Alteration of the bone tissue material properties in type 1 diabetes mellitus: A Fourier transform infrared microspectroscopy study

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A B S T R A C T

Type 1 diabetes mellitus (T1DM) is a severe disorder characterized by hyperglycemia and hypoinsulinemia. A higher occurrence of bone fractures has been reported in T1DM, and although bone mineral density is reduced in this disorder, it is also thought that bone quality may be altered in this chronic pathology. Vibrational microscopies such as Fourier transform infrared microspectroscopy (FTIRM) represent an interesting approach to study bone quality as they allow investigation of the collagen and mineral compartment of the extracellular matrix in a specific bone location. However, as spectral feature arising from the mineral may overlap with those of the organic component, the demineralization of bone sections should be performed for a full investigation of the organic matrix. The aims of the present study were to (i) develop a new approach, based on the demineralization of thin bone tissue section to allow a better characterization of the bone organic component by FTIRM, (ii) to validate collagen glycation and collagen integrity in bone tissue and (iii) to better understand what alterations of tissue material properties in newly forming bone occur in T1DM. The streptozotocin-injected mouse (150 mg/kg body weight, injected at 8 weeks old) was used as T1DM model. Animals were randomly allocated to control (n = 8) or diabetic (n = 10) groups and were sacrificed 4 weeks post-STZ injection. Bones were collected at necropsy, embedded in polymethylmethacrylate and sectioned prior to examination by FTIRM. FTIRM collagen parameters were collagen maturity (area ratio between 1660 and 1690 cm−1 subbands), collagen glycation (area ratio between the 1032 cm−1 subband and amide I) and collagen integrity (area ratio between the 1338 cm−1 subband and amide II). No significant differences in the mineral compartment of the bone matrix could be observed between controls and STZ-injected animals. On the other hand, as compared with controls, STZ-injected animals presented with significant higher value for collagen maturity (17%, p = 0.0048) and collagen glycation (99%, p = 0.0121), while collagen integrity was significantly lower by 170% (p = 0.0121). This study demonstrated the profound effect of early T1DM on the organic compartment of the bone matrix in newly forming bone. Further studies in humans are required to ascertain whether T1DM also lead to similar effect on the quality of the bone matrix.

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Introduction

Type 1 diabetes mellitus (T1DM) is a severe disorder that persists through the entire life of affected individuals. T1DM is characterized by the inability of pancreatic β-cells to secrete insulin and therefore results in hyperglycemia. Several complications including bone loss and elevated fracture risk have been reported in T1DM [1,2]. Increased fracture risk could be partially related to diabetic complications such as retinopathy and neuropathy that increase the incidence of fall [3,4]. Another more likely explanation could reside in a direct effect of diabetes on bone tissue. Bone mineral density (BMD), measured by dual energy X-ray absorptiometry, has been reported to be lower in T1DM patients as compared to age-matched control subjects [5,6]. However, elevated fracture risk among T1DM patients still persists even after adjusting for hip BMD [7]. Furthermore, the low BMD observed in T1DM does not fully explain the high fracture risk in this population [8]. Thus, there is a supposition that diabetic bone is more fragile than non-diabetic bone for a given BMD and that T1DM not only affects bone microarchitecture, but also bone quality. The role of glucose in the formation of advanced glycation end products (AGE) is well

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known, and poorly controlled diabetes is suspected to increase the rate of collagen glycation. As AGE have been associated with a decrease in the toughness of trabecular and cortical bone [9,10], it is plausible that a similar mechanism occurs in T1DM.

Rodent models of T1DM are often generated by injection of streptozotocin (STZ), which is specific toxic to insulin-producing pancreatic beta-cells, and leads to hypoinsulinemia and hyperglycemia [11]. Several studies using STZ-injected rodent models have been published in the last decade and reported inconsistent data on the effect of T1DM on bone tissue material properties, i.e., the mineral and organic component of the bone matrix [12–15]. Previous studies seem suggest that bone microarchitecture deterioration appears early, while modification of intrinsic material, as determined by bone toughness, occurs later [16]. Furthermore, an inherent problem with the STZ model is that this compound is often injected around 10 weeks of age, and the general duration of studies is around 10–12 weeks. As such, most of the bone tissue has been formed before the onset of diabetes. To date, the gold standard method to assess the collagen component of the bone tissue has been formed before the onset of diabetes. To date, the gold standard method to assess the collagen component of the bone matrix [12–15].

