Nondestructive assessment of collagen hydrogel cross-linking using time-resolved autofluorescence imaging

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Abstract. We investigate the use of a fiber-based, multispectral fluorescence lifetime imaging (FLIm) system to nondestructively monitor changes in mechanical properties of collagen hydrogels caused by controlled application of widely used cross-linking agents, glutaraldehyde (GTA) and ribose. Postcross-linking, fluorescence lifetime images are acquired prior to the hydrogels being processed by rheological or tensile testing to directly probe gel mechanical properties. To preserve the sterility of the ribose-treated gels, FLIm is performed inside a biosafety cabinet (BSC). A pairwise correlation analysis is used to quantify the relationship between mean hydrogel fluorescence lifetimes and the storage or Young’s moduli of the gels. In the GTA study, we observe strong and specific correlations between fluorescence lifetime and the storage and Young’s moduli. Similar correlations are not observed in the ribose study and we postulate a reason for this. Finally, we demonstrate the ability of FLIm to longitudinally monitor dynamic cross-link formation. The strength of the GTA correlations and deployment of our fiber-based FLIm system inside the aseptic environment of a BSC suggests that this technique may be a valuable tool for the tissue engineering community where longitudinal assessment of tissue construct maturation in vitro is highly desirable.

Keywords: fluorescence lifetime; collagen; cross-link; nondestructive; hydrogel.

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1 Introduction

The scarcity of quantitative, nondestructive assessment techniques for engineered tissues presents a critical bottleneck in the field of regenerative medicine. Conventional biochemical and mechanical tests are often time-consuming and damaging to structure and function of the sample under test, thereby reducing the available tissue for subsequent implantation. Nondestructive measurement techniques have the potential to lower costs and increase sample throughput, while providing an opportunity for a better understanding of sample maturation via repeated, longitudinal measurements. Optical imaging techniques can perform fast, noncontact measurements on a variety of biological samples. However, prior to using these technologies to evaluate tissue function, quantitative relationships between optical and conventional measurement techniques must be established. In this work, we investigate the suitability of label-free fluorescence lifetime imaging (FLIm) as an indirect technique for nondestructively quantifying the changes in mechanical properties of a collagen matrix as a result of cross-link formation.

Collagen is the most abundant protein in the mammalian extracellular matrix and serves a critical role in establishing and maintaining the mechanical properties of various tissues, including bone, skin, blood vessels, and cartilage. Due to its prevalence in mammals and its well-conserved nature, collagen is widely used as a material for tissue engineering applications. The capacity to modulate the stiffness of engineered scaffolds is a desirable trait for tissue engineering applications, including instructing differentiation of stem and progenitor cells and producing materials that support the physical requirements of the defect site. Moreover, the mechanical properties of a material represent a key element in evaluating the maturation of engineered tissues. Beyond changing the composition, one of the primary methods of controlling the mechanical properties of collagen-based tissue scaffolds is to modify the nature of covalent cross-linking between collagen fibrils.

Gluatraldehyde (GTA) is a widely used chemical cross-linking agent that increases the mechanical properties of a sample. However, this enhancement comes at the cost of increased cell toxicity since GTA indiscriminately reacts with amines, amides, and thiol groups on the cells. GTA cross-linking of collagen is mediated by the reaction of the amine groups of lysine or hydroxylsine residues with the aldehyde groups of GTA. Nonenzymatic collagen cross-links naturally occur in the body under hyperglycemic conditions. The formation of advanced glycation end products (AGEs) following the incubation of collagen in a concentrated solution of reducing sugars, such as ribose, induces the formation of nonenzymatic cross-links between lysine and arginine residues on collagen.

