

Collagen Cross Linking Agents: Design and Development of a Multifunctional Cross Linker

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ABSTRACT

A new cross linking reagent based on the first-generation polyamidoamine dendrimer (G.1 PAMAM) has been synthesized by reaction of the PAMAM with eight equivalents of *p*-nitrophenyl diazopyruvate. The resulting water-soluble octa-diazopyruvoyl PAMAM (8G.1 DAP, 1.3) was shown to undergo Wolff rearrangements upon photolysis in methanol at $\lambda > 300$ nm to yield the methyl esters of the ketenes formed from the loss of nitrogen. 8G.1 DAP also forms strong bonds with dehydrated collagen with glass as high as 36 N cm^{-2} . Collagen to collagen bonds with tensile strengths as high as 92 N cm^{-2} were observed with fully dehydrated tissues. The bonding decreased rapidly with increasingly hydrated tissue possibly due to the increased distance between the collagen fibrils and the competition of H_2O for the free ketene functions.

INTRODUCTION

An effective cross linking agent for Type I collagen to replace sutures or attach tissue to other materials is a research objective of this and other research groups (1–8). However, application of these techniques to tissue cross linking has been particularly challenging because of difficulties arising from biological incompatibility as well as the restrictions on physical and chemical properties which hamper effective covalent bonding to collagenous protein. Commonly bi-functional reagents such as glutaraldehyde (9) or bis-isocyanates (10) have been explored but these reagents have deleterious side effects such as reacting slowly with glycosaminoglycans and other endogenous nucleophiles. Furthermore, diffusion of these cross linking agents and the interstitial penetration have been problematic (11).

Recently, new photochemically activated approaches for cross linking have been reported (12,13). For example, bifunctional naphthalimide reagents (14,15) which can be activated at 457.9 nm by an argon laser cross link human meniscus, human articular cartilage (16) and rabbit skin (17) at tensile strengths as high as 20 N cm^{-2} and human corneal stroma (18) with tensile strengths of 30 N cm^{-2} . Photosensitization with rose bengal (19–22) at 514 nm sealed full-thickness corneal incisions as well as penetrating kera-

toplasties in rabbits (20). Riboflavin has also served as a photosensitizer for collagen cross linking in the treatment of keratoconus and corneal ulcers (23,24). Finally, Pedone and Brocchini showed that polyethylene glycol (PEG) can be conjugated with lysozyme and ribonuclease by a photochemical approach (25).

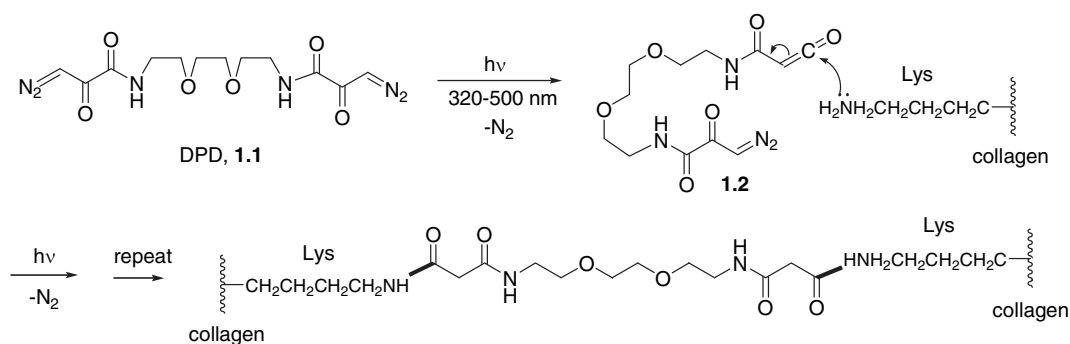
Approaches using light-activated cross linking agents are intrinsically appealing because they provide a greater measure of spatial and temporal control over the bonding event which should minimize the amount of physical trauma to the surrounding tissue. However, the mechanisms of the cross linking processes for most of the photochemical agents are unknown and, more seriously, often produce unknown byproducts.

Our approach has been the design and synthesis of reagents that rely on known, well-characterized photochemical reactions of α -diazopyruvates. This reagent undergoes a clean Wolff rearrangement to form a transparent peptide bond *via* a reactive ketene intermediate (Scheme 1). This approach to cross linking collagen must meet the following criteria. (1) The photochemical activation of the reagent must occur at wavelengths greater than 330 nm to avoid damage to the tissue. (2) The photoactivated chromophore must be completely removed or destroyed by the photolysis reactions. (3) Any reactive intermediates (in this case, and α -ketoketene) must be sufficiently reactive to covalently bond to a functional group or groups on collagen. (4) Any new functional groups that are generated must be transparent in the near-UV visible range. In considering the functional groups available for cross linking Type I collagen, we have focused our attention on the nucleophilicity of γ -amino group on the lysyl side chain of collagen. In previous studies we established (26) that typical 1° and 2° amine fluorescent labeling reagents such as *o*-phthalaldehyde or dansyl chloride can be covalently attached to Type I collagen.

