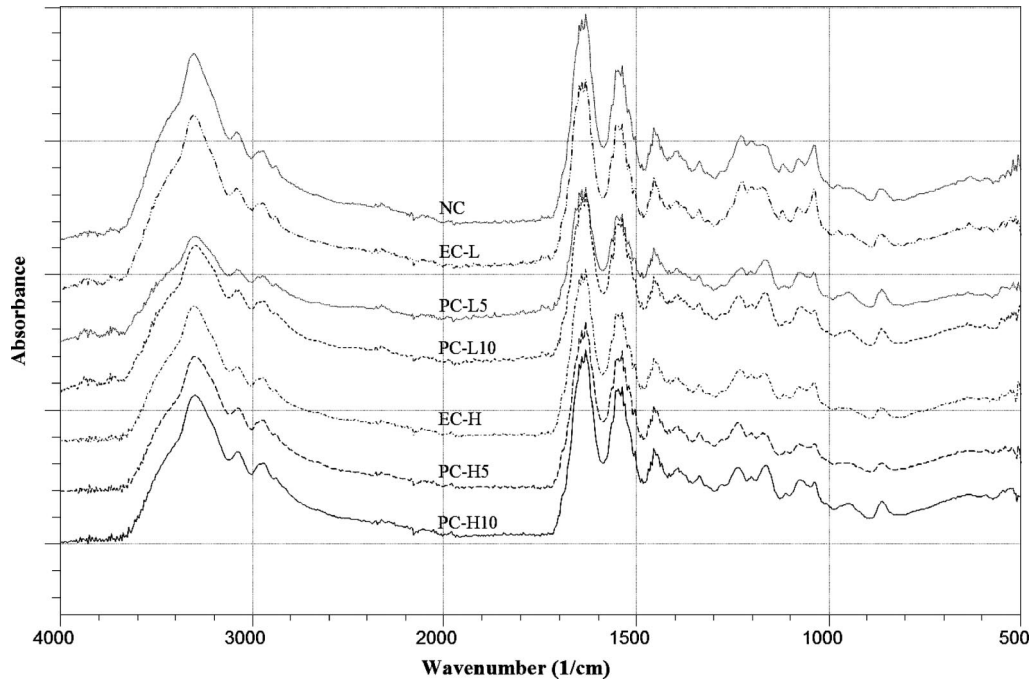

Figure 4. ATR-FTIR spectra of collagen matrices stacked together, showing the position of each amide band.

EC-H matrices, with a higher concentration of EDC, achieved a T_s of 93 °C, approximately 17 °C higher than that for EC-L matrices. Further addition of PAMAM in PC-H5 and PC-H10 matrices reduced the T_s to 86.9 and 80.8 °C, respectively. This pattern of change is similar to that observed in the collagenase assay, which showed that the addition of PAMAM to cross-linked collagen matrices at high concentration of EDC did not confer further stability to the collagen matrices.

Collagen IR Spectra. FTIR spectroscopy involves the measurement of the wavelength and intensity of absorption of IR light through the excitation of molecular vibrations, which provides predictive information about changes in the molecular structure of organic materials. ATR-FTIR has been a useful tool in the prediction of protein structure and cross-linking.[26,62] The representative IR spectra of the various matrices in this study are shown in Figure 4.

The bands at ~3300 and ~1635 cm^{-1} are identified as amide A and amide I bands, respectively. These bands are characteristic of protein structures with each band representing $-NH_2$ and $-CONH-$ bonds, respectively.[62−64]

EDC cross-links collagen by activating the free $-COOH$ groups of the polypeptide to react with the free $-NH_2$ groups to form a $-CONH-$ bond. Hence, with cross-linking, the intensity of the amide I band of the matrices will increase while that of amide A band decreases. Quantitative peak information of the various matrices was obtained using the Hyper-IR software (Shimatzu, Japan). The absorption peak area of the amide I band to that of the amide A band was determined, and their ratio was plotted as a function of the concentration of cross-linker (Figure 5). An

