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RESEARCH ARTICLE

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# Pre-glycation impairs gelation of high concentration collagen solutions

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## Abstract

There remains a need for stiffer collagen hydrogels for tissue engineering and disease modeling applications. Pre-glycation, or glycation of collagen in solution prior to gelation, has been shown to increase the mechanics of collagen hydrogels while maintaining high viability of encapsulated cells. The stiffness of glycated collagen gels can be increased by increasing the collagen concentration, sugar concentration, and glycation time. However, previous studies on pre-glycation of collagen have used low collagen concentrations and/or low sugar concentrations and have not investigated the effect of glycation time. Therefore, the objective of this study was to determine the effects of pre-glycation with high sugar concentrations (up to 500 mM) and extended glycation times (up to 21 days) on high concentration collagen (8 mg/ml). The addition of sugar to the collagen and the formation of advanced glycation end products (AGEs) were quantified. The ability to gel successfully and rheological properties were determined and correlated with biochemical characterizations. Successful collagen gelation and rheological properties of pre-glycated collagen were found to be strongly dependent on the ratio of added sugars to added AGEs with high ratios impairing gelation and low ratios resulting in optimal storage moduli. There is likely a competing effect during pre-glycation of the formation of AGEs resulting in crosslinking of collagen and the formation of Amadori intermediates acting to increase collagen solubility. Overall, this study shows that collagen glycation can be optimized by increasing the formation of AGEs while maintaining a low ratio of added sugar to added AGEs.

**KEYWORDS**
advanced glycation end-products, hydrogels, nonenzymatic glycation, rheology, ribose

## 1 | INTRODUCTION

Collagen is a common biomaterial used for tissue engineering, regenerative medicine, and disease modeling. A main limitation of collagen hydrogels is their poor mechanical properties.[1–6] Collagen hydrogels typically exhibit stiffnesses that range from 0.1 to 10 kPa depending on collagen concentration. These stiffnesses are sufficient for soft tissue applications, but higher stiffnesses are desired to model stiffer tissue types and disease states. For instance, the stiffness of arterial walls is 10–1000 kPa,[7–9] the stiffness of cartilage is 1 MPa,[7–9] the stiffness of bone is 1 GPa,[7–9] and certain breast cancers can have stiffnesses of 1–50 kPa.[7,10] Therefore, there remains a need for stiffer collagen hydrogels for a wider variety of applications.

Additionally, collagen can be used to study diseases such as atherosclerosis and cancer by altering the chemical composition of the hydrogel. For example, calcific aortic valve disease has been studied

  

by seeding cells into collagen gels containing hydroxyapatite[11] and the invasiveness of cancer cells in diabetic patients has been studied by seeding cells in collagen gels glycated with sugar.[12] Stiffer collagen hydrogels that can be chemically tuned could be advantageous for studying diseases such as arthritis, diabetes, atherosclerosis, or cancer.

Methods to increase the stiffness of collagen gels have included mixing with other natural[13] and synthetic biomaterials,[14] increasing collagen concentration,[15,16] enzymatic crosslinking,[17,18] and nonenzymatic crosslinking. Nonenzymatic crosslinkers include glutaraldehyde,[19] carbodiimide,[20] riboflavin,[16,21] and reducing sugars (e.g., glucose, ribose).[22–24] However, except for glycation, these methods are usually limited to acellular applications or applications before cell-seeding due to their risk of cytotoxicity.

Glycation, particularly pre-glycation (i.e., glycation in solution prior to gelation), has been shown to enhance collagen gel mechanics while maintaining a high level of viability of encapsulated cells.[12,22,23,25–28] Glycation is the process by which a sugar reduces in the presence of a lysine residue on collagen to form an Amadori intermediate. Amadori intermediates can then further react with lysine or arginine residues to form advanced glycation end-products (AGEs). These AGEs can then form crosslinks between collagen fibers.[29–31]

Glycation can be used to increase the stiffness of collagen gels and to model conditions such as diabetes and aging, which are characterized by increased sugar content and accumulation of AGEs. Glycation has been used to increase the mechanics of tissue engineered cartilage,[32,33] tissue engineered bone,[34] tissue engineered blood vessels[35,36]; and has been used to create models of diabetes and aging,[24] models for mechanotransduction studies,[23,37,38] models for neovascularization,[39] and models for cancer.[12,26,27]

The earliest approach to glycation, post-glycation, is often limited to applications before cell-seeding.[35,39] This is because for post-glycation, collagen gels must be exposed to high sugar concentrations for extended periods of time. The hyperosmotic environment this creates could be cytotoxic to encapsulated cells. Previous studies have shown that pre-glycation methods can be used to overcome this challenge.[12,22,23,25–28,32] With pre-glycation, collagen is glycated while still in the solution phase. This allows for cells to be encapsulated in the gel at the time of gelation and prevents the need for extended culture with sugar solutions. For example, Roy et al. found that the number of viable cells was significantly reduced when they were encapsulated in a post-glycated collagen gel compared to non-glycated controls. However, there was no difference found in the number of viable cells between pre-glycated collagen gels and non-glycated controls.[32]

Previous studies on glycated collagen have shown that increasing collagen concentrations[27] and increasing sugar concentrations[22,23,27] results in increased equilibrium compressive moduli. Additionally, extended glycation times results in increased tensile moduli.[35] However, previous studies on the effect of pre-glycation of collagen gels have used low collagen concentrations (1–3.5 mg/ml)[12,22,23,25,26] and/or low sugar concentrations (50–100 mM)[27,28] and relatively short glycation time (5 days).[12,22,23,25–28] We hypothesize that stiffer collagen gels, which would be more relevant for tissue engineering or disease modeling applications, could be created by pre-glycating collagen using higher collagen concentrations, higher sugar concentrations, and longer glycation times.