To selectively capture the target γ -amino lysine function, we designed a reagent that photochemically generated ketene through a Wolff rearrangement. Ketenes react rapidly with 1° and 2° amines, thereby providing the desired connectivity to collagen. Lawton and coworkers (27–29) have demonstrated the feasibility of this strategy in earlier studies on photoaffinity labeling of proteins and in at least one instance that cross linking of a protein is possible (27,30). We captured this chemistry by designing a bis-substituted PEG diazopyruvamide, *i.e.* N,N'-bis-(3-diazopyruvoyl)-2,2'-(ethy-

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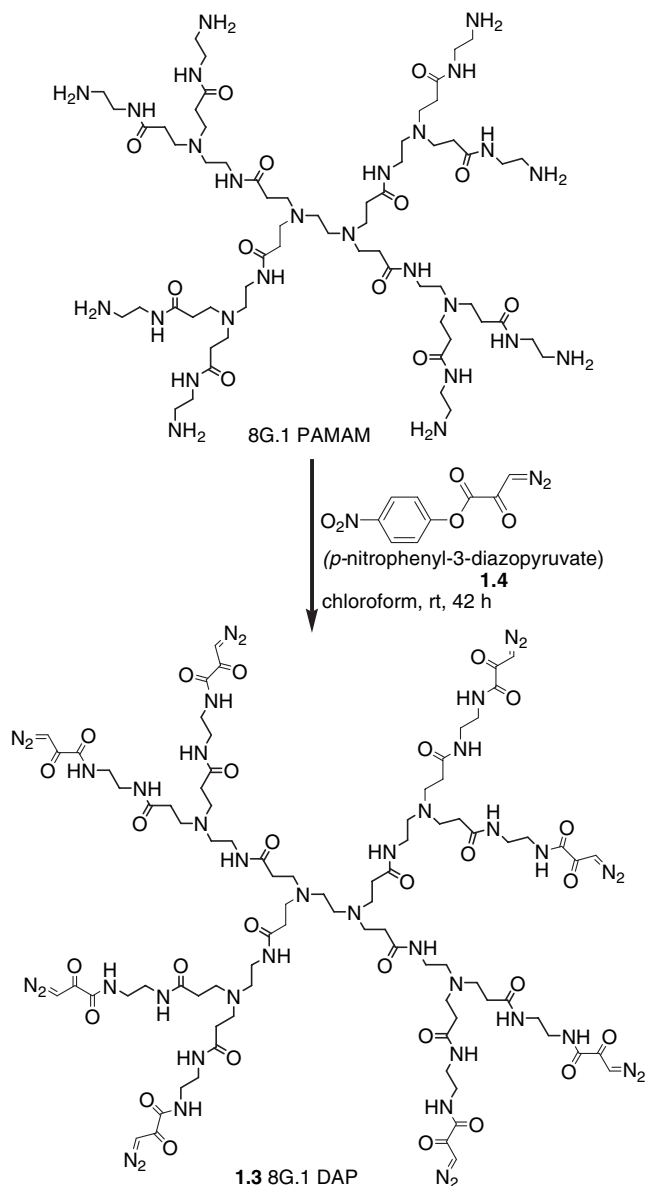
lenedioxy)bis(ethylamine, DPD **1.1**) as our first cross linking agent. The photochemical activation and rearrangement are shown in Scheme 1 (27,31).

Photolysis of DPD **1.1** at 320–550 nm sequentially generates two ketene amides (see **1.2**) through the photo-Wolff rearrangement. Nucleophilic attack on the ketene by a γ -amino group on lysine or hydroxylysine residue forms a malonamide, *i.e.* a peptide bond. We have demonstrated that collagen cross linked by photoreaction with **1.2** strongly binds two strips of dehydrated bovine corneal tissue with tensile strengths up to 46.5 N cm^{-2} (26). We extended the study to include dehydrated corneal tissue strips bonded to gel strips or to glass with comparable strengths. Unfortunately, these initial studies only produced measurably strong bonds with fully dehydrated and partially hydrated tissue, suggesting that further design modifications were required. Critical features such as low aqueous solubility, the short length of the PEG tether, the rather modest selectivity of the ketene for amines over H_2O and hydroxyl groups and the low number of reactive groups per cross linking agent/tether required modification of our original design.

Toward this end, we are now building functionalized poly(amidoamine) PAMAM dendrimers (32) for the tether. These were selected because of their ready availability in a variety of sizes (or generations), the well-known chemistry of dendrimers and the multifunctional nature of the reagents. Dendrimers exhibit good water solubility properties and possess multiple sites for attachment of the diazopyruvyl chromophore that increase exponentially with each generation. Furthermore, the backbone functional structure of the dendrimer is composed of peptide linkages which are near-UV transparent. In fact, recent application of another dendrimer has been reported by Wathier *et al.* (33) employing lysine-based peptide dendrons, to generate hydrogels for repair of corneal incisions. Amino-terminated cysteine dendrons-PEG dialdehyde polymers have also been employed in a hydrogel to seal corneal incisions in an enucleated eye (34). As noted for earlier studies, mechanisms of these wound-sealing processes are not known. In addition, the hydrogel approaches offer little or no temporal and spatial control that are possible with photoinitiated reactions (34).

Based on our previous DPD results (26) and the structure and properties of the PAMAM dendrimers, we report a new, multifunctional PAMAM diazopyruvamide to serve as a more versatile, biocompatible light-activated cross linking agent for Type I collagen. Our initial studies with the multifunctional

diazopyruvyl dendrimers generated from readily available first-generation (G.1) PAMAM dendrimer (Scheme 2) are reported here.



