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Processing of Type I Collagen Gels Using Non-Enzymatic Glycation

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Abstract

This study focuses on the development of a novel method of non-enzymatic glycation of fibrillar collagen gels. In contrast to previous studies in which type I collagen gels were glycated in the solid state, this study presents a method for glycation in solution. Type I collagen in solution or gels was exposed to a range of ribose concentrations from 0–250mM. The binding of ribose to collagen was documented using Fourier Transform Infrared (FTIR) Spectroscopy. Formation of advanced glycation endproducts (AGEs) was quantified by fluorescence measurement. The bulk compressive modulus and viscoelastic time constant of processed gels were determined in stress relaxation studies. Both methods of glycation enhanced ribose addition and AGE formation in a dose dependent manner, with glycation in the gel state being more efficient. Both methods enhanced mechanical properties similarly, with 250mM ribose treatment resulting in a 10-fold increase in bulk modulus.

Keywords

tissue engineering; collagen; hydrogel; scaffold; mechanical properties; FTIR; non-enzymatic glycation

Introduction

Hydrogels made from materials such as collagen have been documented in several studies as scaffolds for tissue engineering [1–4]. Collagen gels have been used in the tissue engineering of skin, cartilage, tendon, blood vessels and other tissue substitutes [5,6]. However, the main limitation of collagen gels in tissue engineering applications is their lack of mechanical strength and the inability of such materials to maintain their shape after the addition of cells [6]. In tissue engineering shape retention is critical for many applications, with previous studies demonstrating that the final shape of implants is dependent on both the initial mechanical properties as well as the degradation rate of the gel [7].

To overcome the obstacles posed by the high innate compliance of such materials, collagen scaffolds have been processed by many different methods. Crosslinking agents such as glutaraldehyde and carbodiimide reagents as well as physical crosslinkers such as dehydrothermal treatment, photo oxidation, microwave and ultraviolet radiation [8–10] have been used to strengthen scaffolds for biomedical applications. However, a drawback to all of these

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methods is their inherent cytotoxicity, and, thus, they are not appropriate for *in situ* crosslinking of cell-seeded constructs [11–13]. This limits the utility of collagen as a cell delivery vehicle for injectable and injection molding applications [1,14–18]. Few studies document methods of collagen crosslinking that are not cytotoxic. An exception to this are enzymatic crosslinkers such as lysyl oxidase or transglutaminase, and non-enzymatic glycation with reducing sugars, which have been introduced as methods to crosslink cell-seeded constructs [1,19–24]. While enzyme crosslinkers may not be cytotoxic, they may be inefficient due to competitive inhibition from the large amounts of proteins synthesized in culture [22,23,25].

Non-enzymatic glycation is the process by which reducing sugars post-translationally crosslink proteins, such as collagen. Glycation has been studied primarily as a phenomena associated with diabetes and aging [26–29]. In the pathogenesis of these conditions, the accumulation of advanced glycation endproducts has been shown to change the mechanical and biochemical properties of tissues *in vivo* [30–33].

The mechanism of non-enzymatic glycation, the Maillard reaction, involves the reaction of the aldehyde group on reducing sugars, such as glucose or ribose, in their open chain form with amino groups on collagen. This reaction produces a Schiff base that can then undergo an Amadori rearrangement to form an Amadori product, which is one of the known early intermediates of the glycation process 26,27. These Amadori products then go on to form heterogeneous products called advanced glycation endproducts (AGEs), under both oxidative and non oxidative conditions, the intermediates of which still remain unclear [34,35]. The large diverse set of AGEs has yet to be completely characterized, however, reaction products are often characterized by the inherent fluorescence of a percentage of the endproducts 26,27,[36–39].

In the field of tissue engineering the process of non-enzymatic glycation has been exploited primarily for skin and blood vessel substitutes. These studies have focused on the ability of glycation to inhibit contraction of cell seeded gels, and to stiffen and strengthen blood vessel tissue equivalents [24,32,40,41]. *In vitro* glycation has been documented to decrease the degradation rate of collagen gels and to change the properties of tissues *in vitro* and *in vivo* 32,40,[42]. The objective of this study was to use the known mechanisms of glycation to develop a technique for processing of collagen in the liquid and solid state for the ultimate use as a biomaterial or scaffold for cell-based tissue engineering techniques. This study focuses on the development of a novel method of non-enzymatic glycation where glycation occurs in solution (pre-glycation), to allow for a build-up of Amadori intermediates, instead of the documented use of glycation to process the solid collagen gel (post-glycation). This study then aims to compare changes in the chemical composition and quantify the enhancement of mechanical properties of collagen gels resulting from glycation in the solid state and the liquid state.

Methods and Materials

Collagen Gels

Acid soluble Type I rat tail collagen (Sigma, St. Lois, MO) was solublized in cold 0.1% acetic acid at a concentration of 3mg/mL for at least 72 hrs at 4°C. To form gels for both the post and pre-glycated conditions, the collagen solution was diluted on ice with 0.1% acetic acid and neutralized with 0.4 M NaOH (pH 10.2) in 10X D-PBS (Invitrogen, CA) and 250mM HEPES (Sigma, St. Lois, MO) buffer to form 1.5 mg/mL gels with final concentrations of 1X D-PBS and 25mM HEPES [43]. A 750 μL aliquot of the neutralized collagen solution was pipetted into the wells of a 24-well plate and incubated at 37°C for 45 minutes to allow for fibril formation. All gels were washed extensively with distilled water prior to analyses.