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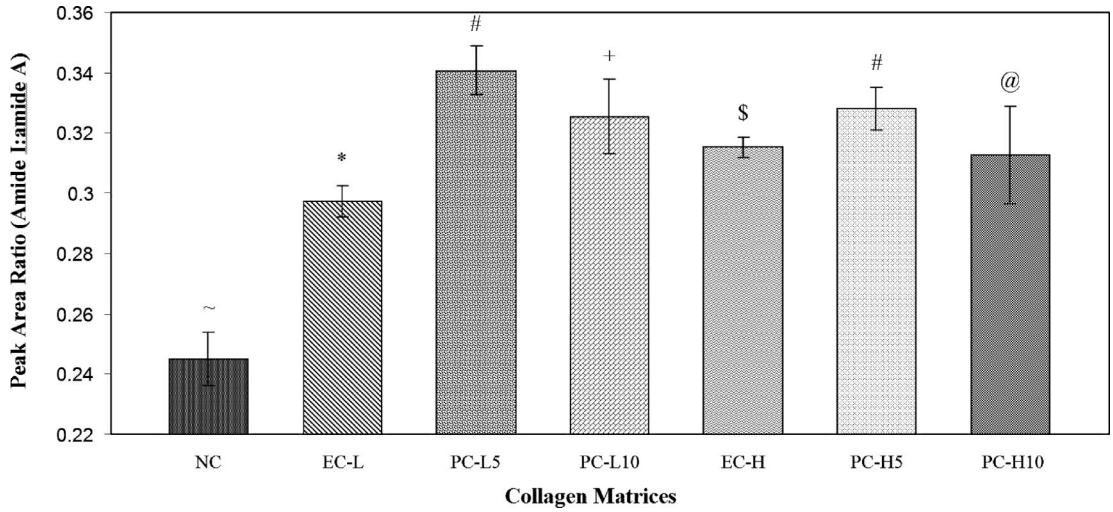

Figure 5. ATR-FTIR peak area ratios of amide I/amide A band vs the group of collagen matrices. Values are mean ± SD ($n = 3$). (~) $p < 0.05$ vs all matrices; (*) $p < 0.05$ vs all except PC-H10; (#) $p < 0.05$ vs NC, EC-L, EC-H; (+) $p < 0.05$ vs NC, EC-L; ($) $p < 0.05$ vs NC, EC-L, PC-L5, PC-H5; (@) $p < 0.05$ vs NC, PC-L5.

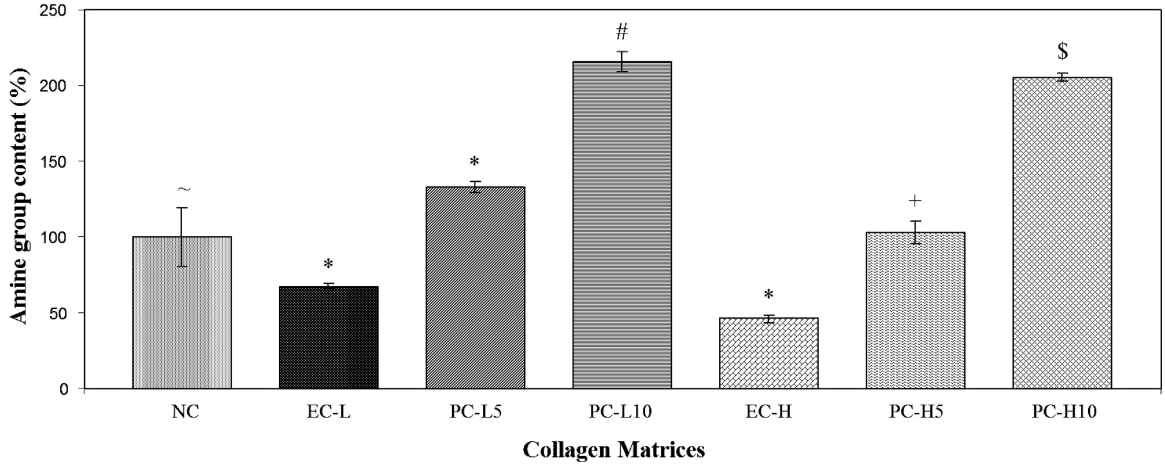

Figure 6. Percentage of amine group content in relation to non-cross-linked collagen among collagen matrices. Values are mean ± SD ($n = 3$). (~) $p < 0.05$ vs all except PC-H5; (*) $p < 0.05$ vs all; (#) $p < 0.05$ vs all except PH-H10; (+) $p < 0.05$ vs all except NC; ($) $p < 0.05$ vs all except PC-L10.