Scheme 2. Synthesis of diazopyruvyl PAMAM (**8G.1 DAP**, **1.3**).

MATERIALS AND METHODS

General. All starting materials were purchased commercially and used without further purification unless otherwise noted. ^1H and ^{13}C NMR spectra were obtained on a Bruker 400 MHz instrument unless otherwise noted. Samples were dissolved in chloroform- d (CDCl_3), dimethyl sulfoxide- d_6 ($\text{DMSO-}d_6$), N,N' -dimethylformamide- d_7 ($\text{DMF-}d_7$) or deuterium oxide (D_2O) and chemical shifts are reported in parts per million (δ). Precoated silica plates from Merck were used for thin layer chromatography and spots were visualized under a UV lamp. Chromatographic purification was effected with flash chromatography using standard grade (32–63 μm) silica gel (Sorbent Technologies, Atlanta, GA). IR spectra were obtained on a Shimadzu FTIR-8400S spectrophotometer; results are reported in cm^{-1} . HPLC analyses were conducted with a C18 Ecosphere 250 \times 4.6 mm analytical column (Altech Associates, Inc., Deerfield, IL) connected to a Rainin dual pump system. UV/Vis spectra were measured on a Cary 100 spectrophotometer (Varian, Inc., Palo Alto, CA). Irradiations were performed with a Novacure 2000 near-UV (320–550 nm) light source (Exfo, Mississauga, ON, Canada) unless otherwise noted.

Synthesis of octa-substituted diazopyruvoyl PAMAM (8G.1 DAP, 1.3). A 10 mL round bottom flask was flame dried and charged with 0.3 mL of a solution of (G.1) PAMAM dendrimer (Aldrich, 20 wt.% in methanol). The methanol was removed under reduced pressure (6–7 h). The resulting residue was weighed (60 mg, 0.042 mmol) and dry chloroform (3 mL) was added, along with *p*-nitrophenyl 3-diazopyruvate (30) (**1.4**, 119 mg, 0.506 mmol) and the solution stirred at RT in the dark. About 36 h later, the solution together with the entire residue on the flask wall were transferred to 125 mL of a separatory funnel. The organic layer was discarded and the water layer was further extracted with 10 mL of chloroform three to six times until the water layer appeared colorless. The aqueous solution was cooled by dry ice for 30 min and lyophilized (24 h) to afford octa-diazopyruvoyl PAMAM (**1.3**, 8G.1 DAP) as a brown solid: 51 mg, 55%; ^1H NMR (400 MHz, D_2O) δ = 6.41 (s, 4.27H), 6.15 (s, 2.65H), 5.53 (s, 0.60H), 5.39 (s, 0.48H), 3.49–3.40 (bm, 9.07H), 3.37–3.24 (bm, 33.4H), 3.17 (bm, 14.4H), 3.06–2.99 (bm, 19.9H), 2.55 (bm, 23.3H); ^{13}C NMR (500 MHz, $\text{DMF-}d_7$) δ = 182.14, 171.63, 171.29, 160.76, 54.91, 54.41, 52.24, 49.81, 38.46; IR (KBr, cm^{-1}) 3304, 3080, 2943, 2122 (C=N=N), 1636 (C=O), 1535, 1364, 1119, 779; UV–Vis (H_2O) λ_{max} ($\log \epsilon$ ($\text{M}^{-1} \text{cm}^{-1}$)) 253 (4.92), 300 (4.76); Mass Spec. Calc'd for $\text{C}_{86}\text{H}_{128}\text{N}_{42}\text{O}_{28}$: 2196.99; Found: ESMS(m/z) (M+H) 2197.53 \pm 0.78; MALDI-TOF m/z 2203.

Photochemistry of octa-diazopyruvoyl PAMAM (8G.1 DAP, 1.3) in MeOH. A 10 mL Pyrex tube was charged with 8G.1 DAP (**1.3**, 1.4 mg, 0.64 μmol) in 5 mL of methanol. After dissolution was complete, the tube was transferred to a Rayonet photoreactor fitted with four RPR-3000 Å lamps and photolyzed for 5 min. Sample aliquots (0.4 mL) were taken after 0, 2 and 5 min of photolysis. The aliquots were transferred to a 10 mL volumetric flask, diluted with methanol to the mark, and analyzed by UV (200–400 nm). The disappearance of the λ_{max} 300 nm ($\log \epsilon$ 4.67) band of **1.3** indicated complete conversion of **1.3** to the methyl ester (see Fig. 3).

Glass slides. Gold Seal[®] Microscope Slides (Clay Adams, Parsippany, NJ) were cut into *ca* 0.5 \times 2.5 cm pieces and cleaned in a Piranha solution (70:30 vol/vol concentrated H_2SO_4 :30% H_2O_2) for 12 h at RT.[†] After cleaning in the Piranha bath, the glass slides were washed in deionized water for at least 12 h and then dried and stored in a petri dish until use.

Bovine cornea. Corneas were dissected from *ca* 2 h postmortem bovine eyes obtained from a local abattoir. Epithelial and endothelial cell layers were removed by scraping, and the corneas were cut into two *ca* 5 \times 15 mm strips. Each strip was cut in half, parallel to the corneal surface using a fine scalpel. The four resulting strips were weighed and placed stromal side up on glass slides, covered with another slide and dehydrated in a vacuum lyophilizer for varying amounts of time prior to use. The partially dehydrated collagen tissue was weighed just before use.

