Scanning X-ray microdiffraction of decellularized pericardium tissue at increasing glucose concentration

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Abstract
Blood glucose supplies energy to cells and is critical for the human brain. Glycation of collagen, the non-enzymatic formation of glucose-bridges, relates to diseases of aging populations and diabetics. This chemical reaction, together with its biomechanical effects, has been well studied employing animal models. However, the direct impact of glycation on collagen nano-structure is largely overlooked, and there is a lack of ex vivo model systems. Here, we present the impact of glucose on collagen nanostructure in a model system based on abundantly available connective tissue of farm animals. By combining ex vivo small and wide-angle X-ray scattering (SAXS/WAXS) imaging, we characterize intra- and inter-molecular parameters of collagen in decellularized bovine pericardium with picometer precision. We observe three distinct regimes according to glucose concentration. Such a study opens new avenues for inspecting the effects of diabetes mellitus on connective tissues and the influence of therapies on the resulting secondary disorders.

KEYWORDS
collagen, diabetes, pericardium, SAXS, scanning microscopy, WAXS

1 | INTRODUCTION

Glucose, the principal monosaccharide used by our body, is present in blood (glycemia) in a concentration around 65 to 110 mg/dL. The impaired fasting glucose (IFG) level is defined by the World Health Organization (WHO) at the limit of 110 mg/dL [1]. These glycemic values are the result of a complex metabolism, which involves the uptake, production, consumption and storage of glucose. Diabetes mellitus (DM) is the group of metabolic disorders characterized by high blood sugar levels over a prolonged period of time, caused by defects in insulin action or secretion. The number of people with DM has risen from 108 million in 1980 to 422 million in 2014 and from current projections DM will be the seventh leading cause of death in 2030, heavily affecting the global economy [2]. There are two
main types of DM: (a) Type 1 or juvenile DM: the pancreas fails to secrete insulin, due to autoimmune damage or destruction of insulin-secreting β-cells in pancreatic islets. Type 1 diabetes is characterized by a cellular-mediated autoimmune disruption that occurs in individuals with a genetic predisposition, in particular children and adolescents, associated with severe hyperglycemia, weight loss and early onset [3]. (b) Type 2, typically featured by hyperinsulinemia and weight gain [4, 5]. This is the most prevalent form of diabetes, characterized by insulin resistance and β-cell dysfunction. DM is associated with microvascular and macrovascular functional complications, including retinopathy, ischemic heart disease, peripheral vascular disease and cerebrovascular disease (macrovascular), resulting in organ and tissue damage in approximately one-third to one-half of people with diabetes [6–9]. Glycation is the main biochemical reaction involved in diabetes and it correlates to the high level of glucose in blood. It is the result of the irreversible covalent bonding of glucose to proteins. This reaction consists of several steps that lead to derivative products called advanced glycation end-products (AGEs). These are involved in intra- and intermolecular nonelastic crosslinks of connective proteins, altering molecular and cellular functions. One of the principal targets of glycation is type I collagen, the main protein of the mammalian extracellular matrix (ECM), which provides structural stability and strength. At the nanoscale the collagen fibrils have a dimension in the range of 30 to 500 nm, and can be described as collagen molecules of diameter of ≈1.1 nm placed in bundles at a molecular distance of ≈1.5 nm [10, 11]. Along the fibril axis, the molecules are regularly packed at distances of 64 to 67 nm [12]. In DM, the alteration of connective tissues correlates to the accumulation of AGES, which leads to a chronic inflammation state of tissues [11]. In order to investigate the mechanisms of physiological and pathological processes of the disease, and to test possible drugs to treat diabetes, animal models have been widely used over decades, despite the ethical and economic issues, together with the fact that the development and progression of pathologies could be different with respect to humans [13]. While indirect, biomechanical effects of glycation have been studied in animal models, little is known on the direct impact of glycation on the nano-structural parameters of collagen. Thus, there is the lack of a direct quantification for the impact of glycation. To overcome these issues, the development of human-based tissue-like constructs, that is, 3D in vitro scaffolds that mimic human organs and tissues [14–19], have generated great interest, especially with respect to the testing of new drugs. Such functional and complex 3D tissues are mimicked by decellularized tissue as models of human tissues. Aim of this paper is to study the direct impact of the glycation of type I collagen in tissues derived from decellularized bovine pericardium. This requires sensitivity to nano and subnano-scale structural parameters with high precision and statistical relevance in the picometer range, while at the same time addressing comparatively large areas of several square millimeters to ensure representativeness of the results obtained. Therefore, we have chosen to investigate the changes in the structure of collagen in a set of tissue analogues with increasing glucose concentration using scanning small angle X-ray scattering (SAXS) at the intermolecular/nanoscale level and wide-angle X-ray scattering (WAXS) at the intra-molecular/atomic level. The scanning approach allows imaging the homogeneity of the samples and, used as statistical ensemble, average parameters and their distribution can be determined with the required precision and statistical relevance.