Post-glycated gels were conditioned with ribose after gel formation. Specifically, these gels were incubated at 37°C and 5% CO_2 with 0, 25, 50, 100, or 250mM ribose in a solution of 1X PBS with 44mM $NaHCO_3$ and 25mM HEPES buffer for 5 days. On the fifth day the gels were washed extensively with distilled water and used for studies immediately.

Pre-glycated gels were conditioned with ribose before gel formation. In this conditioning method, the solution was exposed to ribose for 5 days while the collagen was still in solution. To make pre-glycated gels the cold collagen solution was mixed with ribose in 0.1% acetic acid to final concentrations of 0, 25, 50, 100, or 250mM ribose and incubated at 4°C for 5 days prior to the gelation process, and then gelled on ice as described above. These gels were then washed extensively with distilled water after fibril formation (neutralization and subsequent 45 minute incubation at 37°C) and used for studies immediately. Although cells were not seeded into gels in these studies, it has been demonstrated previously that the brief exposure to hyperosmotic conditions does not affect long term metabolic activity of encapsulated cells [44]..

Fluorescence

Each 750μl collagen gel was digested with 1 mL of 0.0025% papain buffer 45،46. Fluorescence was measured at an excitation of 360nm and emission of 465nm with a Perkin Elmer Bioassay Reader HTS 7000 [39]. Non-fluorescent 96-well assay plates (Nunc, Rochester, NY) were used to reduce background fluorescence. Data was corrected for background fluorescence from papain buffer and normalized to the dry weight of collagen.

FTIR

During glycation there is addition of sugar to the gels which produces the early Amadori product intermediates. To quantify this sugar addition to the gels in both glycation methods, Fourier Transform Infrared (FTIR)-attenuated total reflectance (ATR) spectroscopy was used. FTIR spectroscopy was performed using a Thermo Electron Nicolet 4700 Spectrometer. Spectra recorded with 4cm^{-1} spectral resolution under nitrogen purge. The spectra were baselined, and spectral peaks and areas were determined using Bio-Rad 3.1 FTIR software. Sugar peak areas (900–1100 cm^{-1}) were normalized to amide I (1690 cm^{-1}) areas which serve as a measure of collagen content [47].

FTIR spectra were curve fit using MOFFAT (public domain software) to analyze the underlying components of the amide I band. Initial positions of Gaussian underlying bands were input based on previous studies of the spectroscopic characterization of collagen crosslinks in bone [48]. Spectra were iteratively fit until there was less than a 0.1 change in relative percent area and peak position.

Viscoelastic Properties

Quantification of viscoelastic properties of collagen gels was performed on an Enduratec EL2100 mechanical testing frame using a 1000 gram load cell. Collagen gels 10 mm × 1 mm were placed in a confined compression chamber and ten 50 micron step displacements were imposed on gels and the resultant loads were measured to within 0.1 grams. Stress relaxation data was fit to a standard linear solid of viscoelastic behavior, using the equation, $\sigma_{eq}=A(1-e^{-t/\tau})+B$, where A+B is the equilibrium stress and B is the instantaneous stress, t is time, and τ is the exponential time constant. A, B, and τ were solved for by minimizing RMS error between the model and the data for each step. The calculated equilibrium stress at each step was plotted vs. the imposed strain and the slope of this curve was calculated as the equilibrium modulus [49].

Statistics

Two-way analysis of variance (ANOVA) tests were performed to determine statistical significance between concentrations and method of glycation using SigmaStat software. Analysis of covariance (ANCOVA) tests were performed on SPSS statistical software to determine if there was statistical significance between slopes of regression curves for pre and post-glycated gels. Additional comparisons using a Student's t-test to compare simple linear regression equations was used to confirm ANCOVA results [50]. Statistical significance was assumed for p values less than 0.05.

Results

Fluorescence was used to determine the amount of AGE accumulation. There was an increase in the fluorescence in both the post and the pre-glycated conditioned gels with an increase in ribose concentration during incubation ($p<0.05$ both) (Figure 1). There was, however, a significantly higher amount of fluorescence in the post-glycated case for all concentrations except for controls and 50mM gels ($p<0.01$). In both cases AGE accumulation was linearly related to the amount of ribose used during glycation ($R^2=0.993$ post-glycated and $R^2=0.851$ pre-glycated, $p<0.05$ both).

FTIR spectra were recorded for collagen and ribose to determine if there were isolated peaks that could be used to distinguish the presence of ribose in the collagen gels (Figure 2A & 2B). Peaks were found for ribose between 980–1100 cm^{-1} that were used to quantify amount of ribose attached (Figure 2A). These correspond to C-O, C-C stretching vibrations and the C-O-H and C-C-O bending vibrations of ribose (Figure 2A) [51]. The area under the amide I band (found between 1590–1720 cm^{-1}) has been shown to be indicative of collagen content was used for normalization (C=O stretching) (Figure 2B) [47]. In both the pre and the post-glycated condition the increase in the amount of ribose attached to the collagen (sugar/amide I area) was linearly correlated with an increase in ribose concentration during processing ($R^2=0.808$ post-glycated, $R^2=0.951$ pre-glycated, $p<0.05$ both) (Figure 2C). Post-glycation caused a significantly higher amount of ribose addition compared to pre-glycation ($p<0.05$). In both conditions, there was a significantly higher amount of ribose addition in the 250mM gels vs. controls ($p<0.05$).