increase in the amide I to amide A peak area ratio therefore indicates increased amide linkages and cross-linking, whereas a decrease in the ratio reflects a reduced cross-linking effect. In this study, all the cross-linked matrices showed an increase in the peak area ratios consistent with cross-linking compared to non-cross-linked matrices. There was also an increase in cross-linking with higher concentrations of EDC (EC-H) used compared to EC-L matrices as shown in Figure 5. With the addition of 5 mg of PAMAM (PC-L5, PC-H5), there was a further increase in the peak area ratio compared to their respective EDC cross-linked counterparts. At higher concentration of PAMAM (PC-L10, PC-H10), however, the peak area ratio decreased compared to the lower concentration of PAMAM (PC-L5, PC-H5) used. Also, the increase in the peak area ratio with addition of PAMAM was greater at the lower concentration (5 mM) of EDC than at the higher concentration (25 mM).

Amine Group Content. To provide further insight into the degree of cross-linking, the percentages of remaining free $-NH_2$ groups of all non-cross-linked and cross-linked matrices were studied. As EDC cross-linking involves the reaction between free $-COOH$ groups and $-NH_2$ groups of collagen to form a $-CONH-$ bond, cross-linking reduces the amount of free $-NH_2$ groups in collagen. Also, incorporation of PAMAM (a multi-terminal amine dendrimer) in the cross-linking mixture increases the amount of free amine content in the matrix. Therefore, an estimation of free amine content in the collagen matrix before and after cross-linking is a good indicator of the extent of cross-linking. The amount of free amine content is also an indirect measure of the level of incorporation of PAMAM in the collagen matrices. This free amine content was determined via the ninhydrin assay.

At 5 mM (EC-L) and 25 mM (EC-H) EDC concentrations, the percentage of free $-NH_2$ groups was 67.5% and 46.1%, respectively (Figure 6). The reduction in their $-NH_2$ groups relative to NC matrices is consistent with their degree of cross-linking. EC-H also had significantly less remaining free $-NH_2$ groups than EC-L matrices confirming that it had a higher degree of cross-linking. This finding correlated with the observations of improved collagenase resistance, higher shrink temperature, and increased FTIR peak area ratio of the matrices. All the rest of the collagen matrices with the addition of PAMAM had increased $-NH_2$ groups. The highest content of free $-NH_2$ groups was seen in PC-L10 and PC-H10 matrices (Figure 6). This is followed by PC-L5 and PC-H5 with the percentage of free $-NH_2$ groups at 132.7% and 102.9%, respectively. This finding suggested that PAMAM dendrimer molecules were successfully

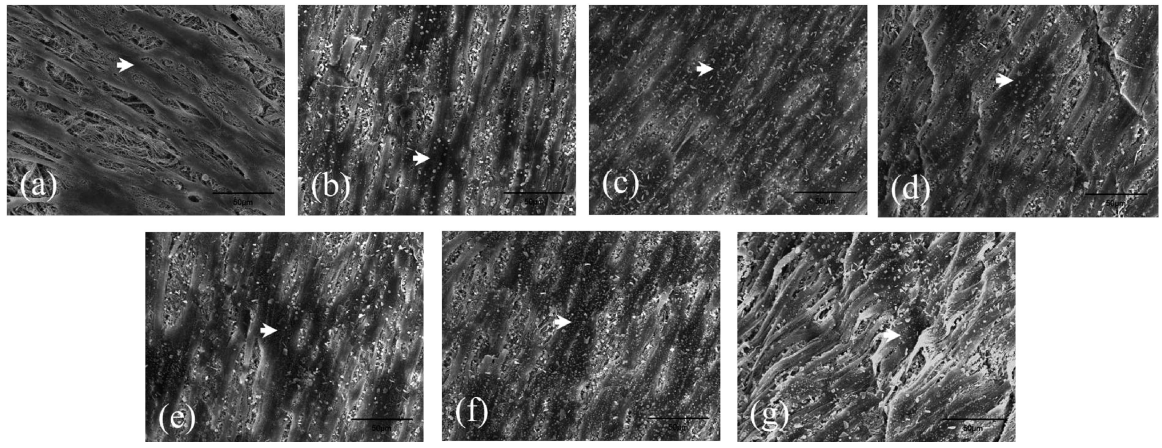

Figure 7. Scanning electron microscopy view of HFFF attachment and proliferation on collagen matrices. Collagen matrices were non-cross-linked (a), cross-linked with 5 mM EDC/5 mM NHS (b) with addition of 5 mg of PAMAM (c) or 10 mg of PAMAM (d), or cross-linked with 25 mM EDC/5 mM NHS (e) with addition of 5 mg of PAMAM (f) or 10 mg of PAMAM (g). HFFFs are indicated by white arrows.