Higher collagen concentrations provide denser matrices and provide more binding sites for AGEs to form.[27] Higher ribose concentrations increase the amount of sugars that can bind to the collagen and, therefore, increase the number of AGEs that form.[22,23] And, as has been shown using post-glycation, longer glycation times allow for more of the sugars to reduce and form AGEs.[35,40] However, the effects of pre-glycation using high ribose concentrations and extended glycation times have not been studied using high concentration collagen. Therefore, the objective of this study was to determine the effects of pre-glycation with up to 500 mM ribose and glycation times up to 21 days on high concentration (8 mg/ml) collagen.

## 2 | MATERIALS AND METHODS

### 2.1 | Collagen preparation

Type I collagen was prepared as described previously.[41] Briefly, tendons from rat tails (BioIVT, Westbury, NY) were soaked in 0.1% acetic acid (Kodak, Rochester, NY) for at least 48 h. The solution was then centrifuged for 90 min at 9000 RPM. The supernatant was then collected, frozen, and lyophilized. A stock collagen solution was then created by reconstituting the lyophilized collagen in 0.1% acetic acid at 15 mg/ml.

Pre-glycation was achieved by mixing stock collagen with ribose (Sigma-Aldrich, St. Louis, MO) in 0.1% acetic acid to final ribose concentrations of 0, 125, 250, 375, or 500 mM. After mixing, these solutions were left at 4°C for up to 21 days. Ribose was chosen as the reducing sugar for this study due to its higher efficiency in glycating collagen compared to other sugars, such as glucose.[35]

To form gels, pre-glycated and control collagen solutions were neutralized with 1× phosphate buffered saline (PBS, Corning cellgro, Manassas, VA), 10× PBS (Corning cellgro, Manassas, VA), and 1 *N* NaOH (Avantor, Center Valley, PA).

These mixtures were then allowed to gel at 37°C before being prepared for Fourier-transform infrared (FTIR) and AGE fluorescence testing. For rheology testing, samples were immediately loaded onto the rheometer after mixing.

### 2.2 | FTIR spectroscopy

FTIR spectroscopy was used to quantify the addition of sugar to collagen due to pre-glycation as described previously.[22] Briefly, pre-glycated gels were rinsed in PBS for 5 min to remove excess ribose, frozen, and lyophilized. Lyophilized samples were then loaded onto a Vertex V80V Vacuum FTIR (Bruker, Billerica, MA) in ATR mode. Spectra were recorded with 6 $cm^{-1}$ resolution under vacuum. Spectra were baselined using OPUS software. Sugar peak areas were defined as those between 900 and 1100 $cm^{-1}$. Sugar peak areas were

normalized to the area of amide I peaks which were defined as between 1590 and 1720 $cm^{-1}$. Peak areas were measured using Spectragryph optical spectroscopy software.[42]

## 2.3 | AGE fluorescence

AGE fluorescence was measured by first freezing and lyophilizing pre-glycated gels as described previously.[22] Briefly, sample dry weights were measured after lyophilizing and then samples were digested in 1 ml of 0.125 mg/ml papain solution for 12–16 h at 60°C. Fluorescence was measured using a BioTek Synergy HT plate reader with an excitation of 360 nm and an emission of 465 nm. Sample fluorescence was corrected for fluorescence from the papain solution and are reported as normalized to sample dry weight.

## 2.4 | Rheology

The storage (*G'*) and loss (*G"*) moduli of pre-glycated collagen before, during, and after gelation were obtained as described previously.[16] Briefly, 25 mm diameter coverslips were coated with glutaraldehyde. These coverslips were then attached to the parallel plate geometry of a DHR3 rheometer (TA Instruments, New Castle, DE). A 1 mm gap was used for all tests. *G'* and *G"* were measured in oscillatory mode at 0.1 Hz and 0.5% stain for 5 min at 4°C and then 30 min at 37°C. All rheological values for pre-glycated collagen are reported as normalized to those of control collagen using the same stock collagen batch.

The addition of sugar to collagen in solution is known to increase the solubility of the collagen.[29,30,43,44] This could interfere with fibrillogenesis and impair collagen gelation. Therefore, the ability to form a gel successfully was also measured. Gelation was deemed successful if *G'* and *G"* crossed and reached a plateau within 30 min with incubation at 37°C.

## 2.5 | Statistical analysis

Linear regressions were performed to determine the effect of ribose concentration on normalized storage modulus before gelation, normalized storage modulus after gelation, normalized dG'/dt, and normalized crossover time. The effect of ribose concentration on sugar: amide I and AGE fluorescence was assessed using a Kruskal–Wallis test with Bonferroni correction. Linear regressions were performed to determine the effect of glycation time on normalized storage modulus before gelation, normalized storage modulus after gelation, normalized dG'/dt, and normalized crossover time. The effect of glycation time on sugar: amide I and AGE fluorescence was assessed by Kruskal–Wallis test with Bonferroni correction. Dose response relationships were calculated by fitting data to a four-parameter sigmoid logistic regression (custom MATLAB code). Data are reported as mean ± standard deviation unless otherwise stated.

# 3 | RESULTS

## 3.1 | FTIR and AGE fluorescence

FTIR spectroscopy was used to quantify the addition of sugar to collagen due to pre-glycation. Non-glycated collagen controls and pre-glycated collagen samples displayed characteristic peaks associated with collagen (Figure 1A). These included peaks for amide I (1630 $cm^{-1}$), amide II (1550 $cm^{-1}$), and amide III (1230 $cm^{-1}$). Pre-glycated collagen exhibited distinct sugar peaks in the 900–1200 $cm^{-1}$ range (Figure 1A). The amount of sugar addition was quantified by dividing the area of the sugar peak by the area of the amide I peak, a method which has been reported previously.[22] Similar peaks were found for collagen pre-glycated with all other ribose concentrations (Figure S1).

The amount of sugar addition increased with increasing ribose concentration (Figure 1B). Collagen pre-glycated with 250 mM ribose and 500 mM ribose had 4 times and 16 times more sugar content than non-glycated controls, respectively.