2 | MATERIALS AND METHODS

2.1 | Tissue preparation

Decellularized bovine pericardium was prepared at increasing glucose concentrations according the following procedure. Bovine pericardium were decellularized following a chemical protocol composed by three solutions: (a) 1 M NaCl, 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent and 25 mM ethylenediaminetetraacetic acid (EDTA). (b) 1 M NaCl, 1.8 mM sodium dodecyl sulfate (SDS) and 25 mM EDTA. (c) 6.4 μM Deoxyribonuclease I from bovine pancreas (Sigma-Aldrich), 0.1 M MgCl and 0.9 M NaCl. The thickness of the pericardium depends on the age of the animal and was below 1 mm in the present case.

Glucose concentrations from 10 mM (180 mg/dL) to 60 mM (1080 mg/dL) are known to be suitable to mimick the diabetic environment [20, 21]. Therefore, we have adopted the following protocol: decellularized matrices were immersed in different glucose solutions, at increasing concentration (from 0 mg/dL up to 4000 mg/dL). The samples were incubated at 37°C for 3 days. At the end of incubation, the actual glucose concentration in the matrices was evaluated through the use of a Glucose Assay Kit (Abnova, Germany). Briefly, a sample of 5 mm was cut from each matrix, and hydrolyzed in 500 μL of a 75 mM NaCl, 25 mM EDTA pH = 8, 1% SDS and 100 μg/mL of proteinase K solution for 16 hours. Then, 50 μL of the hydrolyzed matrix were added to an equal volume of Abnova Assay Reaction Mix, and incubated for 10 minutes, protected from light. At the end of the reaction, samples were analyzed through fluorescence spectroscopy (Victor X4 Multilabel Plate Reader, Perkin Elmer, Italy), with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. In order to measure the adsorbed glucose quantity, concentrations were normalized with analyzed matrices weight: the glucose quantity obtained through spectroscopy was divided by the weight of tissue used for the kit, obtaining the glucose concentration for mg of tissue.
2.2 | SAXS and WAXS scanning microscopy

SAXS and WAXS scanning microscopy data were collected at the cSAXS beamline of the Swiss Light Source (SLS) in Villigen, Switzerland [22]. A monochromatic X-ray beam (\(\lambda = 0.09116\) nm, \(E = 13.6\) keV) was focused down to about 26 \(\mu\)m (vertical) and 38 \(\mu\)m (horizontal) by a bent monochromator crystal and a bent mirror. The tissues, kept in phosphate-buffered saline (PBS) within an Ultralene sachet, were placed perpendicular to the direct beam and raster scanned through the beam spot, with the detector measuring the intensity scattered from the currently illuminated spot on the sample, integrated over the exposure time and across the sample thickness. Step sizes of 40 \(\mu\)m in both the horizontal and vertical directions, and exposure times of 0.2 seconds for WAXS and 0.32 seconds for SAXS, were used. To speed up acquisition, data were recorded in a continuous line-scan mode where the sample is moved at constant speed along a line of the two-dimensional raster scan, with the Pilatus 2 M detector continuously recording data. SAXS/WAXS data were collected at a sample-detector distance of 7089.8/322.84 mm. The transmitted, that is, unscattered X-ray beam was blocked from impinging onto the area detector by a beam stop, placed close to the center of the detector. For the SAXS experiments, part of the combined X-ray fluorescence and scattering signal of the beam stop was detected using a point detector. This enabled measuring the intensity of the transmitted X-ray beam, thus allowing the SAXS data normalization and the generation of X-ray transmission microscopy (XTM) images.