Cross linking with bovine corneal tissue. A plastic snap-cap vial was charged with 8G.1 DAP (**1.3**, 23 mg, 10 μmol) and water (52 μL) and the material was dissolved with gentle warming and shaking in the dark. The solution was kept in an amber jar between experiments to protect the compound from incident light. Three microliters of 8G.1 DAP solution was used for each experiment. For the co-monomer experiments, 2,2'-(ethylenedioxy) diethylamine (EDEA, 1 mL, 6.8 mmol) was dissolved in water (10 mL) in a volumetric flask. In a separate snap-cap vial 12 μL of 0.2 M 8G.1 DAP was mixed with 8 μL of the freshly prepared EDEA solution to provide 2.2 equivalents of EDEA.

Corneal samples were prepared as described earlier. For irradiation and bonding studies, one corneal sample was placed stromal side up on a quartz slide with the other sample placed on top, forming an overlap area of $\sim 5 \times 5$ mm. Three microliters of a solution of 0.2 M 8G.1 DAP in water was instilled between the two corneal samples after which a second quartz slide placed on top. Light fingertip pressure was applied evenly to the quartz slides which were then stabilized for further manipulation with small clamps.

The glass slides were then exposed to UV–Vis irradiations. The exposure time was adjusted to provide an exposure of 333 J for the bonding process in accordance with the calibrated power of the light source. Tensile strength testing was carried out as described previously (31). The overlap areas were defined by a light tan residue from the photoreaction of 8G.1 DAP.

Bonding cornea to glass. The glass slides were prepared as described above. A clean glass (*ca* 0.5 \times 2.5 cm) slide was put on a quartz slide and on one end of it was added 3 μL of an aqueous solution of 0.2 M 8G.1 DAP, then the cornea strip (*ca* 0.5 \times 2.5 cm) was placed on top with the stromal side in contact with the glass, thus creating an overlapping area of $\sim 5 \times 5$ mm². A second quartz slide was applied on the top of the glass slides with two clamps helping to assure full contact of the glass slides. The overlapping area was then exposed to the UV light source for the requisite time to provide an exposure of 333 J for the cornea-to-glass bonding. Tensile strength testing was determined as reported previously (31). The overlap areas were defined by a light tan residue from the photoreaction of 8G.1 DAP.

Bonding glass to glass. The glass slides were prepared as described above. A clean glass slide (*ca* 0.5 \times 2.5 cm) was placed on a quartz slide and 3 μL of an aqueous solution of 0.2 M 8G.1 DAP was applied at one end. A second glass slide (*ca* 0.5 \times 2.5 cm) was placed on top, thus creating an overlapping area of $\sim 5 \times 5$ mm² and a second quartz slide applied on the top of the glass slides with two clamps providing a minimum amount of pressure, to assure surface contact. The overlapping area was then irradiated (333 J). Tensile strengths were measured as reported previously (31) except that O-rings were attached to the nonbonded end of each glass slide with cyanoacrylate glue to provide a clamping surface for the mechanical force measurements. The overlap area measurements are described later.

RESULTS

Synthesis and exploratory photochemistry

Synthesis of 8G.1 DAP shown in Scheme 2 was accomplished using 10–12 equivalents of *p*-nitrophenyl 3-diazopyruvate in chloroform for each equivalent of 8G.1 PAMAM. The reaction was complete within 42 h (^1H NMR) giving 8G.1 DAP in 55% yield.

The incorporation of eight diazopyruvoyl groups per PAMAM dendrimer was established by ^1H NMR and mass spectral analyses. A calculated mass of 2196.99 was exactly matched by the experimental value of 2197 (ESMS). The ^1H NMR, however, was more intriguing. The spectrum of 8G.1 DAP (1.2, Fig. 1) showed four characteristic downfield diazomethine hydrogen singlets at δ 6.41, 6.15, 5.53 and 5.39 p.p.m. in a ratio of 1.0:0.62:0.13:0.11. The chemical shifts are in agreement with the expected ^1H NMR results from similar structures reported by Goodfellow *et al.* (27) and our earlier studies. The unusual number of diazomethine hydrogen

[†]Caution: Piranha solution reacts violently with organic compounds and should be handled carefully behind a blast shield (35).

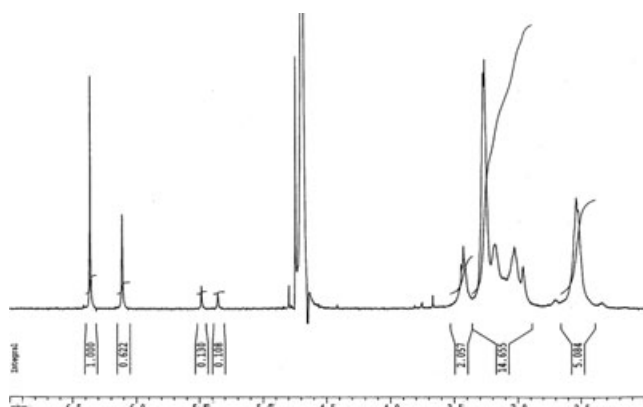


Figure 1. ^1H NMR spectrum of 8G.1 DAP **1.3** in D_2O at 400 MHz (the absorption at 4.9 p.p.m. is due to HOD).