These FTIR measurements are associated with the amount of sugar bound to the collagen but do not provide any information on the formation of AGEs. The amount of AGEs was quantified using fluorescence since a portion of AGEs are known to be fluorescent. AGEs were found to increase with pre-glycation independent of ribose concentration (Figure 1C). Pre-glycated collagen had 12–19 times more AGEs than non-glycated controls but there were minimal differences in AGE content with varying ribose concentration. The amount of AGEs was correlated with the amount of sugar detected by FTIR and a dose response relationship was found (Figure 1D).

Binding of sugar to collagen occurred rapidly (Figure 2A). By 30 min, addition was 70%–75% complete and was effectively at steady state by 1–3 days. At steady state, sugar: amide I was found to vary between 2.0 and 3.3 for pre-glycated collagen compared to 0.5 for non-glycated controls.

Similarly, the amount of AGE formation was also found to occur very rapidly (Figure 2B). The amount of AGEs formed within 30 min were similar to the amount formed with 2 weeks of pre-glycation. For pre-glycated collagen, the amount of AGEs varied between 6300–10,-100 units/mg compared to 530 units/mg for non-glycated controls. When the amount of AGE formation was correlated with the amount of sugar detected by FTIR for these results, a weak positive correlation ($R^2 = 0.44$) was found (Figure 2C). However, this was dominated by the control group. When the control group is excluded from this correlation, no relationship was found ($R^2 = 0.14$), so we cannot confidently report a linear relationship between sugar addition and AGE formation with varying glycation times.

Rheology.

Rheological testing was used to assess the effect of pre-glycation on the mechanics of collagen before, during, and after gelation (Figure 3). Four outcome measures were determined from this testing: the storage modulus before gelation ($G'_0$), storage modulus after gelation ($G'\infty$), the growth rate of *G'* (dG'/dt), and the crossover time of *G'* and *G"* ($t_c$). $G'_0$ was determined by averaging *G'* of the first 5 min of

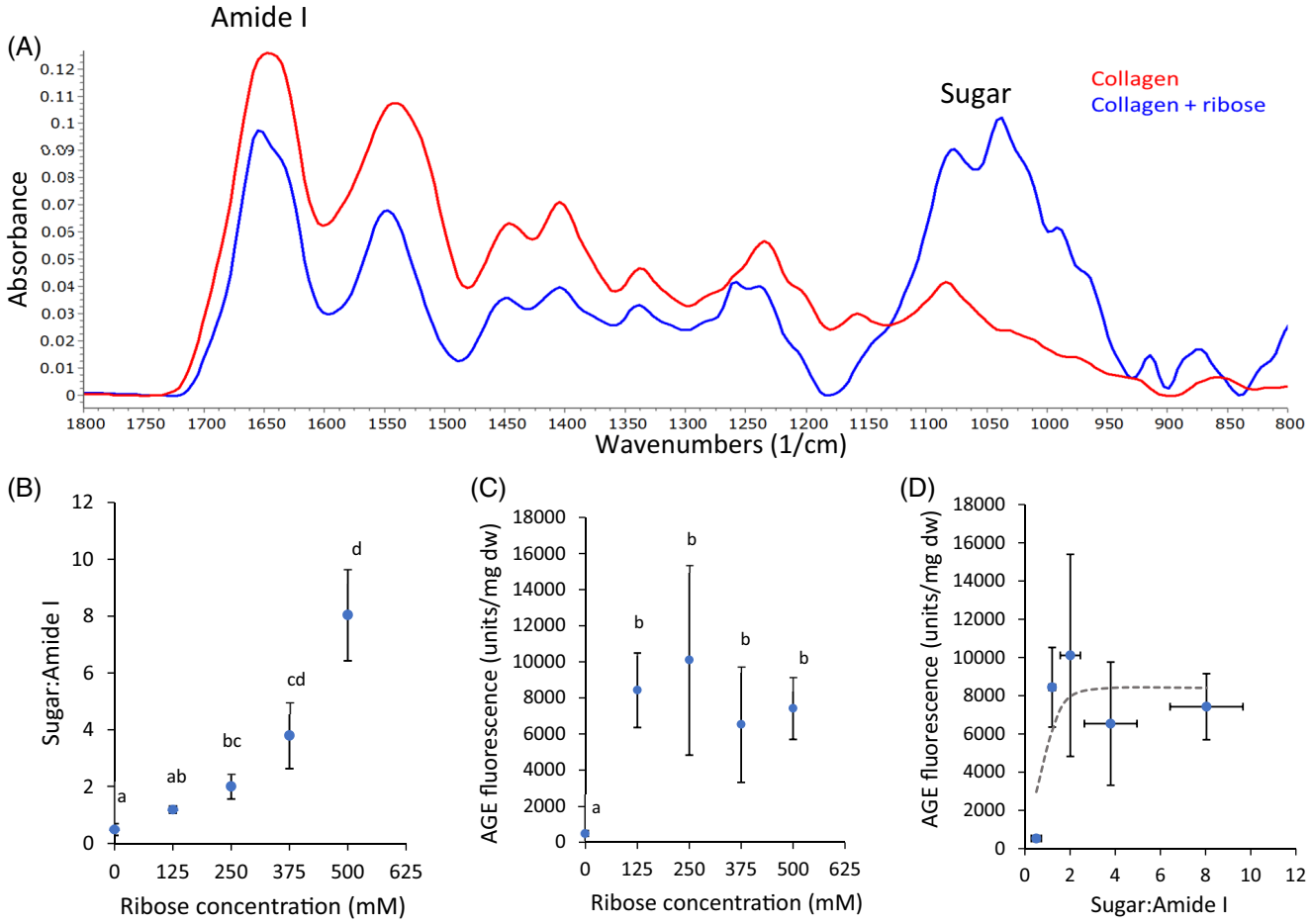


**FIGURE 1** (A) Representative FTIR spectra of non-glycated collagen controls (red) and collagen gels pre-glycated with 250 mM ribose (blue). (B) Ratio of sugar peak area to amide I peak area with varying ribose concentrations. All samples were glycated for 5 days. $n = 15–27$. (C) AGE fluorescence per sample dry weight with varying ribose concentrations. All samples were glycated for 5 days. $n = 12–18$. (D) Relationship between sugar addition and AGE formation appears to follow a dose response curve (RMSE = 2013). Different letters indicate significant difference ($p < .05$). AGEs, advanced glycation end-products; FTIR, Fourier-transform infrared