2.3 | Data analysis of the SAXS and WAXS microscopy data

For the visualization of the data, a variation of a previously published multi-modal approach has been employed [22], combined with peak fitting [23]. Collagen exhibits, along with the SAXS scattering signal, characteristic diffraction peaks in the range of momentum-transfer \(q\) covered by SAXS and WAXS measurements. The peak position corresponds to the characteristic distance \(d = 2\pi/q\) in direct space on which ordering occurs. The peak width corresponds to the length over which the ordering prevails and the peak height to the abundance of ordered material. Furthermore, the scattering and diffraction signals typically exhibit preferential orientation along the azimuth, originating in preferential orientation of the scattering units, that is, collagen fibers, over the X-rays illuminated volume. The analysis allows to capture all these characteristic parameters. Their values were combined in a single “multimodal” plot such that strong isotropic scattering shows up as white, whereas strong asymmetric scattering shows up in a color that identifies the azimuthal scattering direction. If, for example, a \(q\)-range is selected that is dominated by scattering from collagen, the high density of oriented collagen shows up in a bright color that represents the orientation, whereas a high density of collagen with isotropic orientation over the illuminated area shows up in white. While these multi-information plots are quantitative, images of a single quantity are more accessible in case quantitative information shall be read directly from the graphs. Thus, plots of the characteristic distance \(d\) to which the peak position corresponds are shown as well. Treating the distances \(d\) determined at each point of a sample as statistical ensemble, the distribution can be plotted and its main value and spread can be determined. In this way, the spatially resolving aspect of the imaging method is used for achieving high statistical relevance of characteristic values for a whole sample area. This allows determining changes of characteristic distances in the nanometer range, for example induced by exposure to glucose, with precision in the picometer range.

3 | RESULTS

In the following, the known resulting interactions of type 1 collagen with X-rays are described in the SAXS and WAXS regime, before focusing on the spatial distribution of these parameters over the pericardium tissue analog samples as a function of glucose content. The glucose concentration of the control sample, 0.005376 ± 0.000002 \(\mu\)g/mg and very close to the lower limit of the kit's calibration curve has been subtracted as offset from all concentrations. Across the entire set of samples, the samples analyzed by SAXS/WAXS microscopies correspond to the measured glucose concentrations in collagen of: 0.087 ± 0.002, 0.368 ± 0.003, 0.454 ± 0.016, 0.551 ± 0.039 and 0.905 ± 0.008 \(\mu\)g/mg, as specified in Table 1. The error bars refer to the SD of fluorescence spectroscopy values measured on three samples for each condition. The highest and smallest values were found for the samples which received the highest (nominal value = 4000 mg/dL) and the smallest (nominal value = 100 mg/dL) value of glucose in solution.

We remind the reader that a normal physiologic level of glucose in plasma is less than 110 mg/dL [1], that is, 100 mg/dL corresponds to physiologic condition. A linearity was found between the glucose administrated in solution and the glucose found in the tissues, apart for the 2000 mg/dL case, which corresponds to a lower actual concentration in tissue, see plot inset in Table 1 on semilogarithmic scale.