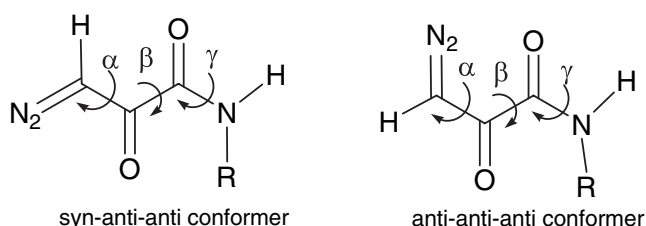


Figure 2. Examples of restricted rotations for the diazopyruvyl moiety.

signals is attributable to four of eight possible rotational isomers arising from the restricted rotation of the conjugated amide, the ketone carbonyl and diazomethane groups (*i.e.* the dihedral angles α , β and γ in $\text{RNH}^\alpha\text{CO}^\beta\text{CO}^\gamma\text{CHN}_2$). We have not attempted to assign these signals to the individual conformers although consideration of the steric and dipole-dipole interactions would suggest that an anti-anti arrangement of the three groups would be preferred (Fig. 2).

The up field region of the spectrum is characterized by a number of broad signals from 2.4 to 3.5 p.p.m., corresponding to the methylene protons of the dendritic backbone. The ratio of the total integration of these signals to those of the four downfield methine protons was approximately 11.7:1, which closely matches the predicted ratio of 12.5:1 (complete derivatization) indicating a yield of 93.6% derivatization of all eight amino groups (see **1.3** in Scheme 2).

The ^{13}C NMR spectrum of 8G.1 DAP showed carbon resonances at δ 182, 172 and 161 p.p.m. corresponding to the diazoketone and amide functions. Other significant spectral features were an intense IR band for the diazo group at 2122 cm^{-1} and the UV absorption (H_2O) maxima at 253 ($\log \epsilon$ 4.92) and 300 nm ($\log \epsilon$ 4.86), similar to those reported by Goodfellow *et al.* (27) and by us (26) for other diazopyruvamide.

The photochemical rearrangement of 8G.1 DAP **1.3** was followed by UV. A sample of 0.13 mM **1.3** in methanol irradiated at 300 nm and monitored at 0, 2 and 5 min (Fig. 3) showed a rapid, efficient disappearance of the diazoketone chromophore. No new absorptions appeared above 220 nm indicating quantitative conversion of 8G.1 DAP to product (Scheme 3).

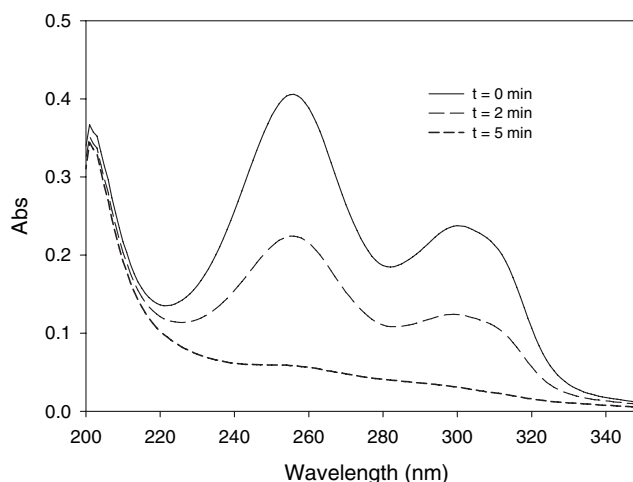
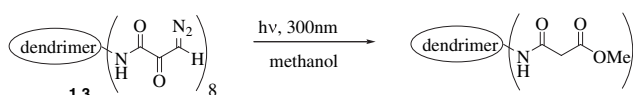


Figure 3. UV spectral analysis of photolysis of 8G.1 DAP **1.3** at 300 nm in methanol.



Scheme 3.

Dendrimer cross linking studies

Unlike the bifunctional DPD, G.1 DAP was moderately soluble in methanol and very soluble in water. The latter solvent was chosen for use in cross linking studies with cornea as it is the more biologically benign of the solvents and would be more compatible with the interfibrillar matrix that surrounds collagen.

Glass-to-cornea bonding with 8G.1 DAP. Corneas were prepared as described earlier for bonding cornea to glass with DPD. Aqueous solutions of 8G.1 DAP were administered between the tissue and glass and then irradiated for an exposure of 333 J. The results of several runs are shown in Fig. 4 and Table 1.

8G.1 DAP glass-to-corneal stroma bonding exhibits the same general trends that were observed for DPD glass-to-corneal stroma bonding, *i.e.* the strongest bonding occurs at lower hydration levels. In addition, all samples tested with 8G.1 DAP produced a measurable bond at low hydration values ($H < 2$).

Glass-to-glass bonding with 8G.1 DAP. The strength of bonding glass-to-glass was also tested. The generation of N_2 was readily observed in these experiments and the effervescence apparently resulted in the formation of small bubbles between the plates as illustrated in Fig. 5. This disruption of the surface contact may be the source of the weak bonding, especially for the glass-to-cornea bonding. Methods for minimizing this effect are under investigation.