Cross-links between collagen fibrils are a major source of autofluorescence. Multiple studies have measured the intensity, wavelength, and temporal dynamics of fluorescence emission attributed to collagen cross-links. Here, we present the first use of a fiber-based imaging system to monitor changes in fluorescence that result from cross-linking treatments. Microscopes and spectrophotometers are extremely powerful...
measurement tools; however, their size and complexity make it challenging to translate these devices into tools that can operate in sterile, space-restricted environments required in the field of tissue engineering. The technique presented here uses a narrow and flexible fiber optic to probe the sample fluorescence, allowing fluorescence lifetime images to be acquired from samples inside a conventional, aseptic biosafety cabinet (BSC). In this work, we investigated the use of FLIm to monitor changes in mechanical properties of collagen hydrogels caused by enhanced cross-link formation. Fluorescence lifetime is a measure of the average time a fluorophore takes to return to its ground state. FLIm is a fiber compatible inherently ratiometric technique that has a high sensitivity to local changes in a sample’s biochemistry. To first order, FLIm is insensitive to factors such as fluorophore density or fluorescence excitation-collection efficiency. These factors can produce artifacts in the images acquired by techniques that rely on fluorescence intensity, especially when imaging in restricted geometries such as inside a tissue bioreactor or as was the case for this work, a BSC.

The goal of this study was to investigate if FLIm could be used as a nondestructive technique to measure changes in collagen hydrogel biochemistry that result from new cross-link formation and to correlate these changes with measurements of hydrogel stiffness. We are specifically and separately targeting two cross-linking agents, GTA and ribose, that are used in the field of regenerative medicine to introduce controlled changes in the mechanical properties of collagen-based biomaterials. We emphasize here that our approach is focused on monitoring the actions of individual cross-linking agents and is not applicable in scenarios where the cross-linking agent is not known a priori. Following cross-linking, the fluorescence lifetime of the hydrogels was recorded using our fiber-based multispectral FLIm system. Fluorescence lifetimes are reported from the spectral channels where the signal-to-noise ratio of the recorded fluorescence exceeds 35 dB. Postimaging, the storage modulus or Young’s modulus of the hydrogels was measured using rheological or tensile testing, respectively. In the final analysis of the data, a matched sample correlation analysis between changes in fluorescence lifetime and mechanical properties was used to assess the utility of FLIm as a nondestructive tool for monitoring processes of interest to the field of tissue engineering.

2 Methods

2.1 Collagen Gel Preparation and Cross-link Formation

Collagen hydrogels derived from rat tail type I collagen were prepared using a commercially available kit (Advanced Biomatex, San Diego, California). Briefly, nine parts of collagen solution were mixed with one part of neutralization solution on ice and allowed to polymerize for 1 h at 37°C in a humidified incubator (5% CO₂, 21% O₂) to yield hydrogels 8 mm in diameter. The hydrogels used for the GTA and ribose (both from Sigma, St. Louis, Missouri) studies had collagen concentrations of 3.9 and 2.25 mg/mL, respectively.

For the GTA cross-linking studies, hydrogels were incubated in four different concentrations 62.5, 125, 187.5, and 250 mM of ribose in phosphate-buffered saline (PBS) with 44-mM NaHCO₃ (Fisher Scientific, Santa Clara, California) and 25-mM HEPES (Fisher Scientific) buffer for 5 days at 37°C in a humidified incubator (5% CO₂, 21% O₂). Control samples were incubated in PBS with 44-mM NaHCO₃ and 25-mM HEPES buffer under the same conditions. Hydrogels were washed 2x with PBS to remove excess sugar prior to imaging. All fluorescence lifetime measurements were conducted with hydrogels immersed in PBS in order to maintain the hydration state and pH of the samples.

Immediately prior to imaging, hydrogels were washed 2x with water to remove excess GTA. For the ribose cross-linking study, hydrogels were incubated in four different concentrations 0.05%, 0.1%, 0.2%, and 1% of GTA for 1 h at room temperature. These concentrations of GTA have been reported to produce significant changes in the viscoelastic properties of collagen hydrogels. Control samples were maintained in water under the same conditions.

