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Solid state NMR investigation of intact human bone quality: balancing issues and insight into the structure at the organic-mineral interface

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Abstract

Age-related bone fragility fractures present a significant problem for public health. Measures of bone quality are increasingly recognized to complement the conventional bone mineral density (BMD) based assessment of fracture risk. The ability to probe and understand bone quality at the molecular level is desirable in order to unravel how the structure of organic matrix and its association with mineral contribute to the overall mechanical properties. The $^{13}\text{C}\{^{31}\text{P}\}$ REDOR MAS NMR (Rotational Echo Double Resonance Magic Angle Spinning Nuclear Magnetic Resonance) technique is uniquely suited for the study of the structure of the organic-mineral interface in bone. For the first time, we have applied it successfully to analyze the structure of intact (non-powdered) human cortical bone samples, from young healthy and old osteoporotic donors. Loading problems associated with the rapid rotation of intact bone were solved using a Finite Element Analysis (FEA) approach, and a method allowing osteoporotic samples to be balanced and spun reproducibly is described. REDOR NMR parameters were set to allow insight into the arrangement of the amino acids at the mineral interface to be accessed, and SVD (Singular Value Decomposition) was applied to enhance the signal to noise ratio and enable a better analysis of the data. From the REDOR data, it was found that carbon atoms belonging to citrate/glucosaminoglycans (GAGs) are closest to the mineral surface regardless of age or site. In contrast, the arrangement of the collagen backbone at the interface varied with site and age. The relative proximity of two of the main amino acids in bone matrix proteins, hydroxyproline and alanine, with respect to the mineral phase was analyzed in more detail, and discussed in view of

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Supplementary materials

Figure S1 Orientation of specimen cylinders in anatomic coordinates; Figure S2 Method of balancing inhomogeneous bone specimens; Figure S3 ^1H - ^{13}C CPMAS NMR spectra of an intact bone sample over time; Figure S4 ^1H - ^{13}C CPMAS NMR spectra of all 5 samples in this study; Figure S5 Schematic representation of the REDOR pulse sequence used; Figure S6 Analysis of the influence of the fraction of the original FID used for the SVD denoising; Figure S7 Analysis of the effect of noise thresholding in the SVD transformation domain; Figure S8 $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR spectra processed by Fourier Transform, after exponential multiplication (EM) apodization of the FID with 40 Hz line broadening, or SVD denoising; Figure S9 $\Delta\text{S}/\text{S}_0$ dephasing in $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR spectra denoised by exponential multiplication (EM) or SVD, before Fourier transformation; Figure S10 The free body diagram representation of rotating bone; Figure S11 Schematic of the problem of buoyancy in rotating liquid; Figure S12 Influence of the baseline positioning on $\Delta\text{S}/\text{S}_0$ dephasing determined from a set of $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR experiments; Figure S13 Comparison of ^1H - ^{13}C CPMAS and $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR spectra (recorded without ^{31}P recoupling pulses) of powdered and intact bone samples.

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glycation measurements which were carried out on the tissues. Overall, this work shows that the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR approach could be used as a complementary technique to assess a novel aspect of bone quality, the organic-mineral interface structure.

Keywords

bone; solid state NMR; organic-mineral interface; collagen; REDOR; balancing

1. Introduction

Fragility fractures associated with osteoporosis are a significant contributor to healthcare costs.¹ To help face this problem, patient screening, followed by an intervention, is desirable.² Bone health is conventionally assessed by X-ray based measurement of bone mineral density (BMD), but several recent studies showed that BMD can not be considered as the only predictor of fracture risk anymore.^{3,4} It would be beneficial to complement the conventional fracture risk assessment by methods that take into account bone quality aspects like bone geometry and shape, microarchitecture, turnover, and mineral and organic matrix properties.