With the amide I band curve fits, the three major sub-bands that were analyzed further were $1660cm^{-1}$, $1683cm^{-1}$, and $1627cm^{-1}$, to determine if there were conformational changes in collagen with glycation. There were no significant differences in the percent area or the peak position for either glycation method for any of the bands. There were also no differences between ribose concentrations within each glycation type for any of these three bands (Table 1). There was, however, a shift in the $1660cm^{-1}$ band to ~$1652cm^{-1}$ in all gels.

Linear correlation analysis was performed on fluorescence and FTIR measurements to find the relationship between the amount of ribose accumulation in the gel with the amount of endproduct formed (Figure 3). There was a significant linear correlation between fluorescence and FTIR (R^2 of 0.82, $p< 0.01$), and there was no statistical difference in the slopes of the lines for the correlations in the pre and post-glycated condition.

For quantification of the impact of glycation on the mechanical properties of the collagen gels, the modulus of the gels was measured in confined compression. Exposure to 250mM ribose increased the modulus ~10-fold over untreated samples. There was a significant increase in modulus in both glycation conditions which is characterized by the linear correlation in modulus vs. ribose concentration during incubation ($p<0.05$) (Figure 4A). The glycation-dependent stiffening of gels was similar for both treatment methods. The stress relaxation behavior was well described by the standard linear model, with RMS errors ranging from 1–

15%, R^2 values ranging from 0.39 to 0.82, and resulting p values ranging from .001 to .05. RMS errors and R^2 values were similar for both glycation methods and all ribose concentrations. The viscoelastic model also included the calculation of an average viscoelastic time constant for each sample, which was then averaged to find a time constant for each ribose concentrations. Ribose exposure did not change the viscoelastic time constant for either pre or post-glycated gels (Figure 4B).

Linear correlation analysis was performed on FTIR and fluorescence data to determine whether or not the mechanical properties were related to the amount of AGE accumulation and ribose addition (Figure 5A & 5B). There was a significant correlation between the modulus and fluorescence (R^2=0.813 post-glycated, R^2=0.996 pre-glycated, $p<0.05$ both) and the modulus and FTIR (sugar/amide I area) (R^2=0.945 post-glycated, R^2=0.970 pre-glycated, $p<0.05$ both) for both glycation conditions indicating that with increases in modulus there were also increases in AGE accumulation or ribose addition, respectively. The slopes of the linear regression curves were statistically different dependent on glycation method ($p<0.01$), with pre-glycated gels having higher slopes in both correlated parameters when modulus was considered the dependent variable (interchanging the dependent and independent variables did not affect significance). This indicates that in the pre-glycated condition less ribose attachment or AGE accumulation was necessary for a similar increase in modulus when compared to the post-glycated condition.

Discussion

This study documents the use of a novel method of non-enzymatic glycation of fibrillar, type I collagen gels, where the collagen is glycated in solution prior to gelation. Glycation in solution has been studied in type IV collagen [52], but to date has not been exploited for use with fibrillar collagen. In previous studies type I collagen gels have been exposed to non-enzymatic glycation in order to change the physical properties of the scaffolds for tissue engineering. Previous studies have also demonstrated that glycation of gels that contain cells is toxic due to high osmolarity, however glycation in solution enables extended viability and maintains high metabolic activity [44]. The current study compares these two different methods of non-enzymatic glycation and the resulting changes in physical properties and biochemical properties of the scaffolds.

Similar to other studies in the literature, fluorescence was used as a measure of extent of glycation. In both the pre and the post-glycated condition the fluorescence increased with ribose concentration in culture. This indicates that there was concentration dependent dose response of AGE formation with ribose in culture. A novel method of quantitatively assessing glycation with FTIR showed a similar concentration dependent dose response in the amount of ribose bound to collagen. When these two parameters were analyzed together, the amount of ribose addition was directly related to AGE formation independent of glycation method. This suggests that the reaction by which AGEs form from glycated collagen has a first order rate constant, and that the ribose that attaches to the gels, or the Amadori intermediates, go on to form the fluorescent endproducts (which are a percentage of the total AGEs formed). It may be possible to calculate the rate constant, k, for the forward reaction, if known concentrations of solid ribose and collagen in a heterogeneous mixtures were used to determine a standard curve using FTIR spectroscopy. These values could then be used to calculate the actual concentrations ribose and collagen that was found in the gels after ribose incubation. It is important to realize, however, that the percentage of fluorescent AGEs vs. non-fluorescent molecules may be very small and has been recently postulated to be only about 1% of the heterogeneous set of crosslinking products that may be formed by glycation under physiological conditions [27,28]. One limitation of the current study is that only fluorescent AGEs were measured. To more precisely determine which AGE crosslinks play a mechanical role, it would be necessary to measure AGE crosslinks

more thoroughly with techniques such as high performance liquid chromatrography and gas chromatrography/mass spectroscopy 53–55.