In this regard, vibrational spectroscopy in the mid-infrared (IR) region can provide molecular structure information about mineralized and non-mineralized connective tissue [17–19]. In addition, Fourier transform IR microspectroscopy (FTIRM), in which the spectrometer is coupled to a light microscope, enables investigators to record spectra at discrete points within thin tissue section. This methodology has several advantages as compared with other approaches, such as X-ray diffraction, nuclear magnetic resonance or HPLC, in (i) allowing the use of the same sample for other investigations (histomorphometry, quantitative backscattered electron imaging, nanoindentation, etc.) and (ii) enabling the investigator to precisely determine tissue material properties in a specific location of a bone sample (between double tetraacycline labels as for example).

Based on IR analyses of single crystals and model compounds, the origin of vibrations observed in the IR has been tabulated for the mineral and protein component of the bone matrix. Several calculated parameters have been validated for the analyses of bone tissue and are reviewed in depth elsewhere [20]. As for example, the 900–1200 cm⁻¹ region, assigned to ν 1 3 phosphate vibrations, is often used to derive information regarding the maturity and size of the bone mineral. The 960–1140 cm⁻¹ region also contains information regarding carbohydrate moieties [21]. However, the presence of the strong ν 1 3 phosphate at the same wave number hampers the accurate determination of carbohydrate vibrations. As such, we hypothesized that investigation of the same bone section in a mineralized state (native state) followed by demineralization and re-analysis at the same location may be interesting to ascertain whether tissue material properties are affected in T1DM. Such an approach necessitates the validation of several IR parameters used in other connective tissue but bone, especially for the determination of collagen glycation and collagen integrity.

Therefore, the aims of the present study were (i) to develop a new approach, based on the demineralization of thin bone tissue section to more accurately characterize the organic component of the bone matrix by FTIRM; (ii) to validate collagen glycation and collagen integrity in bone tissue; and (iii) to better understand what alterations of tissue material properties occur in T1DM.

Materials and methods

Reagents

All chemicals were purchased from Sigma-Aldrich (Lyon, France) unless otherwise stated.

In vitro glycation of type I collagen

Type I collagen gels were prepared as reported elsewhere [22]. Type I collagen solution (4 mg/ml in 20 mM acetic acid) was diluted, on ice, with 0.1% acetic acid and neutralized with 0.4 M NaOH (pH 10.2) in PBS buffer to form 1.5 mg/ml gels. A 750 μl aliquot of the neutralized collagen solution was pipetted into wells of a 24-well plate and incubated at 37 °C overnight to allow for fibril formation. All gels were washed extensively with distilled water prior to a 60-day period of incubation with glucose solution. At the end of the incubation period, collagen gels were dialyzed against milliQ water for a week, and half were deposited onto BaF₂ window and allowed to dry to form a uniform film prior to FTIRM measurement. Eight gels per conditions have been analyzed.

In order to assess the glycation level, AGE-specific fluorescence, in the remaining half of collagen solutions, was determined using an M2 microplate reader (Molecular Devices, St Gregoire, France) with excitation and emission wavelengths set at 380 and 440 nm, respectively. The amount of fluorescence has been calibrated using several quinine sulfate concentrations (ranged 0–10 μg/ml). The amount of collagen in each solution was determined by the BCA method as described elsewhere [23]. AGE-specific fluorescence was then expressed as fluorescence intensity, in arbitrary unit/μg collagen.

Animals

A total of 18 young male Swiss 70 mice (8 weeks old) were randomly allocated to either the saline-injected control (n = 8) or streptozotocin-injected diabetic (STZ-150 mg/kg body weight, n = 10) groups. Six days post-STZ injection, diabetes was confirmed by measurement of blood glucose with an automated glucose oxidase procedure using a Beckman glucose analyzer II (Beckman Instruments, Galway, Ireland). Animals were injected intraperitoneally with calcein (10 mg/kg) 10 and 2 days before necropsy. Sacrifice was then performed 4 weeks post-STZ injection. All experiments were carried out according to UK home office regulations (UK Animals Scientific Procedures act 1986).