3.1 | Molecular description of collagen type I (WAXS)

Figure 1A displays the molecular model of 1CAG type 1 collagen (https://www.rcsb.org/). Figure 1B exhibits the pair...
distribution function $P(R)$ as a function of radius $R$, evaluated from the model by means of the PDFGUI software [24]. Two clear peaks in $P(R)$ are seen in:

1. The peak at 0.288 nm corresponds to the meridional distance $d_1$ between adjacent amino acid residues, projected along the central axis of the helical structure [25], or alternatively, to one-third of the unit height twist of 0.86 nm (Figure 1C). ([26]

2. The peak at 1.5 nm corresponds to the equatorial distance $d_2$ between collagen fibrils in the lateral packing (Figure 1D) [26, 27].

These two atomic distances in real space correspond to the WAXS diffraction peaks at scattering vectors of $q_1 = 2\pi/d_1 = 21.9/nm$ and $q_2 = 2\pi/d_2 = 4.2/nm$ in reciprocal space. The WAXS patterns of all investigated bio-tissues exhibit these two main peaks, marked by arrows in

**TABLE 1** Glucose concentration the decellularized tissue has been immersed in and the measured collagen glycation values, as determined by fluorescence spectroscopy

| Glucose concentration in solution (mg/dL) | μg glucose/mg collagen | SD | Glucose in collagen vs glucose in solution |
|------------------------------------------|------------------------|----|-------------------------------------------|
| 0                                        | 0                      |    |                                           |
| 100                                      | 0.087                  | 0.002 |                                           |
| 300                                      | 0.454                  | 0.016 |                                           |
| 500                                      | 0.551                  | 0.039 |                                           |
| 2000                                     | 0.368                  | 0.003 |                                           |
| 4000                                     | 0.905                  | 0.008 |                                           |

**FIGURE 1** Molecular description of collagen type I. A, molecular model of 1CAG type I collagen. B, Pair distribution function $P(R)$ as a function of radius $R$, that is, independent of molecular orientation, evaluated from the 1CAG model. C, The $d_1 = 0.288$ nm meridional distance (along the fiber axis) as one-third of the indicated 0.86 nm distance. D, The $d_2 = 1.5$ nm equatorial distance between the lateral packing of the collagen fibrils. E, WAXS diffraction peaks, with arrows pointing to the scattering vectors of $q_1 = 2\pi/d_1 = 21.9/nm$ and $q_2 = 2\pi/d_2 = 4.2/nm$, which were measured on all samples.
The other peaks in Figure 1E are attributed to the Ultralene sachet, used as a sample holder, and are thus not of interest in the further analysis.

### 3.2 Inter-molecular description of collagen type I (SAXS)

Figure 2A displays the inter-molecular nanoscale organization of 1CAG type 1 collagen, with the fibrils arranged in a staggered manner, leading to gap \((0.54 \, \text{nm})\) and overlap \((0.46 \, \text{nm})\) regions, which repeat with a periodicity \(d = 65.7 \, \text{nm}\) [28]. This nanoscale periodicity in direct space corresponds to the reciprocal-space series of SAXS diffraction peaks positioned at \(q_i = \frac{i \pi}{d}\) with \(i\) being a positive integer number, marked by vertical bars in Figure 2B. All samples measured by SAXS exhibit this series of peaks. The higher the measured diffraction orders, the higher the achievable resolution for the periodicity. At the same time, higher diffraction orders exhibit lower signal than lower ones. For the data analysis we have selected the ninth order reflection, highlighted by a blue arrow, which corresponds

| Glucose [µg/mg] | 0    | 0.087 | 0.368 | 0.454 | 0.551 | 0.905 |
|----------------|------|-------|-------|-------|-------|-------|
| WAXS - mer     | ![WAXS Image](image1.png) | ![WAXS Image](image2.png) | ![WAXS Image](image3.png) | ![WAXS Image](image4.png) | ![WAXS Image](image5.png) | ![WAXS Image](image6.png) |

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[FIGURE 2](image) Inter-molecular description of collagen type I. A, Inter-molecular nanoscale organization of 1CAG type 1 collagen with the fibrils arranged in a staggered manner, leading to gap \((0.54 \, \text{nm})\) and overlap \((0.46 \, \text{nm})\) regions, which repeat with a periodicity \(d = 65.7 \, \text{nm}\). B, SAXS patterns as measured on all bio-tissues, which show a series of peaks corresponding to the periodicity of \(d = 65.7 \, \text{nm}\) (vertical bars). The arrow points to the ninth reflection, which was employed for data analysis.