2.2 FLIm Apparatus

A fiber-coupled FLIm system broadly based on the principal of a previously reported apparatus (16) was used to acquire the lifetime data. A schematic of this system is shown in Fig. 1(e). The light source for fluorescence excitation is a frequency tripled, Nd:YAG microchip laser (STV-02E-1x0, TEEM Photonics, Grenoble, France) that emits 355-nm, 600-ps pulses at a repetition rate of 4 kHz. The average power of the laser incident on the sample surface is 4.51 mW. Fluorescence excitation and emission are guided to and from the sample using a 400-μm core diameter, all silica multimode fiber that has a numerical aperture of 0.22. The lateral extent of the point spread function (PSF) for this fiber was measured using a 13-μm wire phantom coated in fluorescent paint. A fluorescence lifetime image of the phantom was acquired, and the PSF calculated from the mean full width at half maximum of Gaussian fits to 20 adjacent lines in the image that run perpendicular to the wire length. The result of this measurement was a PSF with a lateral width of 251 ± 12 μm. The GTA cross-linked gels were imaged on the benchtop using a 2-m-long fiber, and the ribose-incubated gels were imaged using a 10-m-long fiber, the distal end of which was installed inside a BSC. A wavelength selection module consisting of four dichroic beamsplitters and bandpass filters was used first to separate fluorescence excitation and emission and then to realize four distinct spectral bands for detection. In this work, we report data from the 390 ± 9 nm (CH1), 435 ± 20 nm (CH2), and 510 ± 42 nm (CH3) spectral bands. Each spectral band is coupled to an optical delay line consisting of a 600-μm core diameter multimode fiber (FT600UMT, Thorlabs, Newton, New Jersey). Each delay line has a different length (1, 13, 25, and 37 m for CH1, CH2, CH3, and CH4, respectively), allowing the fluorescence signals in the four spectral bands to be temporally multiplexed on to a single microchannel plate photomultiplier tube (R3809U-50, Hamamatsu, Honshu, Japan) detector. Following amplification by a broadband, low noise amplifier (AM-1607-3000, Miteq, Hauppauge, New York), the temporal dynamics of the fluorescence signals are digitized at a rate of 12.5 GS/s (PXLe-5185, National Instruments, Austin, Texas). Fluorescence lifetimes are extracted from the raw data in postprocessing using a constrained least-squares deconvolution with Laguerre expansion. Exemplar fits and residuals are shown in Figs. 1(f).

A fluorescence lifetime image is generated by scanning the distal fiber tip over the surface of the sample using a motorized 3-axis translation stage (PRomech LP28, Parker, Cleveland, Ohio). Two 3-axis stages were used in this work, with one being permanently installed inside the BSC to facilitate imaging of the ribose-treated hydrogels, and the other used for benchtop scanning of the GTA cross-linked hydrogels.
2.3 Measurement of Mechanical Properties of Collagen Hydrogels

Rheological measurements were performed using a Discovery HR-2 hybrid stress-controlled rheometer (TA Instruments, New Castle, Delaware) equipped with an 8-mm parallel plate geometry on a stage heated to 37°C as described previously. Briefly, hydrogels were tested at a frequency of 0.5 Hz and a logarithmic sweep up to 4% strain 5 points per decade. The shear storage modulus was determined by averaging at least 5 points in the linear viscoelastic region. Tensile testing was conducted using a 5965 Instron machine (Instron, Norwood, Massachusetts) as described previously. Briefly, hydrogels were cut into dog-bone-shaped samples and imaged using a white-light camera. White-light images were processed using ImageJ (National Institutes of Health, Bethesda, Maryland) to extract the sample thickness and width. Samples were then glued at either extremity to two paper strips with a fixed gauge length of 3 mm. A uniaxial strain to failure test was conducted at a strain rate of 1% of the gauge length per second. Force–displacement curves were normalized to specimen dimension data, and the apparent Young’s modulus was calculated by least squares fitting the linear portion of the resulting stress–strain curve in MATLAB® (MathWorks, Natick, Massachusetts).