The amide I band in the FTIR spectra were curve fit to determine the peak position and percent area of the underlying bands. In these methods, the 1660cm^{-1} band is representative of the collagen triple helix, the 1683cm^{-1} band is representative of reducible crosslinks, and the 1627cm^{-1} band is representative of hydroxyproline. There were no significant differences in these bands with ribose concentration or with either glycation method, suggesting that the conformation of the collagen was not altered by ribose attachment. There was, however, a shift in the 1660cm^{-1} band to about 1655cm^{-1} suggesting less triple helical collagen in these gels.

The mechanical properties of the gels were measured in confined compression to determine a compressive modulus and viscoelastic time constants. There was a significant increase in the modulus of the gels that was dependent on concentration of sugar in culture, but independent of glycation method. These results correlated well with other studies which showed decreased compaction with glycation as a measure of the stiffness [40], although this study is the first to report the compressive modulus of acellular collagen gels. Other hydrogels, such as alginate and agarose have reported moduli at ~5kPa 49,56 and another commonly used scaffold for tissue engineering, PGA mesh, has a reported compressive modulus of ~1kPa 57. The collagen gels were found to be generally weaker than these other materials with the highest moduli ~1 kPa, but there was a 10-fold increase in stiffness with the highest ribose concentrations used compared to control gels. In another study the dynamic elastic modulus for 1% collagen gels was reported to be ~4 kPa, which supports the results in the present study [58]. There was no correlation between the viscoelastic time constant and the concentration of ribose in solution for either the pre or post-glycated conditions. In a study of collagen gel crosslinking it was reported that there were increases in the time constant (τ) (in a Voigt-Kelvin creep compliance model) with increases in concentrations of glutaraldehyde crosslinking [58]. These time constants, however, were determined for a collagen gel solution and not a solid gel matrix such as the ones used in this study. Other studies of glycation of type I collagen gels also show increased resistance enzymatic to degradation both of cell seeded gels and acellular gels, and although similar studies were not included in the current set of experiments, it is likely that these are also more resistant to enzymatic degradation due to increased crosslinking 32,59.

Correlation analysis indicated a linear relationship between gel modulus and both ribose addition (FTIR) and AGE formation (fluorescence) 36,53,59,60. This relationship was dependent on the glycation method. The pre-glycated gels had similar moduli to the post-glycated gels for all concentrations of ribose in the incubating media; however the pre-glycated gels showed a greater increase in modulus (slope) than post-glycated gels for the amount of ribose attachment and AGE formation measured in the collagen gels. The correlation between mechanical properties and AGEs indicates that the set of heterogeneous endproducts formed in the two different processing methods are different and that fluorescence may not be the ideal indicator of AGE formation. The ratio of non-fluorescent to fluorescent crosslinks in pre-glycation and post-glycation are likely different. Because AGEs are a set of heterogeneous products that are not yet completely identified [27,28], it is possible that different molecular crosslinks were produced by the different processing methods, and not all of the crosslinks affect the compressive modulus of the glycated gels. Future studies including the measurement of concentrations of specific AGE crosslinks in each glycation method may account for the difference between the correlations between modulus and ribose addition. The correlation between the mechanical and chemical properties in both glycation conditions is consistent with what would be expected for a polymer network, where the crosslink density is expected to be proportional to the modulus 61,62. This relationship also has been shown in more complicated systems such as cartilage, tendon, and ligament [63–65].

The use of non-enzymatic glycation to condition collagen gels may be important for tissue engineering techniques in that it provides a method to condition scaffolds that is not toxic to cells. The advantages of glycating in solution over glycation in the solid state include the ability to introduce cells into gels, without exposing them to the detrimental influence of a hyperosmotic environment [24,44]. Large osmotic loads have been shown to affect gene expression, intracellular pH and calcium levels, cellular metabolism as well as the physical and viscoelastic properties of the chondrocytes [66–75], making the previously studied method of glycation disadvantageous.

Pre-glycation also allows for the *in situ* crosslinking of cell seeded collagen gels that may be able to be delivered *in vivo* as an injectable. In this novel method the viscosity of the solution remains low, which is central to injection and injection molding techniques [9,10,12,13]. It would be beneficial to be able to modulate or control the properties of collagen gels with the addition of cells for tissue engineering techniques, because in many applications the delivery of cells is necessary for functional tissue regeneration as cells create and remodel the extracellular matrix [1].

These methods to glycate collagen gels *in vitro* are also valuable as they serve as a way of altering the mechanical properties of the collagen, while studying cell behavior in reaction to differences in the mechanics and biochemical nature of the extracellular matrix. These models are essential not only for tissue engineering or applied biomedical applications, but also for the study of the pathology of diabetes and aging [26,76].