Cornea-to-cornea bonding with G.1 DAP (1.3). The results from bonding of two cornea tissue samples are shown in Fig. 6 and Table 2. Hydration levels were varied for the tissue samples as shown in the Table and Figure. The results of the

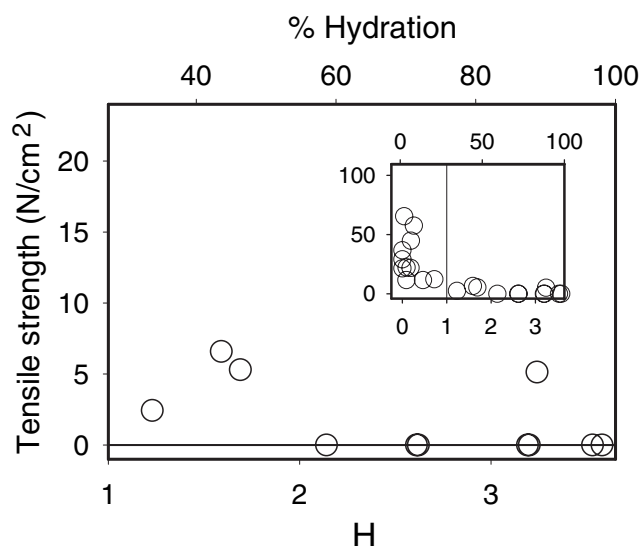


Figure 4. Maximum tensile strengths of glass-to-corneal stroma bonds with 0.2 M 8G.1 DAP 1.3 in H₂O as a function of tissue hydration value. Inset shows that the “dry” tissue ($H < 1$) resulted in bond strengths of 11.5–57.5 N cm⁻². No bonds formed with H₂O only.

Table 1. Tensile bond strengths for glass-to-corneal stroma G.1 DAP 1.3.

Sample no.	H	Tensile strength (N cm ⁻²)
1	0	21.49
2	0	29.11
3	0	36.77
4	0.09	11.57
5	0.09	22.29
6	0.18	21.81
7	0.46	11.54
8	0.72	12.26
9	1.23	2.43
10	1.59	6.59
11	1.69	5.30
12	2.14	0
13	2.61	0
14	2.62	0
15	3.19	0
16	3.20	0
17	3.24	5.13
18	3.53	0
19	3.58	0

H = hydration value of corneal sample.

tensile strength measurements paralleled those of the DPD bonding studies in that the strongest bonds were obtained at lower hydration levels. The maximum tensile strengths with 8G.1 DAP (92 N cm⁻²) were nearly twice the value of 47 N cm⁻² obtained for DPD.

Although the measured tensile strengths at intermediate hydration levels ($H = 1-3$) were slightly higher (6.4 ± 3.9 N cm⁻²), they were within the experimental error identical to those for DPD at similar hydration levels (5.9 ± 6.2 N cm⁻²). Even the addition of 2.2 equivalents of EDEA as a co-monomer with 8G.1 DAP resulted in no significant improvement in the tensile strength (Fig. 6).

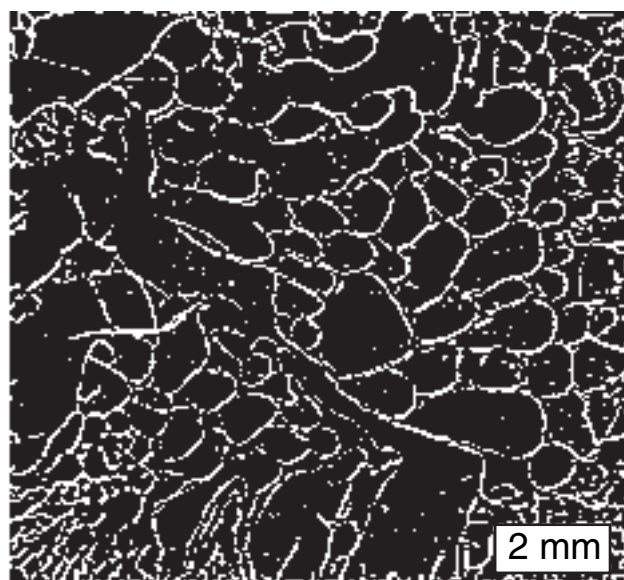


Figure 5. Glass-to-glass bonds with 0.2 M 8G.1 DAP 1.3 in H₂O. The large dark areas are nonbonded regions generated by N₂ from photolysis of the diazo group. The lines outlining the cavities are the bonding regions. The tensile strength is 60 N cm⁻².