2.4 Statistics

For the GTA study, 12 gels were prepared for each of the 5 concentrations of GTA. All gels were imaged using FLIm. Measurements of storage and Young’s modulus were made using n = 6 gels/group. The uncertainty on the mechanical testing data is reported as the standard deviation of each group. Technical difficulties with the tensile testing apparatus meant that the 1% GTA group could not be reliably measured. A total of four gels (n = 2/group) were imaged in the longitudinal GTA study. For the ribose study, due to technical challenges in performing the Young’s modulus measurements of the ribose cross-linked gels, only storage moduli (n = 6/group) are reported here. In the longitudinal ribose study, n = 6 gels were imaged for each of the two groups. The intensity weighted average lifetime and standard deviation of lifetime for individual samples were calculated using the pixel values found inside a circular region of interest centered on the sample and encompassing >75% of the sample area. The fluorescence lifetime attributed to a group in this work was calculated by taking the mean of all individual samples within the group. For all end-point studies, the uncertainty in the fluorescence lifetime of each group was found by taking the standard deviation of all individual samples within the group. For all linear fits, the uncertainty on the mechanical testing (Young’s modulus) was performed using linear regression. To validate the use of linear regression, the Anderson–Darling test was used to test the normality of the fit residuals. For the longitudinal studies, the uncertainty in fluorescence lifetime at each time point is calculated by combining the standard deviation of each sample using standard error propagation.
3 Results

3.1 GTA Cross-linking Increases Collagen Hydrogel Storage and Young’s Modulus and Decreases Mean Fluorescence Lifetime

Mechanical testing data for collagen hydrogels incubated at room temperature for 1 h in solutions of increasing GTA concentration are presented in Fig. 2. These data show that increasing concentrations of GTA led to increases in hydrogel mechanical properties. Specifically, the storage modulus of the 1% GTA group was $1540 \pm 190$ Pa, as compared to $240 \pm 50$ Pa for the control group. Similarly, the Young’s modulus of the 0.2% GTA group was measured as $55.5 \pm 10.1$ kPa, compared with $6.4 \pm 1.3$ kPa for the control group.

The GTA cross-linked hydrogel mean fluorescence lifetimes recorded in CH1, CH2, and CH3 are presented in Fig. 3. These data show that for increased GTA concentration, and so by inference increased chemical cross-linking of collagen, the mean fluorescence lifetime of each spectral channel decreases. The largest difference was observed in CH2 where the mean lifetime of the 1% GTA group was measured as $2.80 \pm 0.06$ ns, as
compared with 3.90 ± 0.23 ns for the control group. Both trends in the data presented in Figs. 2 and 3 are monotonic with generally statistically significant ($p < 0.05$) differences between groups, suggesting a spectrum of mechanical properties and fluorescent lifetimes that should lend themselves to a correlation analysis.

### 3.2 Storage and Young’s Moduli of Glutaraldehyde Cross-linked Collagen Hydrogels Present Strong and Specific Correlations with Mean Fluorescence Lifetime

Figures 4 and 5 show pairwise correlation analyses of mean fluorescence lifetime in three spectral bands plotted as a function of storage and Young’s modulus, respectively. First degree polynomials fitted to these data reported $R^2$ values of 0.75, 0.83, and 0.80 for storage modulus and 0.68, 0.72, and 0.74 for Young’s modulus for spectral bands CH1, CH2, and CH3, respectively. The Anderson–Darling test indicated that all the fit residuals in Figs. 4 and 5 were normally distributed ($p < 0.05$).

### 3.3 FLIm Allows for Longitudinal Monitoring of GTA Cross-linking of Collagen Hydrogels

The nondestructive nature of FLIm was exploited in a longitudinal measurement of GTA-mediated cross-linking in collagen hydrogels (Fig. 6). Fluorescence lifetime images of two gels immersed in PBS and two gels immersed in 0.2% GTA were recorded at 15-min intervals for the duration of the 1-h cross-linking period. The biggest changes were observed in CH2 where the mean lifetime of the 0.2% GTA group decreased from 3.97 ± 0.37 ns at the beginning to 2.89 ± 0.25 ns at the end of the cross-linking period. By comparison, the control group mean lifetime remained relatively constant, decreasing from a starting value of 3.96 ± 0.37 ns to 3.70 ± 0.47 ns over the 1-h period.
3.4 Ribose Treatment Increases Collagen Storage Modulus and Mean Fluorescence Lifetime