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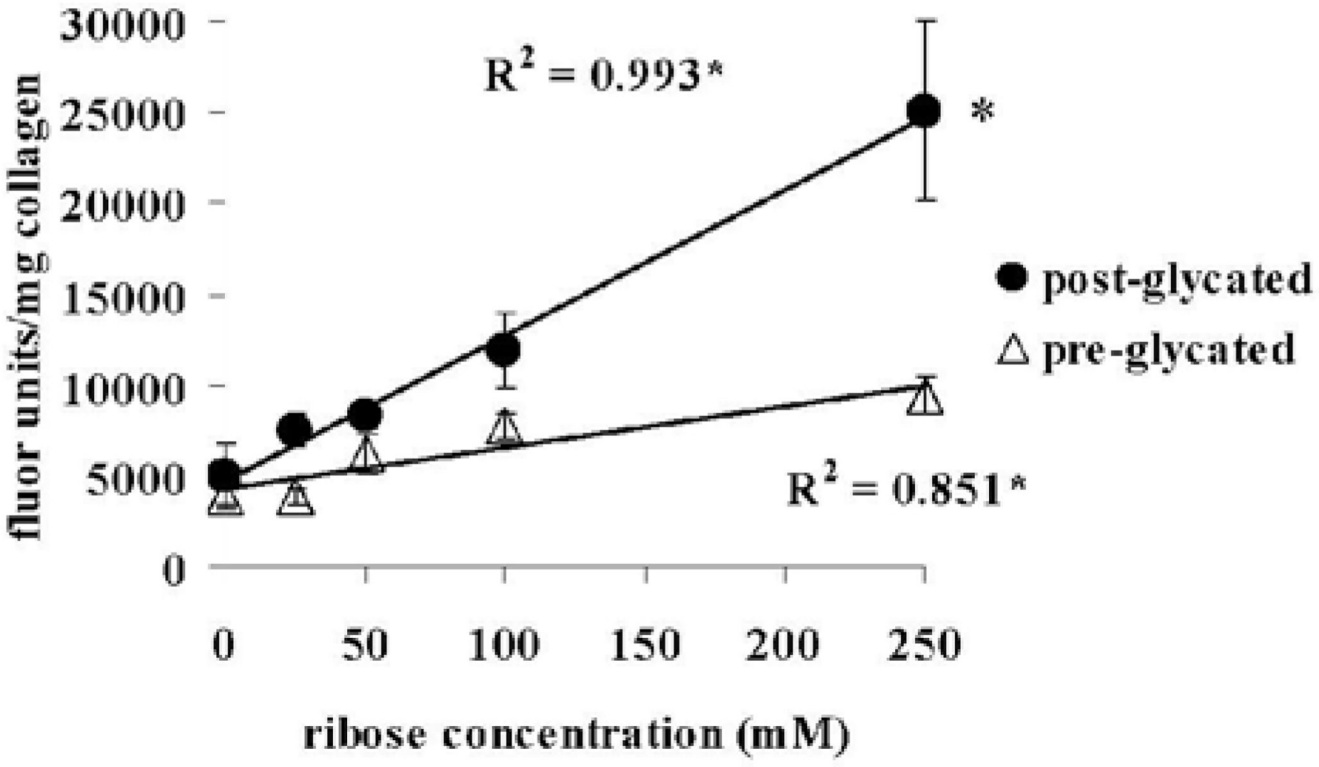


Figure 1.