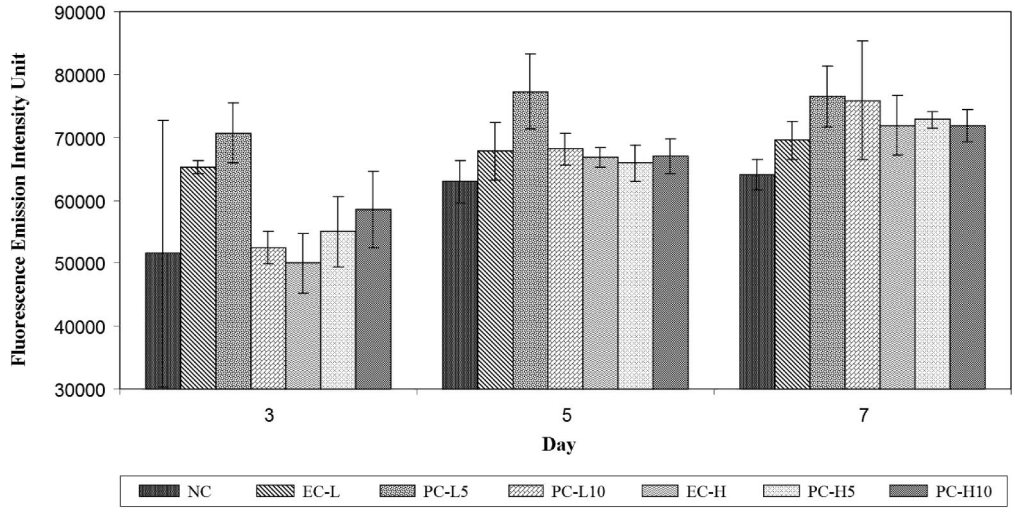

Figure 8. Cell viability assay of HFFFs on day 3, 5, and 7 of incubation on the respective collagen matrices. Values are mean ± SD ($n = 3$).

incorporated in collagen polypeptides through the cross-linking process and unreacted amine arms increased with higher concentration of PAMAM used.

Cell Viability. *Cell Morphology.* The morphology of human fetal foreskin fibroblasts (HFFFs) was studied on the matrices after 7 days of culture. HFFFs adhered to the matrices and aligned themselves when in close proximity to each other to form a monolayer of cells on the surface of the matrix (Figure 7). The cells exhibited a spindle-shaped morphology. There was no morphological difference between HFFFs proliferating on non-cross-linked or cross-linked matrices.

Cell Metabolic Activity. The viability of HFFFs seeded on non-cross-linked matrices and matrices cross-linked with 5 or 25 mM EDC and/or 5 or 10 mg of PAMAM was assessed by Alamar Blue assay on day 3, 5, and 7. HFFFs on all the matrices showed normal comparable metabolic activity levels (Figure 8). Both cell morphology and metabolic activity levels confirmed that EDC cross-linking up to 25 mM concentration and addition of up to 10 mg of PAMAM to the collagen matrices did not impair cellular sustainability of the collagen matrices.

Discussion

Collagen cross-linking with EDC involves the activation of −COOH groups of glutamic and aspartic acid residues and the formation of −CONH− bonds in the presence of $-NH_2$ groups from lysine or hydroxylysine residues. "Zero-length" cross-linking of EDC confers a great advantage because the molecules are directly cross-linked to each other, hence eliminating potential cytotoxic chemical residues.[23] Its drawback, however, is its dependence on the limited number of inherent free $-NH_2$ groups available in collagen polypeptides for reaction. This has significantly restricted the extent of the cross-linking capacity of collagen by this method.[12] With the introduction of the multifunctional PAMAM dendrimer, a large number of free amine groups become available and accessible for cross-linking with activated carboxyl groups.

Collagen consists of tropocollagen molecules with a length and diameter of approximately 300 nm and 1.5 nm, respectively.[65] A G1 PAMAM dendrimer with a size of 1.1 nm should theoretically be able to permeate collagen macrofibrils to enhance intra- and intermolecular cross-linking of collagen fibers.[12,55] The dendrimers become integrated during the cross-linking process and form bridges between the collagen molecules. The large number of amine groups also means that there are more available amine groups for amide bond formation with activated carboxyl groups in collagen. This, in association with "zero-length" cross-linking of EDC, changes the dynamics and

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complexity of the cross-linking process. Furthermore, multiterminal dendrimer molecules could be involved in the cross-linking in multiple ways, such that a single dendrimer molecule with eight terminal amine groups could react to either multiple activated carboxyl groups within a single collagen molecule, or to those of as many as eight different collagen molecules.