[FIGURE 3](image) Scanning WAXS images of the meridional intra-molecular distance \(d_1 = 0.29 \, \text{nm}\). Top row: multimodal maps of the orientation of X-ray scattering by collagen. Color represents orientation according to the color disc. Bottom row: peak-position analysis, position in nm shown as gray-scale. The scanned area is \(4 \times 8 \, \text{mm}^2\) and the length of the scale bar corresponds to \(2 \, \text{mm}\)
to a lattice spacing of \( d_3 = 7.3 \text{ nm} \) and in reciprocal-space to a diffraction peak at \( q_3 = 2\pi/d_3 = 0.86/\text{nm} \) in the SAXS curve. This diffraction order was reliably detected over the full area of all samples.

### 3.3 | WAXS and SAXS imaging

Figures 3 and 6 show the results of the analysis on the \( d_1 = 0.288 \text{ nm} \) meridional intra-molecular and the \( d_2 = 1.5 \text{ nm} \) equatorial inter-molecular distances. Figures 3 and 4 show the scanning-WAXS data obtained as multimodal images (upper row) and peak-position analysis (lower row). The lateral heterogeneity of the samples, spatially resolved across the entire \( 4 \times 8 \text{ mm}^2 \) inspected area, is revealed as color, that is, collagen-orientation variation (upper row) and as variation in the peak position, that is, variation in the precise value of the \( d_1 \) or \( d_2 \) distance (lower row). Both color- and gray-scale maps readily highlight the statistical relevance of collagen molecular orientation and packing. Note that the dark regions, both in color (orientation) and in gray scale (peak shift) maps, are “no signal” regions, rather than “no peak-shift” regions. For obtaining characteristic \( d \)-spacing values for each sample, we treated all points of a sample as statistical ensemble. Figures 5 and 6 show the resulting spatial distribution of \( d_1 \) and \( d_2 \) spacing for each sample. The \( d \)-spacing distributions were fitted with one (full circles in central panel of Figures 5 and 6) or if obviously skew, bimodal, two (open circles in central panel of Figures 5 and 6) Gaussian peaks. The positions and full width at half maximum (FWHM) of the fits are reported in the central panel of each figure. In the case of two Gaussian peaks, the average peak-position, weighted to the area under the peak, was added to the figure as full circle. The peak positions are linked by a full line as guide for the reader’s eyes.

Figures 7 and 8 show the results of an analog analysis at the nanoscale on the \( d_3 = 7.3 \text{ nm} \) meridional inter-molecular distance, which corresponds to the ninth order of the 65.7 nm spacing. Figure 7 shows spatially resolved the multimodal imaging analysis (upper row) and peak-position analysis (lower row) and Figure 8 the resulting spatial distribution of \( d_3 \) spacing for each sample. The \( d \)-spacing distributions, plotted in different colors for each glucose concentration in Figure 8, were fitted with 1 (full circles in

![FIGURE 4](image_url)

**FIGURE 4** Scanning WAXS images of the equatorial molecular distance \( d_3 = 1.5 \text{ nm} \). Upper row: multimodal maps of the orientation of X-ray scattering by collagen. Color represents orientation according to the color disc. Bottom row: peak-position analysis, position in nm shown as gray-scale. The scanned area is \( 4 \times 8 \text{ mm}^2 \) and the length of the scale bar corresponds to \( 2 \text{ mm} \).
central panel) or 2 (open circles in central panel) Gaussians. The positions and FWHM of the fits are reported in the central panel of the same figure as dots and error bars, respectively.