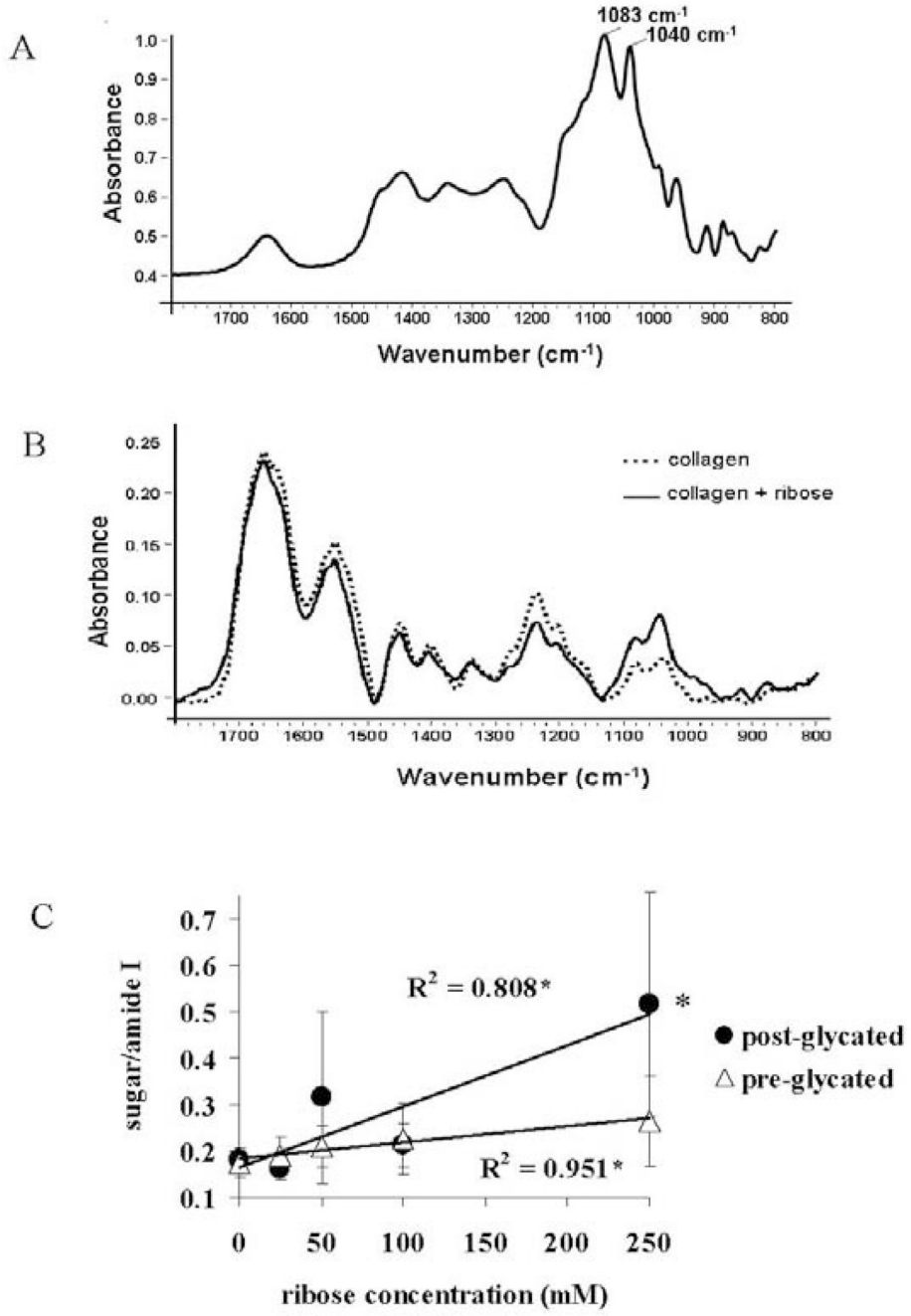
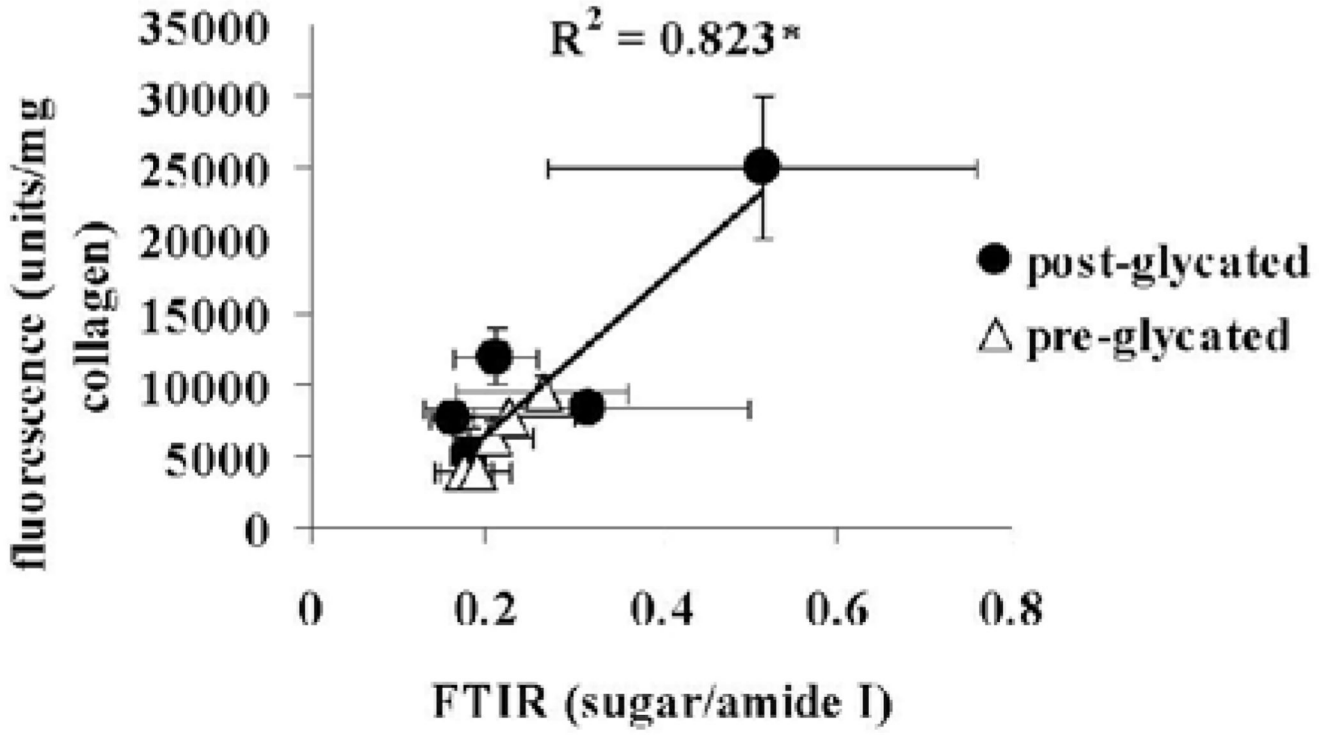


Figure 2.