Bone tissue processing

At necropsy, femurs were harvested, cleaned of soft tissue and fixed with 70% ethanol for 24 h at 4 °C and embedded in polymethylmethacrylate at 4 °C. This fixation/embedding protocol has previously been successfully employed for FTIRM measurements [24]. Cross-sectional sections (4 μm thickness) of the femur mid-diaphysis were cut dry on a heavy duty microtome equipped with tungsten carbide knives (Leica Polycut S). Four bone sections were analyzed for each animal. Each section was separated by 15 μm. Sections were first observed with a fluorescence Olympus AX-60 microscope (Olympus France, Rungis, France) with a 40x magnification with either bright field or fluorescence light for the visualization of calcein double labeling (excitation and emission filters set at 490 nm and 520 nm, respectively), and digital photographs were taken with both illuminations. White and fluorescent pictures were overimposed using Adobe Photoshop CS5 (Adobe, Paris, France) and were used with demineralized bone section only to ensure proper locations of the two calcein lines (See Fig. 1). Sections were then sandwiched between BaF₂ windows and ready for FTIRM data acquisition as detailed below.

FTIRM data acquisition

Spectral acquisitions were obtained on a Bruker Vertex 70 spectrometer (Bruker optics, Ettlingen, Germany) interfaced with a Bruker Hyperion 3000 infrared microscope equipped with a standard single element Mercury Cadmium Telluride (MCT) detector in the range 750–4000 cm⁻¹. The Bruker Hyperion 3000 was also equipped with a mercury lamp, a 455 nm excitation filter and a 520 nm emission filter (Bruker). The FTIR spectrometer was continuously purged with dry
air from air purifiers. Using the fluorescence set up and a 36X IR Schwarzschild objective (NA = 0.5), acquisition of spectrum was conducted with the video measurement mode with a pixel size of 1.1 µm. In this mode, an image is recorded, and markers are manually positioned at the locations where spectra should be acquired, and then due to a motorized stage, spectra are recorded at marked locations with an IR beam size of ~7 µm at 1650 cm⁻¹. Markers were positioned manually in the center of double calcein labels and were spaced by 20 µm. The number of markers per double calcine regions was variable depending on calcine labeling length. At least one spectrum was acquired in each double-labeled surface. At the meantime, marker locations were also added on the Photoshop-processed images taken above. A minimum of 40 IR spectra was recorded for each sample at a resolution of 4 cm⁻¹, with an average of 32 scans in transmission mode with the Opus software (Bruker, release 6.5). Background spectral images were collected under identical conditions from the same BaF₂ windows at the beginning and end of each experiment to ensure instrument stability.

**FTIRM data processing**

The spectral region 850–1800 cm⁻¹ was used for analysis. Each spectrum, obtained from a pMMA section, was first corrected for polymethylmethacrylate (pMMA) contribution by subtraction of pMMA background after normalization of the peak at 1730 cm⁻¹. The 40 spectra of each sample were baseline corrected with an elastic correction method and vector normalized on the range 1495–1720 cm⁻¹. Spectra were subjected to curve fitting using a commercial available software package (GramsAI 8.0, ThermoFisher scientific, Villebon sur Yvette, France). Briefly, the second derivative spectrum was used to determine the number and the position of the bands constituting every fingerprint region. Second derivative were overimposed, and subband was positioned, with a gaussian shape, at minimum intensities observed on the second derivative. Within each subband, a gaussian peak was fitted with an area and a height. All parameters in Table 1.