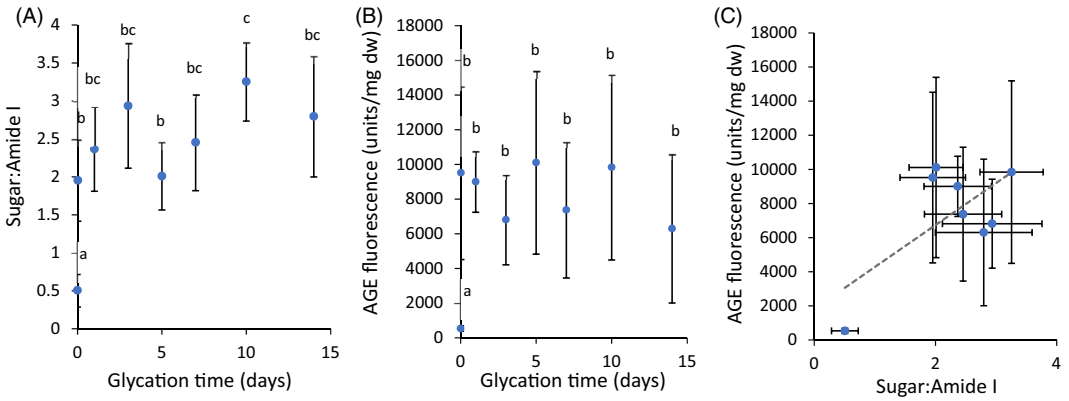


**FIGURE 2** (A) Ratio of sugar peak area to amide I peak area with varying glycation times. All samples were glycated with 250 mM ribose. $n = 15–27$. (B) Advanced glycation end-product fluorescence per sample dry weight with varying glycation times. All samples were glycated with 250 mM ribose. $n = 12–18$. (D) Relationship between sugar addition and AGE formation. A linear correlation was not found when the control group was excluded. Different letters indicate significant difference ($p < .05$)

testing at 4°C. G'∞ was determined by averaging the final plateau region of testing at 37°C. dG'/dt was the maximum slope of G' after the temperature was raised to 37°C. $t_c$ is the time at which G' started to exceed G".

Collagen samples that reached a plateau in G' with G' greater than G" within 30 min of incubation at 37°C were deemed to have gelled successfully. This success rate was found to decrease with increasing ribose concentrations and with longer glycation times (Figure 4A,B). Collagen pre-glycated with 125 and 250 mM ribose gelled successfully in 90% or more of trials whereas collagen pre-glycated with 375 and 500 mM ribose gelled successfully in less than 15% of trials. Collagen pre-glycated for up to 7 days gelled successfully in 75% or more of trials whereas collagen pre-glycated for 9 days or more gelled in less than 50% of trials. Collagen pre-glycated for 17 and 21 days did not successfully gel in any trials.

Successful gelation was found to be correlated with the ratio of added sugar to added AGEs in a dose-dependent manner (Figure 4C).

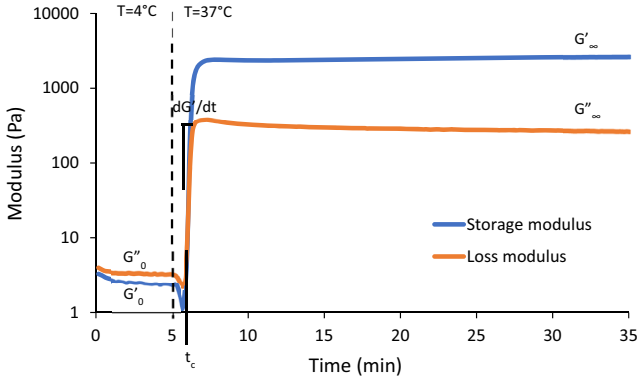


**FIGURE 3** Representative results of rheological testing of collagen before, during, and after gelation. Labels indicate how outcome measures were determined

Added sugar and added AGEs were calculated by subtracting the values of non-glycated controls from those of each glycation condition and dividing by the values of non-glycated controls. Collagen with higher ratios of added sugar to added AGEs were less likely to gel successfully. A very steep drop in success rate was found around 0.4. At 0.37 and below, gelation was successful in 90%–100% of cases, but at 0.47 and above, fewer than 10% of samples gelled.

G' of collagen before gelation increased slightly with increasing ribose concentration ($p = .04$, Figure 5A). Normalized $G'_0$ values were 40% greater for collagen pre-glycated with 500 mM ribose compared to non-glycated controls. G' after gelation had no linear correlation with ribose concentration ($p = .87$, Figure 5B). G' after gelation appeared to reach a maximum with 250 mM ribose, but with 500 mM ribose G' after gelation was similar to non-glycated controls. There were no statistical changes in growth rate of G' ($p = .21$), but there were large variations with ribose concentration from 0.35 to 1.25 (Figure 5C). However, while the maximum growth rate during gelation was unaffected by ribose concentration, the crossover time, or the time at which G' first exceeded G", did vary with pre-glycation. The crossover time increased moderately with increasing ribose concentration with values for pre-glycated collagen being 1.5–3 times higher than non-glycated controls ($p < .01$; Figure 5D). Rheological data did exhibit some high variability in glycated groups. However, we were able to find some statistically significant linear relationships between ribose concentration and G' before gelation ($R^2 = 0.91$, $p = .04$) and between ribose concentration and crossover time ($R^2 = 0.99$, $p < .01$).