The results of the WAXS/SAXS microscopies are summarized as follows:

1. The \( d_1 = 0.29 \) nm meridional intra-molecular distance of collagen obtained from WAXS analysis (coded as color in Figure 3, upper row) follows a similar orientation as the \( d_3 = 7.3 \) nm inter-molecular distance measured by SAXS (Figure 7, upper row). This is expected considering that both periodicities are along the same fiber axis (compare Figures 1C and 2A).

2. The orientation of the \( d_2 = 1.5 \) nm equatorial molecular distance in WAXS (Figure 4, upper row) is perpendicular (see directions in the color wheel) to the \( d_1 = 0.29 \) nm meridional molecular distance in WAXS (Figure 3, upper row), as expected from the relative orientation of the \( 0.29 \) nm and \( 1.5 \) nm lengths (see Figure 1C,D).

3. Samples are laterally heterogeneous. The spatially resolved peak-position images in the lower row of Figures 3–4 and 7 show that the \( d_1 = 0.29 \) nm, \( d_2 = 1.5 \) nm and \( d_3 = 7.3 \) nm peaks change position both across the investigated areas and among different samples.

4. Some macroscopic bands (several mm in length) are visible both in WAXS and SAXS, for example, for \( 0.551 \) \( \mu \)g/mg glucose concentration the right-angle hook-like feature from the bottom right to the top left and from there continuing to the top-right, visible well in Figure 4.

5. Glucose has an effect on collagen at both intra- and inter-molecular scale. Indeed, it is worth to remember

**Figure 5** Spatial distribution of the \( d_1 = 0.29 \) nm intra-molecular distance. The spatial distribution of the \( d_1 \) spacing, evaluated across the scanning WAXS maps reported in the lower row of Figure 3, was fitted by one or two Gaussian peaks for each glucose concentration. The latter is specified as “%glu” label in \( \mu \)g/mg. The positions and FWHM of the fits are reported in the central panel using dots and error bars, respectively. In the case of two Gaussian peaks in the distribution, the average peak-position is indicated as full circle.
the reader that collagen molecules have a diameter of \(\approx 1.1 \text{ nm}\), that is, comparable to the size of glucose molecules of \(\approx 1 \text{ nm}\) [10].

6. We observe the same trend in the results of the SAXS and WAXS analysis, that is, both for intra- and inter-molecular spacing: an expansion \((0 \leq \text{[glu]} \leq 0.087 \mu\text{g/mg})\); a contraction \((0.087 \mu\text{g/mg} \leq \text{[glu]} \leq 0.551 \mu\text{g/mg})\); stagnation or minor expansion for one data point beyond \((\text{[glu]} \geq 0.551 \mu\text{g/mg})\).

7. The relative structural changes in the spacing are two to three times larger for the inter-molecular distances than for the intra-molecular one.