### Table 1 FTIRM parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral phase</td>
<td></td>
</tr>
<tr>
<td>Degree of mineralization</td>
<td>Area of 900–1200 cm⁻¹ phosphate band/area of amide I band (1585–1720 cm⁻¹)</td>
</tr>
<tr>
<td>Carbonate substitution</td>
<td>Area of 850–890 cm⁻¹ band/area of phosphate band</td>
</tr>
<tr>
<td>Mineral crystallinity</td>
<td>Area of 1030 cm⁻¹ subband/area of 1020 cm⁻¹ subband</td>
</tr>
<tr>
<td>Protein phase</td>
<td></td>
</tr>
<tr>
<td>Collagen maturity</td>
<td>Area of 1660 cm⁻¹ subband/area of 1690 cm⁻¹ subband</td>
</tr>
<tr>
<td>Collagen integrity</td>
<td>Area of 1338 cm⁻¹ subband/area of amide II band (1585–1405 cm⁻¹). Reduction in this parameter suggests collagen denaturation.</td>
</tr>
<tr>
<td>Tissue secondary structure</td>
<td>Relative area of subbands located at 1694 cm⁻¹ (β-turn), 1661 cm⁻¹ (α-helix), 1647 cm⁻¹ (unordered), 1638 cm⁻¹ (triple helix) and 1626 cm⁻¹ (β-sheet).</td>
</tr>
<tr>
<td>Collagen glycation</td>
<td>Area of 1032 cm⁻¹ subband/area of amide I band</td>
</tr>
</tbody>
</table>

Parameters in italic can only be assessed after the demineralization of the bone matrix.

**Demineralization protocol**

Sections were demineralized by immersion in a solution of 0.5 M ethylenediaminetetraacetic acid (EDTA) in Tris buffer pH 7.4 for 7 days. The EDTA solution was replenished at day 3. At the end of the 7-day period, sections were rinsed 8 times with Tris solution followed by 6 washes with milliQ water. Sections were then allowed to dry at 37 °C for 48 h. Sections were then sandwiched between BaF₂ windows and reexamined by FTIRM. As calcine labels were no longer present after EDTA treatment, suggesting demineralization. Indeed, EDTA analysis confirmed that calcium concentration was drastically reduced and phosphorus below the detection limit. Furthermore, C₀mean, which represents the mean mineral concentration, decreased to 0 after EDTA treatment, suggesting a mineralization state equivalent to osteoid tissue and as such the absence of mineral (Fig. 2B). FTIRM spectra recorded before and after EDTA treatment revealed dramatic changes (Fig. 2C). First, the large band observed at 900–1200 cm⁻¹, trypsin (Lonza, Verviers, Belgium) at 37 °C for 10 min. Sections were then rinsed 6 times in milliQ water and allowed to dry at 37 °C for 48 h. prior to FTIRM reexamination.

**Quantitative backscattered electron imaging (qBEI)**

Quantitative backscattered electron imaging was employed to determine the bone mineral density distribution (BMD) of the section before and after demineralization [25]. Sections were observed with a scanning electron microscope (EVO LS10, Carl Zeiss Ltd, Nanterre, France) equipped with a five quadrants semi-conductor backscattered electron detector and operated at 20 keV with a probe current of 250 pA and a working distance of 15 mm. The backscattered signal was calibrated using pure carbon (Z = 6, mean grey level = 25), pure aluminum (Z = 13, mean grey level = 225) and pure silicon (Z = 14, mean grey level = 253) standards (Micro-analysis Consultants Ltd, St Ives, UK). Analysis was performed as previously reported [26]. C₀mean, representing the mean calcium concentration, was determined. The composition of the bone section was investigated by energy-dispersive X-ray analysis (EDX) using an Inca Xmax device (Oxford Instruments, Oxford, UK). The detection limit of this EDX system was of 0.01% atomic percentage.

**Statistical analysis**

Results were expressed as mean ± standard error of the mean (SEM). Student’s t-test with Levene’s test of homogeneity was used to compare the differences between the CTRL and STZ groups using the Systat statistical software release 13.0 (Systat software Inc., San Jose, CA). Differences observed in vivo assays were assessed using Levene’s test followed by ANOVA and Bonferroni post hoc test. Differences at p < 0.05 were considered significant.