All of these rheological properties ($G'_0$, $G'_\infty$, dG'/dt, and $t_c$) were compared to the amount of sugar addition, AGE formation, and the ratio added sugar to added AGEs (Table 1). Sugar: amide I and the ratio of added sugar to added AGEs were most predictive of rheological properties. G' before gelation was found to increase moderately and crossover time was found to increase strongly with both increasing sugar: amide I and increasing added sugar/added AGEs

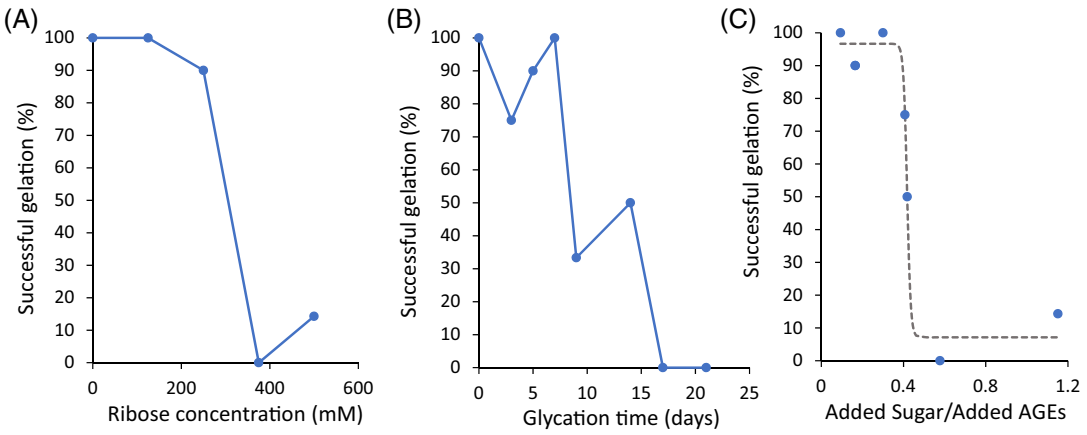


**FIGURE 4** (A) Effect of ribose concentration on successful gelation rate where success was defined as the ability for the collagen to completely gel within 30 min. There were 3–30 attempts per ribose concentration. (B) Effect of glycation time on successful gelation rate. There were 2–30 attempts per time point. (C) Correlation between success rate and the ratio of added sugar to added advanced glycation end-product (AGE) appears to follow a dose response curve (RMSE = 4.91). Both sugar:amide I and AGE have been normalized by subtracting the values of control gels and dividing by the control values.

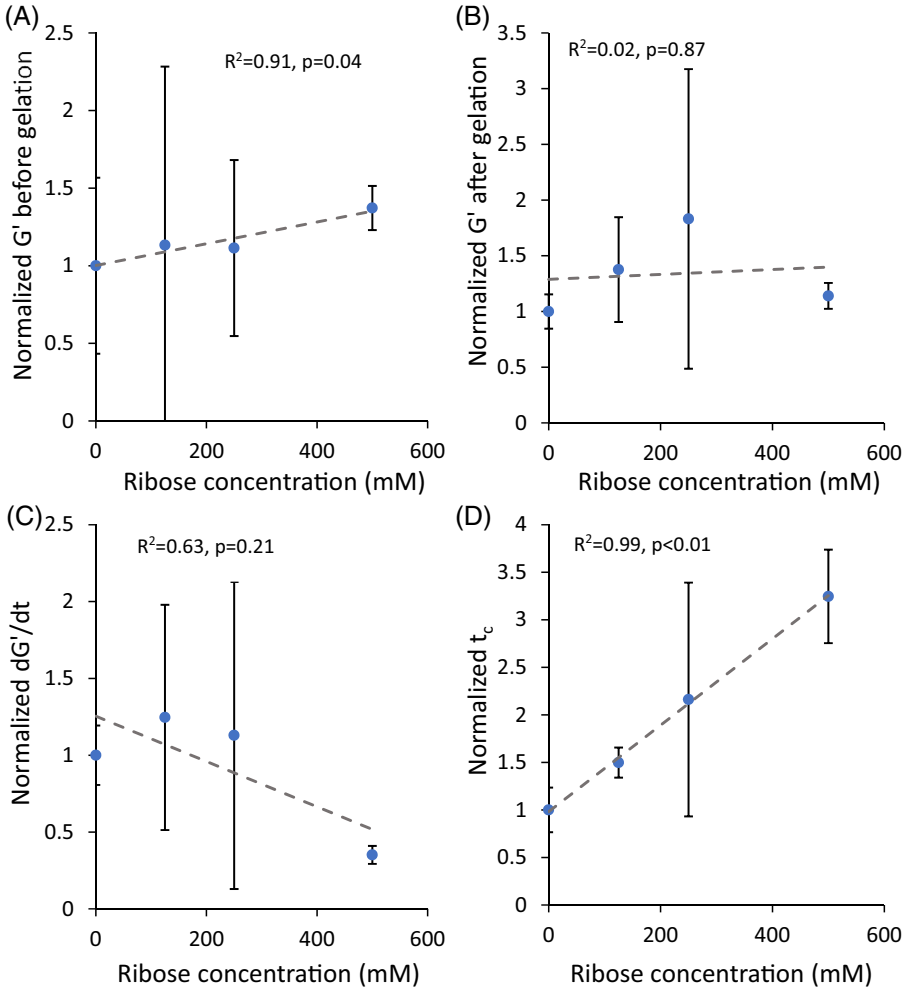


**FIGURE 5** Rheological properties of collagen before, during, and after gelation with varying ribose concentrations. All properties are reported as normalized to those of the control group for the same batch of collagen. (A) *G'* of collagen at 4°C before gelation. (B) *G'* of collagen after complete gelation at 37°C. (C) Maximum growth rate of *G'* after temperature was increased. (D) Crossover time of *G'* and *G"* after temperature was increased. $n = 2$–30. Dotted lines represent linear regressions of the averages of the data.

| | $G'_0$ | | $G'_\infty$ | | dG'/dt | | $t_c$ | |
|---|---|---|---|---|---|---|---|---|
| | Δ | $R^2$ | Δ | $R^2$ | Δ | $R^2$ | Δ | $R^2$ |
| Sugar:Amide I | 0.37 | 0.93 | 0.14 | 0.04 | −0.65 | 0.87 | 2.25 | 0.89 |
| AGE | 0.11 | 0.22 | 0.83 | 0.64 | 0.13 | 0.01 | 1.16 | 0.30 |
| Added sugar/added AGEs | 0.24 | 0.98 | −0.24 | 0.52 | −0.90 | 1.00 | 1.75 | 0.90 |

**TABLE 1** Correlations between rheological properties from testing varying ribose concentrations and sugar addition, AGE formation, and the ratio of added sugar to added AGEs

*Note*: For each rheological property, the maximum change (Δ) is reported as well as the goodness of fit ($R^2$) of the correlation. The maximum change was measured as the difference in the rheological property values for the data points with the minimum and maximum sugar:amide I, AGE, and added sugar/added AGEs.