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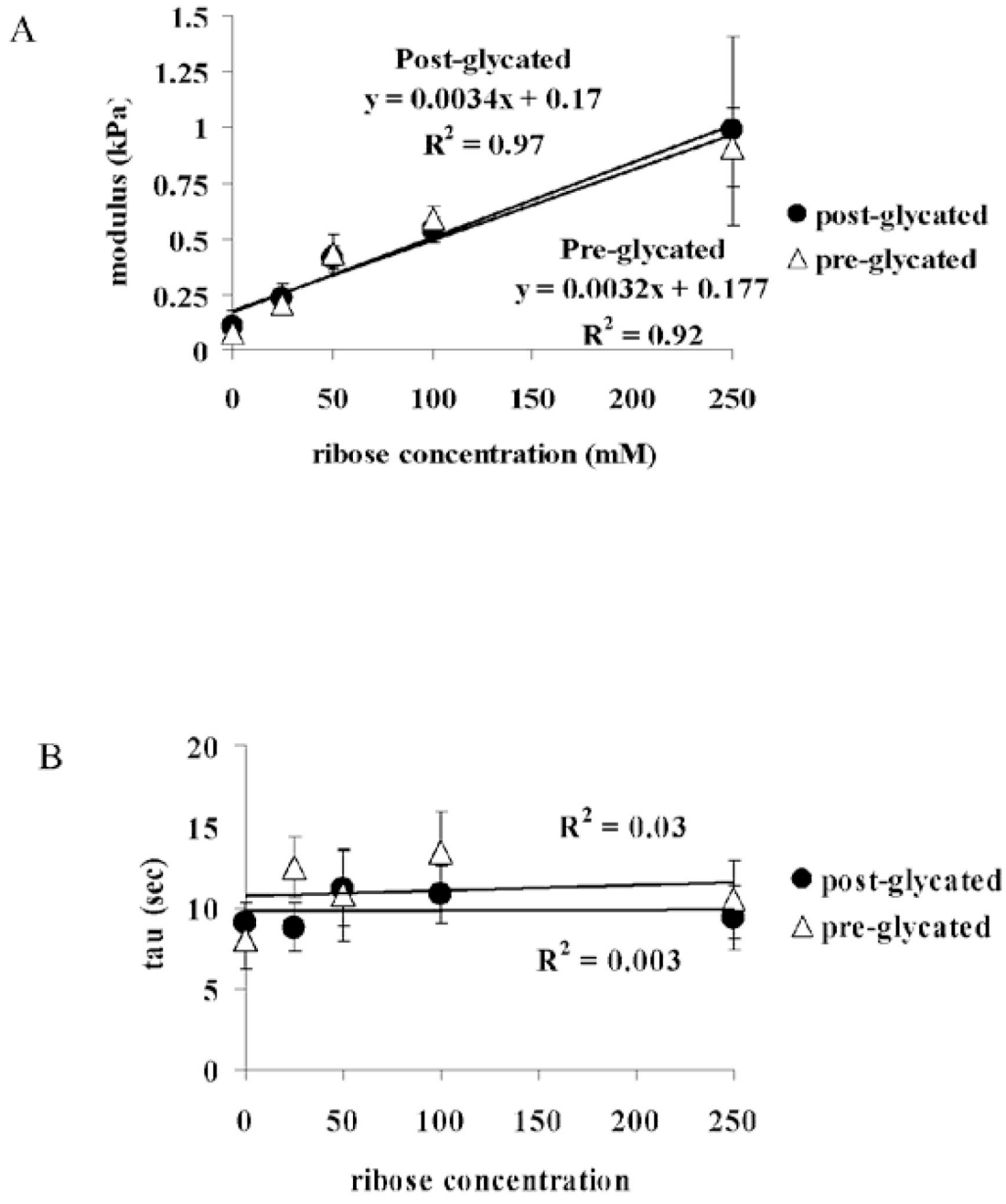

Figure 4.