4 | DISCUSSION

We start with a consistency and robustness test of the methods applied. The expected and observed parallel orientation of (a) the \(d_1 = 0.29 \text{ nm}\) intra-molecular and of the \(d_3 = 7.3 \text{ nm}\) inter-molecular meridional distances as well as (b) the perpendicular orientation between the meridional \(d_1 = 0.29 \text{ nm}\) and the equatorial \(d_2 = 1.5 \text{ nm}\) molecular distances provides clear evidence for the robustness and reliability of the data analysis. This is an important test considering that one single map in Figures 3–4 and 7 is the result of the analysis of about 20 000 WAXS or SAXS 2D data frames of 2.4 million pixels each. Using the ensemble of all WAXS or SAXS frames recorded for each sample to determine the main value and spread of an intra- or inter-molecular spacing enabled achieved significantly higher precision than in previous studies based on single or few measurements for each sample. For example, the distribution of the \(d_1 = 0.29 \text{ nm}\) intra-molecular distance reported in Figure 5 has a width of about 0.2 pm, that is, 0.07%, limited by the homogeneity of the sample rather than the resolving power of the method.
We try to integrate the structural findings into a hypothetical model of the structural aspects of glycation. As structural parameters along and perpendicular to the fiber axis exhibit the same trend as a function of increasing glucose concentration, we interpret the results as a swelling of the collagen fibers in all directions, followed by shrinkage, followed by stagnation or minor re-swelling. That the magnitude of this trend is larger for inter-molecular distances than for the intra-molecular one is consistent with collagen molecules as building units that are stiff in comparison with their supra-molecular arrangement. The swelling trend can be explained based on a speculative working model taking into account factors like alteration of electrostatic interaction, cross-linking and steric effects [29]. The initial increase in volume upon going from 0 to 0.087 µg of glucose/mg of tissue may be attributed to steric effects, pushing the collagen molecules apart upon glycation. This could potentially ease access for water, both due to the volume becoming available and glucose being polar and thus hydrophilic. The result would be an amplification of the swelling effect caused by the initial glycation. While it is widely accepted that AGE cross-linking has the effect of stiffening the tissue, a phenomenon adopted on purpose in tissue engineering to stiffen and strengthen tissue equivalents [30], less is known on the nano-scale structural implications. We speculate that increased cross-linking is not just reinforcing the collagen fibers, but also reducing the measured structural distances. Thus, increasing the level of sugar in the engineered tissue, the lattice dilatation process is counter-balanced by the formation of more AGEs. At even higher sugar concentrations or extended exposure to sugar, excess AGE formation may then have no further effect on structural parameters, or even lead to a minor re-increase of the distance between the multiply cross-linked collagen molecules due to frustration of the competing cross-linking forces. While this model is speculative, it motivates the importance of parameters like the size and polarity of the sugar molecules that have thus far been frequently ignored. If, for example, one chooses ribose rather than glucose to increase the reaction rate and thus reduce the duration of the glycation, then this will lead to different structural parameters, and potentially also different trends for these parameters. This casts doubts on the full
equivalence of the exposure to different sugars, even if demonstrated in bio-mechanical experiments for a few points of the vast parameter space.

We compare these results with values reported for human tissue as a function of age [31, 32], as a function of exposure to ribose [33], and for rat-tail tendon as a function of exposure time to different sugars in a pioneering study published years ago 25 [34]. For human tissues with increasing age of the donor, a decrease of the molecular repeat distance along the collagen fibrils and an increase of the pentosidine fluorescence signal as marker for glycation have been reported [31]. This is in accordance with the decrease of $d_3 = 7.3$ nm with increasing glucose content observed in the present study. For rat-tail tendon no effect has been observed, rather a change in peak intensity ratios. For the inter-molecular distance $d_2 = 1.5$ nm an opposite behavior has been observed: a decrease with increasing glucose content in the present study and a weak but statistically relevant increase with increasing age for human tissue [31, 32], as well as an increase with increasing exposure time to ribose for rat-tail tendon. In view of the pronounced effect of mechanical strain [31], the hydration state on the structural parameters of collagen [34], the complexity of AGE formation in the human body [35], and the wide variety of experimental protocols (hydration, exposure time, sugar used), we can only speculate about the reasons for differing results of these studies.

Furthermore, only the present study aimed at quantifying the glucose content of the tissue on an absolute level and both intra- and inter-molecular structural parameters of collagen at the same time. This situation complicates direct comparisons of studies. The main conclusion we derive from this comparison is thus the chance to disentangle and quantify the factors that influence structural parameters of

**FIGURE 8** Spatial distribution of the $d_3 = 7.3$ nm inter-molecular distance, the ninth order of the 65.7 nm inter-molecular spacing. The spatial distribution of the $d_3$ spacing, evaluated across the images reported in the lower row of Figure 7, was fitted by one or two Gaussian peaks for each glucose concentration. The latter is specified as “%glu” label in μg/mg. The positions and FWHM of the fits are reported in the central panel using dots and error bars, respectively. In the case of two Gaussian peaks in the distribution, the average peak-position is indicated as full circle.
collagen using WAXS/SAXS microscopy, provided precise control on the sample preparation is given. Studies of human tissue are clearly required as reference. However, for such tissue it is impossible to have precise knowledge on the complex, multi-parameter history an individual sample has seen. This can be considered as a strong argument for the use of engineered tissue-analogs or standardized decellularized tissue for well-controlled studies of effects and therapies. Finally, the undoubted limitations of the present experimental work performed without replicate treatments of the material, will be overcome as we plan to step further with the investigation of collagen glycation, and apply the same kind of microscopy and relative data analysis to investigate the effect of glycation on the same collagen tissue (pericardium), due to different sugars (glucose, ribose, galactose) and for different incubation times (from few days up to 3 months).