In the present study, we compared the effect of different concentrations of PAMAM dendrimer in cross-linking collagen with two different concentrations of EDC. Low, 5 mM, and high, 25 mM, concentrations of EDC were chosen to examine their respective effects on the integration of G1 PAMAM dendrimer into collagen matrices in cross-linking. To understand the nature of incorporation of G1 PAMAM dendrimer and its effect on cross-linking as well as on cellular sustainability, two different concentrations (low, 5 mg and high, 10 mg) of PAMAM were used with each concentration of EDC. The effective incorporation of dendrimer in the cross-linking of collagen was evaluated using structural scanning electron microscopy, collagenase degradation assay, shrink temperature assessment, ATR-FTIR spectral analysis, and free $-NH_2$ group assay. The effect of dendrimer incorporation on cellular sustainability of the collagen matrices was studied with *in vitro* analysis of HFFF morphology and viability. The results were compared with samples of collagen cross-linked with EDC alone and with non-cross-linked collagen as controls.

G1 PAMAM dendrimer is a multifunctional (eight free amines per molecule) chemical agent, which was used as an adjunct to the EDC/NHS cross-linking of collagen. In order to form effective bridging cross-links, each dendrimer molecule must form at least two amide bonds to bridge the polypeptides. An initial increase of $-NH_2$ groups by the presence of PAMAM dendrimer allows more bridging cross-link formation with the $-COOH$ groups in collagen, thereby increasing the extent and degree of cross-linking. When an excess quantity of PAMAM dendrimer was present, competition for $-COOH$ groups occurred, leading to fewer effective bridge cross-links between the polypeptides. This observation was also reported by Chan et al.[61] The authors found an NH_2/COOH ratio of between 2 and 14 to be optimal for achieving stability with collagen scaffolds using a similar cross-linking system of EDC/NHS.[19,61]

Structural scanning electron micrographs revealed a denser organization with fused arrays of collagen fibers among the cross-linked collagen matrices compared to controls. There was no discernible difference, however, between matrices cross-linked with either concentration of EDC, with or without PAMAM dendrimer, due to saturation of cross-linking reaction as seen on the exposed surfaces of the matrix under the experimental conditions.

From collagenase degradation assay and shrink temperature analysis, it is evident that the highest cross-linking reaction was obtained with a higher concentration of EDC at 25 mM. This was further supported by a higher FTIR peak area ratio and lower $-NH_2$ group content compared to those cross-linked with lower (5 mM) EDC concentration. This is due to the increased number of $-COOH$ groups being activated for reaction with $-NH_2$ groups in collagen molecules to form amide linkages between the polypeptides. In addition, when a high EDC concentration such as 25 mM is used for collagen cross-linking, it may induce ester bond formation between the polypeptides.[66] This may further contribute to their stability. A high degree of cross-linking was also achieved in matrices cross-linked with a lower (5 mM) EDC concentration by the addition of 5 mg of PAMAM. The addition of PAMAM dendrimer rendered the dendrimeric matrices capable of achieving the level of cross-linking comparable to that of matrices cross-linked at high concentration of EDC through provision of extra amine groups for reaction with activated carboxyl groups. This finding is supported by the improved collagenase resistance, higher shrink temperatures, and higher FTIR peak area ratio in these matrices. Although there was also an increase in free $-NH_2$ groups compared to non-cross-linked controls as observed in the ninhydrin assay, this was expected, as it is due to the remaining unreacted functional $-NH_2$ groups provided by the G1 PAMAM dendrimer following the heightened cross-linking level of the collagen molecules. All these observations support the hypothesis that the successful incorporation of G1 PAMAM dendrimer will improve the cross-linking of collagen biomaterials with free $-NH_2$ groups available for further molecular tethering.