**Results**

**Demineralization efficiency**

In order to validate our demineralization protocol, we first looked at the mineral presence by qBEI. As shown in Fig. 2A, EDTA treatment led to a dramatic reduction of signal, suggesting demineralization. Indeed, EDX analysis confirmed that calcium concentration was drastically reduced and phosphorus below the detection limit. Furthermore, C₀mean, which represents the mean mineral concentration, decreased to 0 after EDTA treatment, suggesting a mineralization state equivalent to osteoid tissue and as such the absence of mineral (Fig. 2B). FTIRM spectra recorded before and after EDTA treatment revealed dramatic changes (Fig. 2C). First, the large band observed at 900–1200 cm⁻¹,

\[ \text{RMSnoise} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (a_i - p)^2} \]

where n is the number of data point in the fitted region, a is the true absorbance value and p is the predicted absorbance value determined by the Levenberg–Marquardt (LM) algorithm. Initial trace and second derivative were overimposed, and subband was positioned, with a gaussian shape, at minimum intensities observed on the second derivative spectrum. The LM algorithm was run, and the position and area of subbands were obtained. Evaluated IR spectral parameters are presented in Table 1.

**Enzymatic treatment**

Demineralized sections of control animals, already examined by FTIRM, were incubated in 1 mg/ml type IA-S collagenase or 0.25 mg/ml.
due to phosphate ν1,ν3 band, disappeared as a consequence of EDTA treatment, confirming demineralization. Second, the band located at −870 cm−1, representing ν2 carbonate vibration, is no longer present in the demineralized bone section. Third, the band located at 1350–1500 cm−1, representative of ν3 carbonate and CH2 wagging, was dramatically modified by demineralization, to reveal two peaks located respectively at 1451 cm−1 and 1400 cm−1 and corresponding to CH2 and CH3 wagging of collagen side chain. The band centered at 1338 cm−1, representing the vibration of amino acid side chain, was better resolved in demineralized sections.

Assessment of the extent of collagen glycation in collagen gel

Next, to study collagen glycation and identification of suitable peak to measure glycation, we used collagen gels that were incubated in glucose solution. As depicted in Fig. 3A, the main consequence of incubation with 25 mM glucose was a large band appearing between 960 and 1140 cm−1 on the FTIR spectra and representative of C-O-C, C-OH and C-C ring vibrations. Curve fitting of this region, based on second derivative analysis, revealed the presence of seven underlying bands located at 995 cm−1, 1012 cm−1, 1032 cm−1, 1049 cm−1, 1064 cm−1, 1082 cm−1 and 1101 cm−1 (Fig. 3B). In order to ascertain whether one of these bands could be used to monitor collagen glycation, we looked at their respective evolution in the presence of 0, 25 and 50 mM glucose. The subband located at 1032 cm−1 was the biggest (39.1%) in the region 960–1180 cm−1 at a glucose concentration of 50 mM (Table 2). Furthermore, the 1032 cm−1 subband was the only subband that showed an increase over the three tested glucose concentrations (Table 2). As such, we decided to use this subband for determining collagen glycation.

Validation of collagen glycation and collagen integrity parameters

Collagen glycation was determined by calculating the area ratio of the 1032 cm−1 subband over the Amide I region (Fig. 4A). This ratio augmented significantly in a dose-dependent manner by 10- and 34-fold with 25 and 50 mM glucose incubations, respectively. Collagen glycation was also determined by assessing the fluorescence emission at 440 nm (Fig. 4B). Similarly, the fluorescence intensity (arbitrary unit/μg collagen) was significantly and dose-dependently augmented by 5- and 12-fold with 25 and 50 mM glucose incubations, respectively. These two methodologies examine collagen glycation and were significantly correlated with an R2 value of 0.9992 (p = 0.013). However, no significant modification of collagen secondary structure was evidenced after glycation (Fig. 4C) although a subband located at −1674 cm−1 was present in 5 of the 8 collagen gels incubated with 50 mM glucose.

In order to assess whether collagen integrity was affected by collagen glycation, we measured this parameter in the presence of 0, 25 and 50 mM glucose. Collagen integrity was unaffected by collagen glycation (Fig. 4D). We then assessed collagen integrity in demineralized bone section of control animals before and after enzymatic treatment. The ratio 1338 cm−1/Amide II was significantly reduced after treatment with collagenase but not with trypsin (p = 0.008 and p = 0.540 respectively, Fig. 4E). Secondary structure of bone tissue was dramatically altered after collagenase treatment with significant augmentations in β-turn and α-helix and significant reductions in unordered and triple helix (Fig. 4F). On the other hand, trypsin did not significantly alter tissue secondary structure.