5 | CONCLUSION

To quantify the direct, nano-structural impact of glycation on collagen, decellularized bovine pericardium-based tissues were studied by combined scanning SAXS/WAXS, a technique suitable for imaging the intra- and inter-molecular lattice parameters of collagen with picometer precision over comparatively large and thus representative areas of 32 mm² for each sample in the present case. Three distinct trends were observed: an initial expansion of both intra- and inter-molecular distances, due to a swelling process, from unphysiologically low glucose concentration toward a physiological one; a contraction of the same distances up to glucose concentrations in collagen of 0.551 μg/mg; a stagnation or minor re-expansion at an even higher concentration. This study provides a first step toward the vision of using engineered tissue or abundantly available decellularized farm animal tissue—rather than animal models—for well-controlled studies of the effects of glucose on connective tissues. We propose further studies of intra- and inter-molecular parameters of collagen in such tissues as a function of sugar type (glucose, ribose, fructose), concentration and incubation time up to several months. Based on these parameter-structure relationships, biomechanical studies must follow to establish structure-property relationships. These studies under well-controlled conditions would support disentangling the complex parameter space of glycation in human tissue as complex and not well controlled sample system. This information might be included in new models of the effects of diabetes and thus help to identify new diagnostic or therapeutic approaches for pathologies related to glucose metabolism.

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AUTHOR BIOGRAPHIES

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REFERENCES

[1] American Diabetes Association, Classification and Diagnosis of Diabetes, Diabetes Care 2016, 39(Supplement 1), S13.
[2] C. D. Mathers, D. Loncar, PLoS Med. 2006, 3(11), e442.
[3] H. Moole, V. Moole, A. Mamidipalli, S. Dharapu, R. Boddireddy, D. Taneya, et al., J. Community Hosp. Intern. Med. Perspect. 2015, 5(5), 28709.
[4] L. A. Henríquez-Hernández, O. P. Luzardo, P. F. Valerón, M. Zumbado, L. Serra-Majem, M. Camacho, L. D. Boada, Sci. Total Environ. 2017, 607-608, 1096.
[5] P. Zimmet, K. G. M. M. Alberti, J. Shaw, Nature 2001, 414, 782.
[6] WHO Mortality Database [online database]. Geneva: World Health Organization; 2019. https://www.who.int/healthinfo/mortality_data/en/.
[7] M. Kozakova, C. Palombo, Int. J. Environ. Res. Public Health 2016, 13(2), 201.
[8] I. M. Stratton, A. I. Adler, H. A. W. Neil, D. R. Matthews, S. E. Manley, C. A. Cull, D. Hadden, R. C. Turner, R. R. Holman, BMJ 2000, 321, 405.
[9] A. K. Harris, J. R. Hutchinson, K. Sachidanandam, M. H. Johnson, A. M. Dorrance, D. W. Stepp, S. C. Fagan, A. Ergul, Diabetes 2005, 54(9), 2638.
[10] J. Jeevanandum, A. Barhoum, Y. S. Chan, A. Dufresne, M. K. Danquah, Beilstein J. Nanotechnol. 2018, 9, 1050.
[11] G. Fessel, Y. Li, V. Diederich, M. Guizar-Sicairos, P. Schneider, D. R. Sell, V. M. Monnier, J. G. Snedeker, PLoS One 2014, 9 (11), e110948.
[12] S. J. Wilkinson, D. W. L. Hukins, Radiat. Phys. Chem. 1999, 56 (1–2), 197.
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