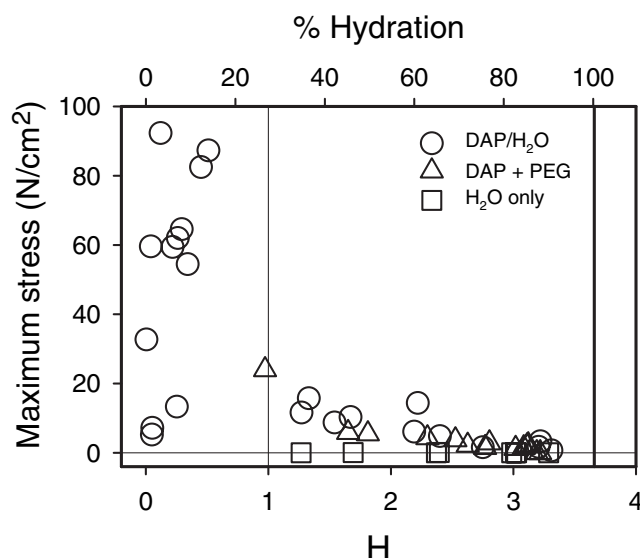


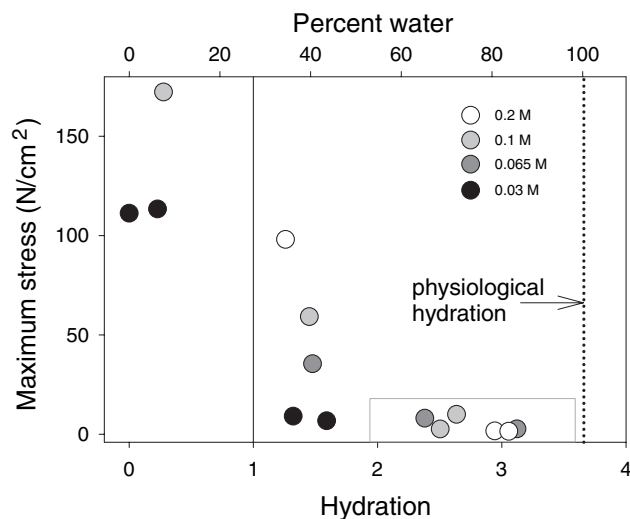
Figure 6. Maximum stress of cornea-to-cornea bonding with aqueous 0.2 M 8G.1DAP 1.3 versus tissue hydration value (H). Circles (○) indicate level of hydration. Triangles (△) indicate sample pairs in which the bonding solution contained 2.2 equivalents of the co-monomer EDEA (2,2'-(ethylenedioxy)diethylamine). Squares (□) indicate H₂O control runs.

Concentration dependence of cornea-to-cornea bonding. In order to optimize the bonding, the concentration of 8G.1 DAP was varied. For these studies, two cornea strips (about 0.5 cm wide) were cut into two pieces lengthwise, thus giving two strips of half the original width with the same hydration level. As shown in Fig. 7, the tensile strengths of the bonded strips of cornea decreased with increasing hydration. For $1 < H < 2$, the data clearly showed 0.2 M 8G.1 DAP gave better bonding than 0.03 M concentrations. For $H > 2$, the tensile strengths remain low. With H -values between 2 and 3.1,

Table 2. Tensile bond strengths for corneal stroma-to-stroma 8G.1 DAP 1.3.

Sample no.	Tensile strength (N cm ⁻²)	H1	H2
1	1.7	1.94	3.21
2	3.3	3.22	3.1
3	0.67	3.31	3.23
4	59.56	0.04	0.04
5	59.42	0.22	0.05
6	13.3	0.05	0.25
7	64.58	0.07	0.29
8	32.71	0.00	0.00
9	92.33	0.00	0.12
10	7.19	0.05	0.05
11	5.26	0.04	0.05
12	14.38	0.73	2.22
13	10.32	1.31	1.67
14	1.61	2.08	2.75
15	4.82	2.26	2.4
16	8.73	0.96	1.54
17	11.61	0.54	1.27

HI, H2 = hydration value for each member of a corneal pair.

**Figure 7.** Maximum tensile stress of cornea-to-cornea stromal bonds versus tissue hydration for DAP concentrations of 0.03 M–0.2 M.

bonding ranged from a high of 10 N cm⁻² ($H = 2.635$) using 0.1 M 8G.1 DAP to a low of 1.50 N cm⁻² ($H = 3.06$) using 0.2 M 8G.1 DAP.

DISCUSSION

Expanding on our previous studies employing diazopyruvamides to effect cross linking of collagenous proteins, we have explored the use of PAMAM dendrimers as the tether. These initial studies have demonstrated that the synthetic and photochemical Wolff rearrangements can be duplicated and, further, the dendrimer is an improvement on the solubility properties and the multiplicity of reactive functional groups for cross linking vis-à-vis the PEG-based agent.

The biocompatibility of selected dendrimers has been addressed by Shaunak *et al.* (36) who found that carboxyl terminated G3.5 PAMAM dendrimers that are coupled to aminosaccharides were effective in preventing scar tissue

formation after glaucoma filtration surgery on rabbit eyes. The PAMAM dendrimers possessing terminal carboxyl groups were chosen because the carboxylate groups are deprotonated (at physiologic pH), showing low toxicity in *in vivo* studies (37–39).

The use of a first-generation PAMAM dendrimer (G.1 PAMAM) has served as our proof of concept as a cross linking agent. The synthesis of diazopyruvyl PAMAM dendrimer (8G.1 DAP, 1.3) was accomplished by essentially the same chemical transformations employed with DPD (31) with minor modification to the reaction workup and reagent preparation.[‡] To maximize the yield of 1.3, 10–12 equivalents of *p*-nitrophenyl diazopyruvate/G.1PAMAM were used. The excess reagent and *p*-nitrophenol was easily removed by extraction of an aqueous solution of the crude product with diethyl ether. Lyophilization of the aqueous layer gave the powdery octa-diazopyruvyl PAMAM in 55% yield.