T1DM did not alter the mineral component of the bone matrix but increased collagen maturity

Structural parameters were assessed by FTIR in mineralized sections of control and diabetic mice. No major difference was evidenced in FTIRM spectra (Fig. 5A). The degree of mineralization, carbonate substitution and mineral crystallinity were not significantly different between the two groups of animal (Fig. 5B–D). On the other hand, collagen maturity was significantly increased by 17% (p = 0.0048) in diabetic animals as compared to controls (Fig. 5E).

T1DM resulted in dramatic alterations of collagen structural feature

Structural collagen parameters were also investigated in demineralized bone sections of control and diabetic animals. Again, no major differences in spectral features were observed between the two animal groups, except in the region 1000–1050 cm−1, with an
increase in diabetic mice (Fig. 6A). Collagen glycation was significantly higher in diabetic mice by 99% \((p = 0.0121, \text{Fig. 6B})\). Collagen integrity was significantly lower in diabetic animals by almost 170\% \((p = 0.0121, \text{Fig. 6C})\). Interestingly, we observed significant modifications in tissue secondary structure in diabetic mice with a significant higher amount of \(\beta\)-turn (2-fold, \(p < 0.001\)) and significant reductions in unordered (3.8-fold, \(p = 0.018\)) and triple helix (4.25-fold, \(p < 0.001\)) (Fig. 6D).

**Discussion**

Worldwide, the number of people who develop T1DM is increasing. While living with T1DM is manageable with diet, insulin therapy and exercise, diabetic complications still occur. Among them, bone fracture represents a severe complication that greatly alters the quality of life. Despite the need to prevent fracture occurrence, there is a paucity of mechanistic information on how hypoinsulinemia and hyperglycemia
associated with T1DM affect bone tissue, and especially tissue material properties. In the present study, an innovative FTIRM analysis of bone tissue in a mineralized state was used followed by analysis at the same location upon demineralization. We validated several FTIRM parameters (collagen glycation, collagen integrity and tissue secondary structure) that were useful in assessing structural modifications of the bone tissue. Furthermore, we demonstrated a significant augmentation of collagen glycation in diabetic bone tissue and also collagen denaturation (reduction in collagen integrity and breakdown of the triple helix) that could potentially alter bone toughness.