Abbreviation: AGEs, advanced glycation end-products.

(Figure S2A,C,J,L). The growth rate of *G'* was found to decrease with increasing sugar: amide I and added sugar/added AGEs (Figure S2G,I).

*G'* before gelation decreased with increasing glycation time ($p = .04$; Figure 6A). Normalized *G'* before gelation was 60% lower for collagen pre-glycated for 14 days compared to non-glycated controls. No linear trends were found between normalized *G'* after gelation and glycation time ($p = .76$; Figure 6B). Collagen pre-glycated for 7 days exhibited a peak in normalized *G'* after gelation that was five times higher than that of non-glycated controls. Normalized *G'* after gelation also tended to be higher for pre-glycated collagen than non-glycated controls. Normalized dG'/dt also did not exhibit a linear trend with glycation time ($p = .78$; Figure 6C). Collagen pre-glycated for 7 days exhibited a peak in growth rate that was over 2.5 times higher than that of non-glycated controls. Normalized $t_c$ also did not exhibit a linear trend with glycation time ($p = .39$; Figure 6D). However, $t_c$ tended to be greater for glycated collagen than non-glycated controls. For the case of varying glycation time, the only linear relationship between glycation time and rheological measurements was found for *G'* before gelation ($R^2 = 0.69$, $p = .04$).

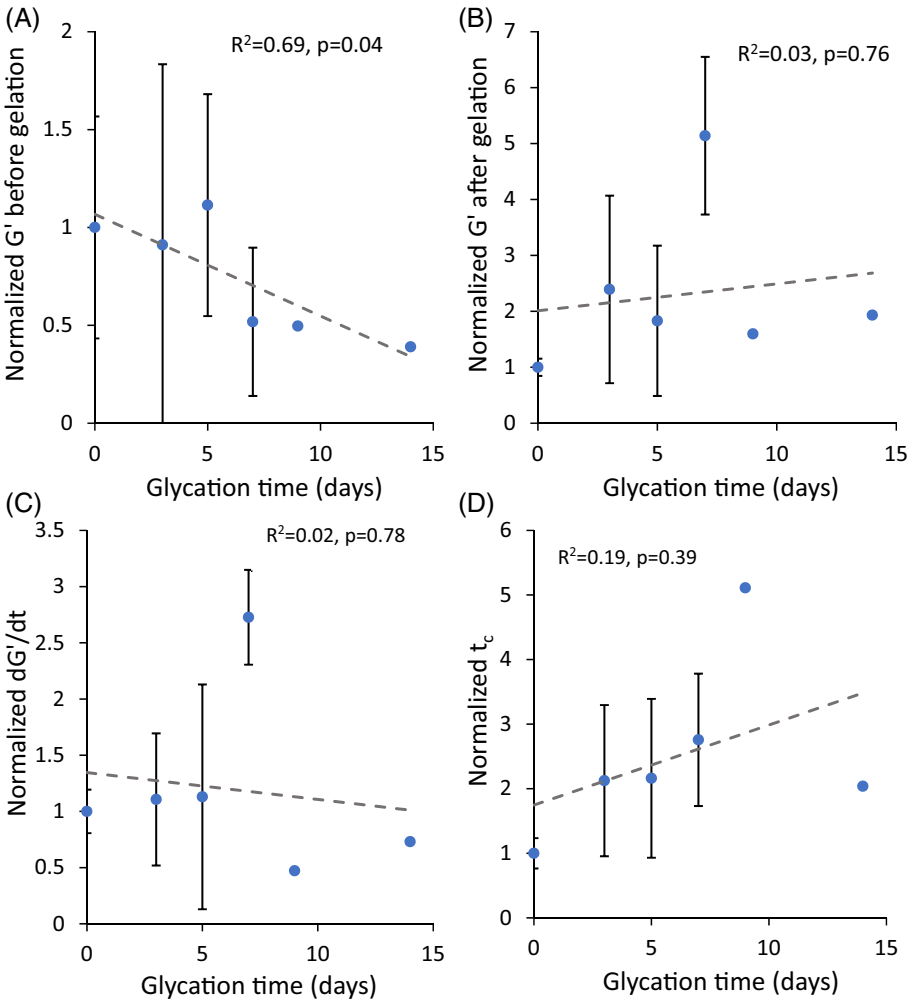


**FIGURE 6** Rheological properties of collagen before, during, and after gelation with varying glycation times. All properties are reported as normalized to those of the control group for the same batch of collagen. (A) G' of collagen at 4°C before gelation. (B) G' of collagen after complete gelation at 37°C. (C) Maximum growth rate of G' after temperature was increased. (D) Crossover time of G' and G" after temperature was increased. $n = 1–30$. Dotted lines represent linear regressions of the averages of the data.

**TABLE 2** Correlations between rheological properties from testing varying glycation times and sugar addition, AGE formation, and the ratio of added sugar to added AGEs

| | $G'_0$ | | $G'_\infty$ | | dG'/dt | | $t_c$ | |
|---|---|---|---|---|---|---|---|---|
| | Δ | $R^2$ | Δ | $R^2$ | Δ | $R^2$ | Δ | $R^2$ |
| Sugar:Amide I | −0.09 | 0.26 | 1.39 | 0.22 | 0.11 | 0.02 | 1.12 | 0.63 |
| AGE | 0.11 | 0.002 | 0.83 | 0.16 | 0.13 | 0.05 | 1.16 | 0.66 |
| Added sugar/added AGEs | −0.72 | 0.37 | 0.10 | 0.001 | −0.40 | 0.06 | −0.12 | 0.06 |

*Note*: For each rheological property, the maximum change (Δ) is reported as well as the goodness of fit ($R^2$) of the correlation. The maximum change was measured as the difference in the rheological property values for the data points with the minimum and maximum sugar:amide I, AGE, and added sugar/added AGEs.
Abbreviation: AGEs, advanced glycation end-products.