The storage modulus of 2.25 mg/mL collagen gels incubated in increasing concentrations of ribose solution or PBS for 5 days is presented in Fig. 7. Rheological measurements were only able to detect a statistically significant difference in storage modulus of the highest ribose concentration group (550 ± 320 Pa) over the PBS control group (130 ± 30 Pa). Conversely, as shown in Fig. 8 in each spectral band, FLIm reported large differences between the control and any ribose-treated group, but only small differences between ribose-treated groups. In CH2, the mean fluorescence lifetime of the control group was 4.21 ± 0.07 ns, as compared with 5.63 ± 0.07 ns and 5.70 ± 0.12 ns for the 62.5- and 250-mM ribose groups, respectively. A monotonic increase of mean fluorescence lifetime as a function of ribose concentration is observed in CH1. However, no statistical significant differences were recorded between the 62.5- and 125-mM ribose groups or the 187.5- and 250-mM ribose groups.

3.5 Storage Modulus of Ribose-Treated Collagen Hydrogels does not Correlate with Mean Fluorescence Lifetime

Figure 9 shows a pairwise correlation analysis of mean fluorescence lifetime in three spectral bands plotted as function of storage modulus for the ribose-treated collagen gels. The $R^2$ values for the first degree polynomials do not exceed 0.22 in any of the spectral bands. Furthermore, the Anderson–Darling test
indicated that the residuals for all of the fits in Fig. 9 were not normally distributed ($p > 0.05$).

### 3.6 FLIm Allows for Longitudinal Monitoring of Ribose-Treated Collagen Hydrogels

All ribose-treated gels were imaged using a 10-m-long fiber optic, the distal end of which was installed inside a BSC. This was particularly important for the longitudinal monitoring of the ribose treatment where repeated measurements of the same gels in an unsterile environment risked contamination of the ribose solution. Fluorescence lifetime images of the same 6 gels incubated in PBS or 250-mM ribose solution were acquired each day for a 5-day period. The mean fluorescence lifetimes of the two groups in the three spectral channels are shown in Fig. 10. The biggest changes were observed in CH3 where the mean lifetime of the 250-mM ribose group increased from 3.98 ± 0.39 ns at the beginning to 5.79 ± 0.97 ns at the end of the cross-linking period. By comparison, the control group mean lifetime was highly consistent, with initial and final values of 4.24 ± 0.48 ns to 4.25 ± 0.30 ns, respectively, over the 5-day period.

### 4 Discussion

Fast, nondestructive optical imaging techniques have the potential to lower costs and improve throughput in the field of tissue engineering by replacing slow and destructive conventional testing techniques used for evaluating engineered construct properties. The end-point studies in this work were conducted by acquiring nondestructive FLIm measurements followed by rheological or tensile testing of the same set of collagen hydrogels. Rheology and tensile testing were selected as direct methods to probe changes in the stiffness of the hydrogels. Both methods have been shown to be sensitive to the changes in collagen matrix stiffness caused by enhanced cross-link formation.

Since the goal of the study was to correlate functional changes such as mechanical properties to optical parameters, we did not directly measure the formation of collagen cross-links as a result of GTA or ribose treatment.

The different concentrations of GTA produced a set of hydrogels with a spectrum of mechanical properties, with higher GTA concentrations generally producing stiffer hydrogels. A similar distribution of mean fluorescence lifetimes was observed, and the resulting matched sample analysis showed strong correlations between lifetimes and hydrogel storage and Young’s moduli. The combination of the strength of the correlations in Fig. 3 and the demonstration in Fig. 7 that changes can be monitored longitudinally without perturbing the cross-linking process suggests that fiber-based FLIm could be used as an effective tool for nondestructive monitoring of changes in sample biomechanics caused by GTA-mediated cross-linking. In the ribose study, the different concentrations of ribose failed to produce a set of samples with a spectrum of mechanical properties as had been reported previously. Instead, statistically significant differences in storage modulus over the control group were only observed for the highest concentration of ribose solution. The storage modulus data did not correlate with mean fluorescence lifetime of the ribose-treated hydrogels. Glycation of collagen catalyzes the production of AGEs, which include a number of fluorescent collagen cross-links, and leads to increased stiffness of the collagen matrix. One postulated explanation for the lack of correlation between the mean fluorescence lifetime and storage modulus of ribose-treated collagen hydrogels is that the AGEs responsible for modifying the native collagen fluorescence are not the same or are not formed at the same rate as the AGEs responsible for conveying the new mechanical properties. Several studies in cancellous bone have reported that increasing abundances of pentosidine, a widely studied AGE, do not correlate with changes in mechanical properties for those tissues. To test this hypothesis, a detailed biochemical analysis, using
high-performance liquid chromatography or mass spectrometry, would be necessary to uniquely identify which AGEs are formed, when, and how they affect the sample fluorescence and functional properties.