First of all, the demineralization of bone sections led to modifications of spectral feature with biggest modifications observed in the 1500–1350 cm⁻¹ and 1200–900 cm⁻¹ regions. In mineralized section, the 1500–1350 cm⁻¹ region was assigned to ν3 carbonate and ν2 wagging. Demineralization led to removal of carbonate moieties as evidenced by the absence of the ~870 cm⁻¹ ν2 carbonate band. It is legitimate then to assume that the modifications of the 1500–1350 cm⁻¹ observed in demineralized section also reflect the absence of the ν3 carbonate vibration and allows a better resolution of underlying peak representative of CH₂ and CH₃ wagging of collagen side chains. The demineralization of bone sections also highlighted changes in the region 1200–900 cm⁻¹ with the removal of ν3 ν1 phosphate vibrations and the apparition of underlying peak assigned to carbohydrate moieties. However, prior to the assignment of any demineralized subbands to collagen properties, several validations were required. In vitro glycation of type I collagen gel demonstrated that the relative area of the 1032 cm⁻¹ subband of the carbohydrate region was dose-dependently augmented during glycation of collagen. This correlated with the fluorometric assessment of collagen glycation, a methodology validated for the assessment of AGE in collagen matrix. Taken together, these data suggest that the 1032 cm⁻¹ subband might be useful in determining collagen glycation in demineralized bone tissue. However, before further validation is made to fully ascertain that the 1032/amide I ratio is a marker of AGE formation, this parameter should be considered as only indicative of collagen glycation. One might wonder why we did not choose the entire carbohydrate region (960–1140 cm⁻¹) or any of the other subbands as markers of collagen glycation. As evidenced in the present study, the carbohydrate region (960–1140 cm⁻¹) is composed of seven underlying bands. Some of these subbands (995 cm⁻¹, 1012 cm⁻¹, 1049 cm⁻¹, 1064 cm⁻¹ and 1101 cm⁻¹) were reduced after trypsin treatment (data not shown), suggesting that these subbands were generated by non-collagenous proteins. Indeed, this region has been proposed in other connective tissue as a marker of proteoglycan content [27]. The 1064 cm⁻¹ band was recently reported by Rieppo and co-workers as a marker of sulfated proteoglycan in cartilage [21]. These authors showed that enzymatic removal of proteoglycan dramatically affected this peak. The extracellular bone matrix contains several proteoglycans and, as such, assignment of this peak in bone tissue is more difficult. The 1082 cm⁻¹ subband can be assigned to the O–O stretching vibration of carbohydrate but also to the symmetric PO₄ stretching vibration [28]. The extracellular matrix of the bone tissue contains several phosphorylated proteins (siblings, fibronectin, vitronectin, etc.) that could potentially influence the area of the 1082 cm⁻¹ subband and although phosphorus was not identified with EDX analysis (detection limit of 0.01 % atomic percent), we cannot rule out the presence of phosphorus trace in the organic matrix that could have modified the 1082 cm⁻¹ subband. Furthermore, Rieppo and co-workers did not observe dramatic alteration of the 1032 cm⁻¹ peak upon enzymatic removal of proteoglycan, suggesting that this peak, although located in the carbohydrate region, might be an indicator of collagen glycation rather than glycosaminoglycan [21]. In our study, no reduction of the 1032 cm⁻¹ peak was observed upon trypsin digestion, suggesting that this peak could not be assigned to non-collagenous proteins and/or proteoglycans. Recently, Guilbert et al. reported in an elegant spectroscopic study that the intensity ratio of the 1032 cm⁻¹ peak over the amide I peak was increased upon collagen glycation, supporting the use of the 1032 cm⁻¹ band as a marker of collagen glycation [29].

In the current study, we also introduced and validated a parameter called collagen integrity. In earlier FTIR studies, absorbance of the 1338 cm⁻¹ band was shown to decrease in intensity as collagen denatures [28]. The 1338 cm⁻¹ band is assigned to the CH₂ wagging vibration of collagen amino acid side chains [28]. The 1338 cm⁻¹/amide II ratio was first introduced by West et al., and these authors evidenced a smaller ratio in osteoarthritic joint as compared to normal [30]. Further work to elucidate the molecular origin of this change confirmed that reduction in the 1338 cm⁻¹ absorbance was linked to collagen degradation [31]. In the present study, we reported similar finding in demineralized bone where the use of collagenase significantly reduced collagen integrity. On the other hand, neither glycation of type I collagen nor trypsin treatment altered this parameter. This suggests that collagen integrity in bone tissue is a marker of collagen degradation.

Protein secondary structure was also determined by second derivative and peak fitting of the amide I region as previously reported [32,33]. For collagen gels, the amide I region reflects the secondary structure of type I collagen. However, the bone matrix is a mixed between type I collagen (~90%) and non-collagenous proteins (~10%). As such, information obtained from the amide I region of bone tissue reflects the overall secondary structure of this tissue. Nevertheless, collagen is distinct from other proteins in that this protein comprises three polypeptide chains (α-chains), which assemble together to form a triple helix with a characteristic band at ~1638 cm⁻¹ [34]. In the present study, tissue secondary structure was dramatically altered by collagenase but not by trypsin. We failed to evidence any significant alteration of collagen secondary structure upon glycation or trypsin treatment, although a subband located at ~1674 cm⁻¹ was evidenced in 5 out of 8 collagen gels incubated with 50 mM glucose. However, collagenase treatment led to a dramatic change in the bone tissue secondary structure with a significant alteration of the collagen matrix as represented by the decrease in the triple helix.