These rheological findings were also compared to the amount of sugar addition, AGE formation, and the ratio of added sugar to added AGEs (Table 2). G' before gelation was found to be best correlated with the ratio of added sugar to added AGEs with modulus decreasing with increasing added sugar/added AGEs, though this was a weak correlation (Figure S3C). G' after gelation was best correlated with the amount of sugar added with higher moduli resulting from more sugar added, but this was only a weak correlation (Figure S3D). dG'/dt was found to have no correlations with sugar added, AGE formation, or the ratio of added sugar to added AGEs (Figure S3G–I). $t_c$ was moderately well correlated with both sugar: amide I and AGE fluorescence (Figure S3J,K). Crossover time slowed with higher amounts of sugar and with higher amounts of AGEs.

## 4 | DISCUSSION

The objective of this study was to determine the effects of pre-glycation on high concentration collagen using high ribose

concentrations and extended glycation times. This study shows that, unlike for low concentration collagen solutions, pre-glycation of high concentration collagen solutions can impair gelation, particularly with high ribose concentrations and long glycation times. The ability for high concentration collagen solutions to gel successfully was found to be strongly dependent on the ratio of added sugar to added AGEs. When this ratio was low, almost all samples gelled successfully; whereas, when this ratio was high almost no samples gelled successfully. The concentration of ribose used during pre-glycation was found to affect the amount of sugar added to the collagen; however, it had little effect on the formation of AGEs. AGEs were found to have formed very rapidly, with 70%–75% forming within 30 min and reaching a steady-state by days 1–3. The storage modulus after gelation was found to increase slightly with increasing ribose concentrations and increasing glycation times and appeared to reach a maximum at 250 mM ribose and 7 days of pre-glycation. Increasing ribose concentrations and increasing glycation times also resulted in increased crossover times. The rheological properties of collagen solutions and gels were found to be best correlated with the ratio of added sugar to added AGEs.

The amount of sugar added to collagen, as measured by FTIR, was found to be dependent on the concentration of ribose used during pre-glycation. This dose-dependent response is similar to the results of a previous study, which found that sugar: amide I increased linearly with increasing ribose concentrations up to 250 mM for 1.5 mg/ml collagen gels.[22] With 8 mg/ml collagen, the current study also shows a linear relationship between sugar: amide I and ribose concentration up to 250 mM ribose. However, this trend appears more exponential when the ribose concentration increases to 500 mM. In addition, the data appears to be much more variable at these higher ribose concentrations. It is unclear if the previous study on 1.5 mg/ml collagen would have found a similar exponential trend if higher ribose concentrations had been tested or if the phenomenon is unique to higher collagen concentrations. Additional experiments are needed to further determine the mechanism behind sugar reduction to collagen, particularly, at high ribose concentrations and whether the relationship between ribose concentration and sugar addition is truly linear or exponential.

The previous study using 1.5 mg/ml collagen also found a linear relationship between ribose concentration and the amount of AGEs formed.[22] With 8 mg/ml collagen, this study found little relationship between ribose concentration and the amount of AGEs. Compared to controls for each study, more AGEs were formed in 8 mg/ml collagen than 1.5 mg/ml collagen. This difference could be because the 1.5 mg/ml controls had much higher fluorescence than the 8 mg/ml controls in this study. This could mean that there were fewer binding sites available for Amadori products to form on the 1.5 mg/ml collagen.

The previous study using lower collagen concentrations and lower ribose concentrations found a linear relationship between the amount of sugar added and AGE fluorescence.[22] Using 8 mg/ml collagen, a dose response relationship was found where small amounts of added sugar resulted in a large increase in AGE fluorescence which then plateaued with increasing sugar. This discrepancy could be due to the low values of sugar: amide I measured with 1.5 mg/ml collagen (all below 1), which all fall within the linear region (0–2) of the dose response curve found in this study. These results suggest that when using high (≥250 mM) ribose concentrations, a saturation point was reached in the amount of AGEs formed and that above this point additional sugars bound to the collagen are not able to reduce to AGEs.

Previous studies have pre-glycated collagen of varying collagen concentrations (1–10 mg/ml) for 5 days[12,22,23,25–28,32] with no investigation into other glycation times. Studies of post-glycated collagen have found increased mechanics with increasing glycation time,[33,35] but no such studies have been done on pre-glycated collagen. For 8 mg/ml collagen, sugar addition and AGE formation occurred very rapidly. Most of the sugar bound to the collagen and reduced to AGEs within 30 min and reached a saturation point by days 1–3. Once this saturation point was reached, no more sugar was able to bind to the collagen and reduce to AGEs, likely because all of the binding sites on the collagen had been occupied. These data suggest that future studies with any collagen concentration can likely use shorter glycation times, though this needs to be verified.

High ribose concentrations (>250 mM) and extended glycations times (>7 days) were both found to impair gelation in 8 mg/ml collagen solutions. Collagen with a higher ratio of added sugar to added AGEs were found to have lower probabilities of gelling successfully within 30 min. The inability to gel is due to an increase in the solubility of the collagen monomers in solution caused by a buildup of Amadori intermediates that inhibited fiber formation and gelation. If the ratio of added sugar to added AGEs is low, most of the ribose has fully reduced and formed AGEs and AGE crosslinks. However, if the ratio of added sugar to added AGEs is high, most of the ribose has not fully reduced to AGEs and remains bound to the collagen as an Amadori intermediate. Sugars and Amadori intermediates act to increase solubility of the collagen monomers[43,44] and could also impair fibrillogenesis by blocking binding sites and reducing flexibility.[29,30,44,45] Amadori intermediates formed on the telopeptides of collagen fibers, which are more likely with pre-glycation than post-glycation, could be particularly effective at hindering early fibrillogenesis.