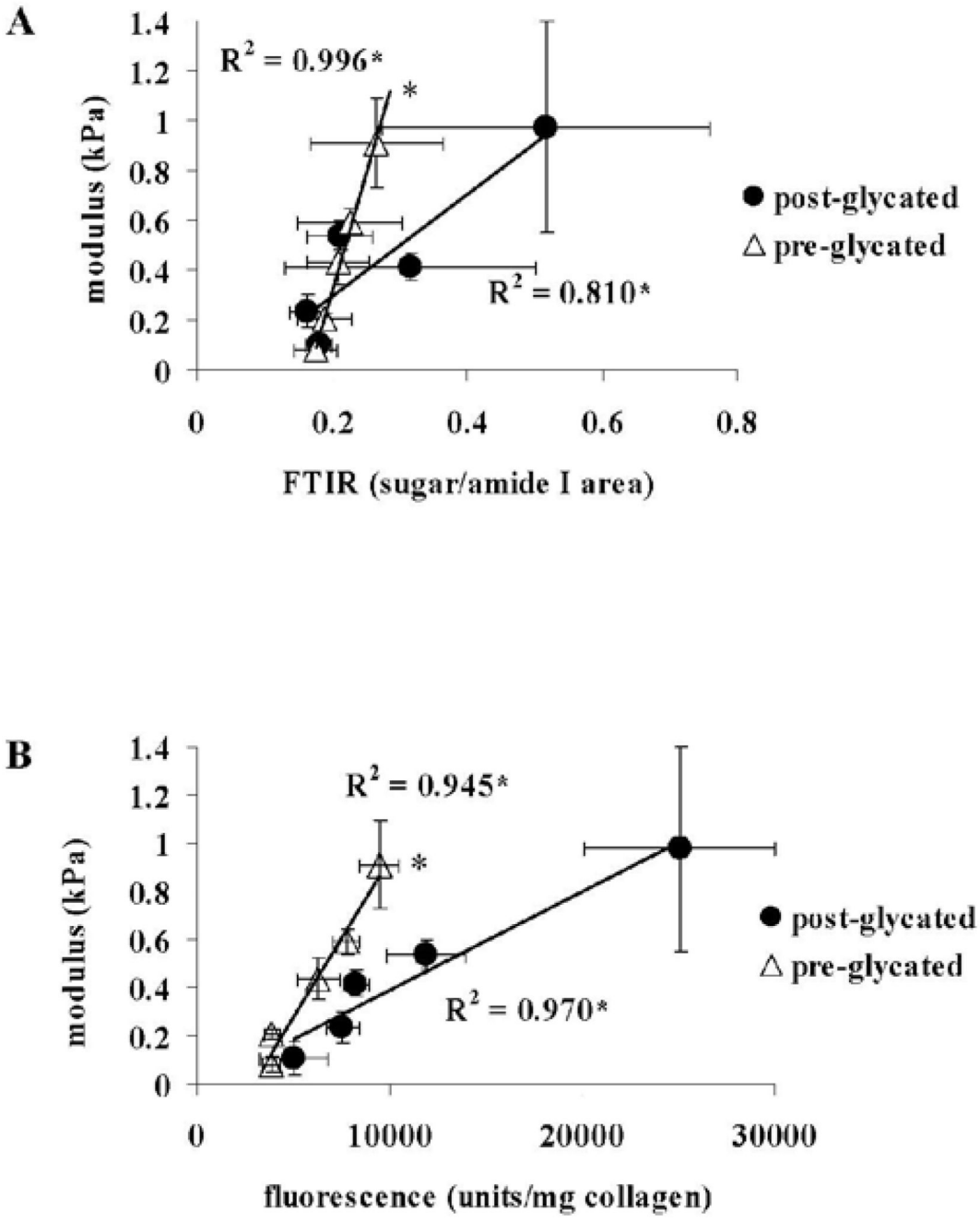

Figure 5.

Table 1

Effect of ribose concentration on peak position and % area of FTIR curve fits

Curve fits of amide I band showed that the peak position of the three major bands (a) 1660cm^{-1}, (b) 1683cm^{-1}, and (c) 1627cm^{-1} did not differ significantly between or within glycation methods. There was a shift in the 1660cm^{-1} band to about 1652cm^{-1}, suggesting less triple helical collagen, (a) The underlying 1660cm^{-1} band of amide I had relative percent area of ~40%. (b) The band at 1690cm^{-1} had a relative percent area ~3–4%. (c) The band at 1627cm^{-1} had a relative percent area of ~10%. There were no statistical differences in any of the percent areas with either method of glycation. n=4–6 ± stdev

a)	Band I - 1660cm^{-1}				
	Conc (mM)	Peak Position		% Area	
		Post-glycated	Pre-glycated	Post-glycated	Pre-glycated
	0	1650.6 ± 2.3	1650.6 ± 2.8	36.0 ± 6.6	41.4 ± 5.0
	25	1650.3 ± 1.6	1649.3 ± 2.4	44.3 ± 1.2	41.8 ± 2.3
	50	1653.4 ± 1.6	1649.9 ± 2.6	35.1 ± 5.5	41.5 ± 3.1
	100	1650.2 ± 1.2	1652.3 ± 1.6	42.3 ± 0.4	38.5 ± 4.9
	250	1653.7 ± 1.3	1652.2 ± 2.5	36.0 ± 5.4	36.6 ± 8.2

b)	Band II - 1683cm^{-1}				
	Conc (mM)	Peak Position		% Area	
		Post-glycated	Pre-glycated	Post-glycated	Pre-glycated
	0	1687.0 ± 6.0	1690.7 ± 2.0	5.4 ± 3.8	2.8 ± 1.5
	25	1692.1 ± 0.3	1691.0 ± 0.1	2.0 ± 0.3	2.2 ± 0.3
	50	1688.5 ± 3.0	1690.8 ± 0.9	4.6 ± 1.8	2.6 ± 1.0
	100	1691.1 ± 0.4	1689.7 ± 2.1	2.3 ± 0.3	3.9 ± 1.3
	250	1690.2 ± 1.6	1688.0 ± 3.8	3.4 ± 0.7	4.4 ± 1.8

c)	Band III - 1627cm^{-1}				
	Conc (mM)	Peak Position (cm^{-1})		% Area	
		Post-glycated	Pre-glycated	Post-glycated	Pre-glycated
	0	1627.3 ± 1.5	1627.9 ± 1.6	12.1 ± 4.8	8.7 ± 3.5
	25	1628.5 ± 0.7	1626.7 ± 1.7	6.2 ± 0.5	8.9 ± 2.9
	50	1628.2 ± 1.0	1627.8 ± 1.1	13.3 ± 4.9	7.9 ± 2.2
	100	1627.7 ± 0.4	1628.0 ± 0.5	8.2 ± 0.8	11.2 ± 3.6

c)

Band III - 1627cm^{-1}					
Conc (mM)	Peak Position (cm^{-1})		% Area		
	Post-glycated	Pre-glycated	Post-glycated	Pre-glycated
250	1627.9 ± 1.1	1628.2 ± 1.3	12.9 ± 4.9	13.0 ± 6.1