8G.1 DAP (1.3) is a stable, water-soluble solid which shows characteristic absorption maxima at 256 and 300 nm corresponding to the diazocarbonyl chromophore (Fig. 3, $t = 0$ min). The mass spectrum and ¹H NMR indicate that nearly all of the terminal NH₂ groups on the G.1 PAMAM dendrimer were converted to pyruvamide functional groups. The NMR further shows that the diazopyruvamide functional groups are a mixture of *s-cis* and *s-trans* conformers. It has been suggested that the photochemical reactions of the two conformers may differ, the *s-cis* undergoing the Wolff rearrangement (possibly through a concerted process) whereas the *s-trans* forms a trappable α -ketocarbene (40–42). Exploratory photochemistry of 8G.1DAP in methanol at 300–350 nm (Scheme 3) showed that 1.3 was converted rapidly and completely to the methyl ester through the Wolff rearrangement following the loss of N₂ which could be detected visually during the course of the reaction by its effervescence from the solution. No indication of a methoxy ketone from a trapped carbene was found (UV and mass spectra).

Cross linking studies with 1.3 on dehydrated collagen with glass and with collagen itself gave results similar to those found earlier with DPD. Our results with cornea-to-cornea bonding with 8G.1 DAP were encouraging as samples with low hydration levels ($H < 1$) gave strong bonding, some even exceeding the strength of DPD cornea-to-cornea bonds. In fact, the maximum tensile strength ($n = 17$) was 92 N cm⁻² or nearly twice the maximum ($n = 17$) of 47 N cm⁻² found for DPD. At higher degrees of hydration ($H > 3$), however, bonding was much weaker, paralleling that for DPD.

Although our earlier studies with DPD (31,43) had shown that an added co-monomer, EDEA, improved the DPD mean bonding tensile strength for rabbit Achilles tendons, it had no effect on the corneal collagen with 8G.1 DAP at intermediate hydration levels.

Glass-to-corneal stroma bonds with 8G.1 DAP at low to moderate hydration levels gave tensile strengths of 37 N cm⁻² which closely parallel the results with DPD (34 N cm⁻²).

These results suggest that 8G.1 DAP is capable of producing stronger cornea-to-cornea bonds and cornea-to-glass bonds at low hydration values compared to DPD. At

[‡]Note: Attempts to derivatize the PAMAM directly using the commercial methanol solutions were unsuccessful.

intermediate degrees of hydration, the bonding capabilities of both 8G.1 DAP and DPD diminish to nearly the same level.

The fact that strong bonds were only obtained at low hydration values implies that the effective length of this generation of the dendrimer as a tether falls short of the length required to effectively crosslink neighboring collagen molecules in hydrated tissue. These results strongly suggest that later generations of PAMAM which provide a longer, more densely functionalized tether will increase the "reach" of the reagent to better breach the gap between collagen fibrils in fully hydrated tissue as well as increasing the probability of covalent attachment to the individual collagen fibrils. As 8G.1 PAMAM has a diameter of *ca* 20 Å (10 Å radius) in the gas phase (44), the overall length of the 8G.1DAP tether was not sufficient to subtend this gap. While PAMAM dendrimers increase in size in aqueous media due to swelling (45), the separation of collagen fibrils is generally estimated to be over 200 Å, much too great a distance for this generation G.1 analog. However, by dehydrating the tissue, the collagen fibrils pack much closer together (46,47) and this, coupled with the increased number of reactive sites using the functionalized dendrimer, allowed cross linking with partially hydrated and dehydrated collagen.

CONCLUSION

A new, multifunctional diazopyruvyl dendrimer cross linking agent has been synthesized and its photochemistry and bonding capabilities explored. 8G.1 DAP successfully cross links dehydrated corneal stromal tissue with glass and with corneal stroma. The limitations due to the level of hydration are similar to those found with DPD.

In accord with a model of corneal stroma dehydration proposed by Fratzl and Daxer (47), the greater distance between collagen fibrils lies at the heart of the low bonding strength. Multifunctional diazopyruvamide derivatives such as 8G.1 DAP provide new specific, target-based bonding of nucleophilic domains through enhanced reactivity of photogenerated ketenes. Such an approach affords covalent binding of the cross linking agent under conditions of both spatial and temporal control through a light-activated generation of an active electrophile. The success of 8G.1 DAP and the promise of higher generation dendrimers as suitable biomaterials for use in tissue bonding continues to be a major thrust of our studies.

One approach that addresses the limitation due to the separation of the corneal stroma is to extend the "reach" of the dendrimer. Higher generations of G.X PAMAMs are being explored as well as the incorporation of 1,*n*-diamines to tether G.X DAP derivatives. Both approaches also have their limitations, however. For example, Maiti *et al.* (44,45) showed that PAMAMs above Generation 10 become highly strained, thus much less flexible. Generation 10 PAMAMs have a gas phase radius of 70 Å, well below the 200 Å separating the fibrils. The use of 1,6-diaminohexanes with diazopyruvyl cross linkers has been explored earlier by us (31) with modest success. We have synthesized a G.3 DAP analog which is currently being examined and are exploring the incorporation of 1,6-diaminohexanes with our 8G1 DAP derivative.

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