For collagen matrices cross-linked with 25 mM concentration of EDC, the addition of 5 mg of PAMAM had little effect on their collagenase resistance and shrink temperature properties. There was, however, an increase in the FTIR peak area ratio and $-NH_2$ group content, thus indicating successful incorporation of PAMAM dendrimer into the matrices. This observation can be explained by the higher rate of cross-linking at a higher concentration of EDC; hence, a more pronounced reaction had taken place on the surface of the collagen matrices.[13,67] It is possible that rapid initial cross-linking of the exposed surface of the matrices restricts further permeation of PAMAM and prevents uptake of PAMAM throughout the whole matrix.

With further increase in the concentration of PAMAM to 10 mg at either concentration of EDC, the cross-linking reaction decreased as shown by reduced collagenase resistance, lower shrink temperatures, and lower FTIR peak area ratios. There was a significantly higher level of $-NH_2$ groups present in this group of matrices as observed in the ninhydrin assay. This indicates a decrease in the effective bridge cross-linking formation among collagen molecules with increased unreacted number of $-NH_2$ groups. The $-NH_2$ group increment despite an increasing number of covalently bonded PAMAM molecules can be explained by the phenomenon where interdendrimer interactions occurred, leading to the formation of oligomeric aggregates of PAMAM molecules. These oligomeric aggregates in turn contributed to the increase in the number of pendant $-NH_2$ groups as demonstrated by the ninhydrin assay. At high concentrations of PAMAM, the molecules become involved in the formation of oligomeric aggregates by both nonspecific repulsive interactions (steric plus electrostatic) as well as shorter range attractive interactions.[52,68] Thus, at high concentrations, the high molecular weight PAMAM oligomeric aggregates will compete for the free $-COOH$ groups on collagen. In addition, the bulkiness of PAMAM oligomers covalently bonded to the collagen presents a steric hindrance, resulting in reduced cross-linking and underutilization of $-COOH$ groups of collagen molecules.

In the cell viability study, cell morphology and metabolic activity were maintained in all non-cross-linked and cross-linked matrices. Although EDC has been reported as an ideal cross-linker due to its zero-length cross-linking mechanism, it is cytotoxicity was observed when greater than 10 mM EDC was used for collagen cross-linking.[13,16,69] One possible explanation of EDC cytotoxicity at high concentration was the inadequate

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release of any unused EDC molecules or their reaction byproducts from the matrices through simple washing techniques. Similarly, although PAMAM dendrimers have been used in many polymers in order to achieve novel functional materials, it does exhibit concentration and generation dependent toxicity as shown by Roberts et al.[36] In the present study, the cellular morphology of all the non-cross-linked and cross-linked matrices exhibited no discernible morphological difference, and a comparable metabolic activity with the non-cross-linked controls was observed. The successful sustenance of cells on these matrices indicates that the integration of G1 PAMAM dendrimer in cross-linking reaction in collagen matrices can be a safe alternative for improving the extent and density of cross-linking of collagen polypeptides.

Conclusions

Collagen cross-linking by EDC was significantly enhanced with the addition of PAMAM dendrimers. By using 5 mg of PAMAM dendrimer in collagen cross-linking mediated by 5 mM EDC, the resultant collagen matrices achieved degradative resistance and thermal properties comparable to those achieved with higher cross-linker concentration. These results support the strategy of the use of dendritic macromolecules to control collagen properties. It also provides an alternative of modulating collagen properties through EDC cross-linking without changing its concentration. This beneficial effect of PAMAM dendrimers, however, was limited to using low concentration of either agent. When the 10 mg concentration of PAMAM was used, the resultant collagen matrices did not achieve high quality cross-linking with its collagenase degradation and thermal stability being similar to those of the 5 mM concentration of EDC used alone. This showed that there is an upper limit to the efficient involvement of PAMAM dendrimers in enhancing collagen cross-linking. It is also noted that no further collagen stability was attained when PAMAM dendrimers were included in collagen cross-linking reactions mediated by a high concentration of EDC due to the rapid cross-linking reaction and ester bond formation at high EDC concentration. In this study, the ninhydrin assay results showed that PAMAM dendrimers were successfully incorporated into collagen polypeptides as demonstrated by the significant increase in free amine groups following addition of PAMAM in the reactions. The successful fabrication of cell sustainable dendrimeric collagen matrices demonstrates the versatility and utility of PAMAM dendrimers. The enhancement of amine terminals through incorporation of G1 PAMAM dendrimer in collagen matrices will allow a dynamic interaction between the matrices and their surrounding environment *in vivo* through provision of a multivalent and modular base for future design of substrate-tethered collagen polymers.

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