Several pilot human studies have investigated the change in fluorescence lifetime of the eye and skin of type 2 diabetic patients due to increased AGE concentration. Each paper reported an increase in the lifetime of fluorescence emitted at wavelengths >420 nm for the diabetic patients compared to healthy patients. Furthermore, recently published results have shown that ribose incubation of decellularized matrices leads to a decrease in fluorescence lifetime at emission wavelengths <390 nm and an increase for wavelengths >390 nm. It is interesting to note that in Ref. [2], the authors report a decrease in fluorescence lifetime as a function of collagen hydrogel glycation time. We postulate that this difference from our study may result from the fluorescence emission being sampled from different spectral regions.

The authors of Refs. [26, 27] demonstrated that fluorescence and second-harmonic generation intensity-based techniques could also be used to monitor changes in collagen-based samples resulting from a modification of the sample cross-links. The dynamic range of these intensity-based measurements generally exceeded that of the lifetime-based measurements presented in this work. Collagen and its cross-links are the main extracellular component that resists tension. While both ultrasound elastography and optical coherence tomography have shown utility in nondestructively monitoring gel compressive properties, they have not been used to monitor collagen tensile properties. In this study, we were able to correlate both tensile and compressive properties of collagen gels to FLIm parameters. It is our belief that lifetime remains a promising candidate for nondestructive monitoring of engineered tissue maturation. The inherently ratiometric nature of lifetime measurements makes them well-suited to imaging in nonideal environments such as inside tissue bioreactors or BSCs, where intensity-based techniques may suffer from artifacts.

5 Conclusion

The present study investigated the use of a fiber-based FLIm system to monitor functional changes in collagen hydrogels associated with the application of cross-linking agents GTA and ribose. The nature and extent of collagen cross-linking has a significant impact on tissue stiffness, a key criterion in the generation of engineered tissues. GTA treatment at different concentrations produced collagen hydrogels with a spectrum of mechanical properties and mean fluorescence lifetimes, which revealed strong correlations when plotted against one another. A longitudinal experiment involving the repeated imaging of four hydrogels showed that FLIm could be used to nondestructively probe the progression of the GTA cross-linking process. Both findings suggest significant promise for fiber-based FLIm as a fast and effective tool to nondestructively infer the mechanical properties of collagen-based materials undergoing GTA cross-linking. Ribose treatment failed to produce a measurable spectrum of collagen hydrogel mechanical properties or mean fluorescence lifetimes. Furthermore, the two datasets showed no clear evidence of correlation, suggesting that additional studies need to be performed before a conclusive statement about the utility of fluorescence lifetime for monitoring ribose-mediated nonenzymatic cross-linking can be made. All ribose-treated hydrogels were imaged using a 10-m-long optical fiber, the distal end of which was installed inside a BSC, indicating that fiber-based FLIm can be deployed in environments where taking measurements using conventional microscopes is highly challenging. In summary, we have demonstrated that FLIm can provide a nondestructive approach to monitoring changes in functional properties of samples undergoing specific, controlled biochemical treatments that are known a priori. We believe these findings have significant implications for the field of regenerative medicine. Nondestructive analysis of tissue properties has the potential to increase sample throughput by avoiding destruction of engineered tissues during measurement, monitor sample maturation over time, and serve as a quality control for engineered samples at various stages of their development.

Disclosures

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References


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