The mineral component of the bone matrix seemed unaffected in T1DM, despite previous reports of lower mineralization degree in STZ-induced T1DM [15,35]. However, in these publications, the study period post-STZ injection as well as the STZ dose were different as compared to the present study, and it is plausible that mineralization default arises from this difference. Another possible explanation could reside in the mouse strain. In the current study, we used Swiss TO mice based on the fact that the extent of beta-cells destruction in the pancreas, in response to STZ injection, is similar to what is observed in humans suffering of T1DM. However, the spectral signature of the phosphate band appeared peculiar and the absence of mineral modifications might only be valid in this animal model. On the other hand, collagen maturity, determined on mineralized bone sections, was significantly increased in the diabetic mice. Previously, Saito et al. reported a significant reduction in immature but not in mature collagen crosslinks in the WBN/Kob diabetic rat model [36]. As collagen maturity is calculated as the ratio of the 1660 cm⁻¹/1690 cm⁻¹ subbands, respectively, assigned to mature and immature collagen crosslinks [37], one could expect an increase in collagen maturity in diabetic animals as observed in the present study.
Fig. 4. Validation of demineralized parameters. Collagen glycation was determined by (A) FTIRM and (B) fluorescence readings at 440 nm. (C) Collagen structure was assessed by curve fitting of the amide I region in the presence of 0 mM, 25 mM and 50 mM glucose. (D) Collagen integrity was determined in the presence of 0 mM, 25 mM and 50 mM glucose. (E) Collagen integrity was computerized before and after enzymatic treatment. (F) Tissue secondary structure was tabulated after enzymatic treatment. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. no glucose. FTIRM parameters are defined Table 1.
However, at the present, we have no data that could suggest that collagen glycation and AGE formation could affect the FTIR-determined collagen maturity. Nevertheless, the most notable difference in tissue material properties was observed for the collagen component. As expected, T1DM led to a dramatic and significant 2-fold increase in collagen glycation. Previous studies have shown that T1DM results in increased collagen glycation, which may contribute to the development of diabetic complications. Previous studies have suggested that glycation can affect the mechanical properties of collagen, leading to increased stiffness and decreased elasticity. Additionally, collagen glycation has been linked to increased matrix stiffness and decreased matrix compliance, which may contribute to the development of diabetic complications. Overall, these findings highlight the importance of considering collagen glycation as a potential contributor to the development of diabetic complications.
studies have also reported a higher AGE accumulation in the bone matrix of diabetic animals supporting our results [14,36]. Collagen integrity was significantly lower in diabetic animals and accompanied by profound changes in tissue secondary structure. As reported above, collagen integrity was not significantly affected by transphy treatment, suggesting that the 1338 cm⁻¹ vibration is strictly related to collagen amino acid side chain. The decrease in collagen integrity and secondary structure (fall in triple helix) suggests a denaturation of type I collagen in T1DM. These modifications cannot be explained by the augmented collagen glycation but rather by enzymatic degradation of matrix collagen. Interestingly, further reports have been made regarding higher matrix metalloproteinase (MMP) activities, mainly MMP-2, MMP-9, MMP-13 and MMP-14, in connective tissues, other than bone, in type 1 diabetes [38–42]. To the best of our knowledge, MMP activities have never been assessed in bone tissue of diabetic animals. However, it is plausible that MMP activities could be increased in T1DM due to the chronic inflammation state, and this could potentially participate to the reduced collagen integrity by cleaving the triple helix. Furthermore, all FTIR/M measurements have been performed between double calcine labeling, suggesting that the observed alterations of collagen were not due to a difference in tissue age and that they appeared fairly quickly after the onset of diabetes. As such, it is reasonable to assume that the observed reduction in bone toughness seen in other rodent models of STZ-induced T1DM could be due to the modification of the collagen component of the bone matrix as evidenced in the present study. In conclusion, in the present study, we highlighted that investigation of the bone matrix in a demineralized state brings important structural feature of the bone tissue. Indeed, several alterations of collagen quality (collagen glycation, integrity and secondary structure) in the newly forming bone matrix of diabetic animals have been observed and could lead to the observed reduction in bone toughness. Further studies are now required to demonstrate whether these findings are transposable to bone tissue of individuals affected by T1DM.

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References