Crosslinking prior to gelation could cause the formation of fewer, thicker fibrils. Recent studies have applied rigidity percolation theory to understand the mechanics of collagenous networks and tissues.[46–48] This framework describes a phase transition that occurs when a sufficient number of fibrils exist to generate a spanning network. Our current data show what appears to be a phase transition that occurs with the addition of ribose. One explanation of this observation is that ribose crosslinking reduces the number of fibers in favor of thicker fibers. Thus, more highly glycated solutions are unable to generate a sufficient number of fibers to gel the network. This hypothesis is supported by SEM images comparing collagen architecture with varying pre-glycation conditions (Figure S4). These images demonstrate a sparser matrix with thicker fibers at 500 mM ribose compared to non-glycated controls. This is consistent with the theory that excess glycation could prevent the generation of a percolating

network. Notably, for conditions that gelled successfully, shear modulus was relatively insensitive to glycation. These relatively small changes are also consistent with rigidity percolation theory, which predicts large changes in mechanics when networks are near the threshold for connectivity, but smaller changes in networks that are close to fully percolated.

Other studies have not directly reported the ratio of added sugar to added AGEs or how this measurement correlates with gelling behavior. However, based on the data published in the 1.5 mg/ml collagen study,[22] the ratio of added sugar to added AGEs appears to be around 0.3 for ribose concentrations of 50, 100, and 250 mM. This value is below the maximum of about 0.4 found in this study for successful gelation of 8 mg/ml collagen. Therefore, the ratio of added sugar to added AGEs could be indicative of gelation behavior independent of collagen concentration, though further studies are needed to confirm this.

When pre-glycated collagen gelled successfully, no significant linear relationship was found betweeen ribose concentration and storage modulus after gelation. Previous studies have found that 1.5 mg/ml collagen gels pre-glycated with up to 250 mM ribose showed a linear increase in compressive modulus with increasing ribose concentration.[22,23] 5 and 10 mg/ml collagen pre-glycated with up to 100 mM ribose also showed a relatively linear increase in compressive modulus with increasing ribose concentration, but the slopes of these trends were less steep than those of 1.5 mg/ml collagen.[27] Based on all of these findings, the effect of pre-glycation on collagen mechanics seems more pronounced at lower collagen concentrations than higher collagen concentrations.

Crossover time, or the time at which the collagen first changes from a liquid to a solid, increased with increasing ribose concentration up to 500 mM. This was similar to results seen with 1.5 mg/ml collagen where collagen pre-glycated with 250 mM ribose gelled almost four times slower than non-glycated controls.[23] These findings of decreased kinetics may be because Amadori intermediates are increasing the solubility of the collagen and hindering fibrillogenesis. This idea is supported by the fact that crossover time was best correlated with the ratio of added sugar to added AGEs. Pre-glycated collagen with high sugar content compared to AGE content exhibited higher crossover times meaning that these collagen solutions took longer to form a gel when there were more Amadori intermediates than AGEs.

The effect of glycation time on rheological properties was not explained well by the sugar content or the amount of AGEs formed. This lack of correlation is in part because pre-glycated collagen exhibited very similar sugar and AGE content regardless of glycation time, because the sugar and AGEs reached saturation points very rapidly. Therefore, the differences in rheological properties could be related to the types of Amadori intermediates and AGEs being formed at different time points during glycation. Different Amadori intermediates could increase solubility to differing degrees. Therefore, the percentage of fluorescent AGEs that form and are quantifiable could vary with glycation time.

Collagen glycation is an extremely useful technique which has been utilized for a wide range of applications, such as creating stiff engineered tissues,[32–34] studying mechanotransduction in healthy and diseased cells,[23,26,27,37,38] studying the effects of aging and diabetes.[12,24,39] Therefore, it is important to understand the possibilities and limitations of glycating collagen to better inform researchers on how to create the gels and matrices for their given applications.

Based on this study, pre-glycation of high concentration collagen with high ribose concentrations or extended glycation times may not be a reliable method of modeling stiffer or diseased tissues. The high variability in successful gelation is likely because of the build-up of Amadori intermediates that increase collagen solubility and impair fibrillogenesis. Post-glycation may be a more reliable method to create models of stiff tissues or disease states with high collagen concentrations since post-glycation better mimics the natural formation of AGEs during aging and disease. However, the use of post-glycation would limit these studies to only cell-seeding rather than studying encapsulated cells. Therefore, future work should explore the mechanics of high concentration collagen pre-glycated with high ribose concentrations or extended glycation times when given more time to fully gel. This approach could allow for the determination of optimum pre-glycation conditions (collagen concentration, ribose concentration, glycation time) for creating collagen gels with highest mechanical properties and highest rates of gelation.

## 5 | CONCLUSIONS

Overall, this study found that pre-glycation of high concentration collagen with high ribose concentrations and for long glycation times can impair gelation. The ability for pre-glycated collagen solutions to gel successfully was found to be strongly dependent on the ratio of added sugars to added AGEs. When the ratio is high and there are excessive amounts of sugar, gelation is impaired. However, when the ratio is low and most sugars have reduced to AGEs, gelation is successful. There is likely a competing effect during pre-glycation of the formation of AGEs resulting in crosslinking of collagen and the formation of Amadori intermediates acting to impair collagen fibrillogenesis. The rheological properties of pre-glycated collagen that gelled successfully were also most strongly correlated with this ratio of added sugar to added AGEs. The storage modulus after gelation and the kinetics of gelation were both found to decrease when this ratio increased, meaning that preferred rheological properties resulted from conditions with low ratios of added sugar to added AGEs. Future work on collagen glycation should focus on increasing the formation of AGEs while maintaining a low ratio of added sugar to added AGEs.

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## CONFLICT OF INTEREST

Dr. Bonassar is a co-founder of and holds equity in 3DBio Corp and is a consultant for Fidia Farmaceutici, SpA and Histogenics, Inc.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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