Bone is a composite material where the mineral hydroxyapatite (HA) platelets provide stiffness, the organic matrix provides toughness, and water mediates their interface and is related to the overall strength.^{5–7} The organic matrix consists predominately of type I collagen, but it also contains non-collagenous proteins (NCPs) such as osteocalcin and osteopontin,⁸ and other organic compounds such as glucosaminoglycans (GAGs)^{9,10} and citrate.^{11,12}

There are several approaches to assess of bone quality. Modern biochemical approaches can determine femtomolar concentrations of rare biomarkers^{13,14} and overcome microscale heterogeneity by analyzing microscopic volumes of tissue.¹³ Microindentation can help evaluate the fracture resistance of bone by inducing damage in microscopic volume of bone.¹⁵ Because microindentation does not cause major tissue-level damage or pain, it is feasible to perform in clinical setting.¹⁶ In non-X-ray based measurements, it was shown that the analysis of signals of bone water in low field Nuclear Magnetic Resonance (NMR) spectrometers can supply information on its microarchitecture (by monitoring the signal of “free water”, which is inside the in canalicular/lacunar network) and on organic matrix hydration (by measuring the signal of collagen bound water).^{17,18}

The bone hydration state presents a relevant measure of bone quality. Wilson *et al.* observed tightly mineral-bound water in the organic-mineral interface of bone⁵ and proposed its role in mechanical properties of the tissue.⁶ Nalla *et al.* showed using a fracture mechanics approach that in the related bionanocomposite dentin, exchanging water by weaker hydrogen bonding solvents like methanol, ethanol and acetone, increases stiffness and toughness.⁷ In human bone, Nyman *et al.* measured the bone hydration state on larger sample sizes by an NMR technique that, in principle, can be used in clinical Magnetic Resonance Imaging (MRI).¹⁷ It was shown that the amount of collagen-bound water decreases with age¹⁷ and correlates to the yield and peak stress and pre-yield toughness of bone.¹⁸ Because hydration influences mobility of amino acids in collagen, and particularly of the C-4 carbon atom of hydroxyproline¹⁹ located on the periphery of the triple helix and of the fibril, it is likely that the organic-mineral interface is affected as well.

High-field solid state NMR has been used to track the consequences of dehydration on the ^{13}C NMR signature of amino acids in intact animal bone.²⁰ However it is only recently that an attempt has been made by Rai *et al.* to use $^{13}\text{C}\{^{31}\text{P}\}$ Rotational Echo Double

Resonance (REDOR) solid state NMR pulse sequence to measure the spatial distances between organic and mineral phases of intact animal bone under dehydration.²¹ Rai *et al.* determined that the distances from mineral surface of the C-2 carbon atoms of glycine and alanine, C-3 of proline and C-4 of hydroxyproline are in the range of 8.7–9.9 Å. Whether aging of bone affects the organic-mineral interface structure is however still unclear

Besides the hydration changes, the aging of bone is commonly associated with collagen crosslinking. The enzymatic crosslinking, controlled by enzyme lysyl oxidase, increases with the tissue maturity and contributes positively to the mechanical properties of bone. In contrast, cross-linking due to the non-enzymatic glycation (NEG) process occurs spontaneously in presence of reducing sugar, and it increases with age causing bone fragility. NEG process (also referred to as glycation) involves reaction of glucose with the amino group on lysine in collagen to form a Schiff base (glucosyl-lysine).²² The resulting product then undergoes Amadori rearrangement and forms fructose-lysine. The Amadori product can further oxidize and fragment. Intermolecular links (advanced glycation endproducts, AGEs) are formed for example when arginine from neighboring collagen molecules is covalently bound. The influence of the added covalent constraints on the properties of collagen are well established, but it remains unknown whether glycation also alters the organic-inorganic interface.

Thus, there is a clear need to analyze the structure of the organic-mineral interface and investigate its modifications. The $^{13}\text{C}\{^{31}\text{P}\}$ REDOR solid state NMR pulse sequence^{23,24} is uniquely suited to address these questions.²⁵ In bone, most carbon atoms are in the organic matrix while most phosphorus is present in the form of calcium phosphate mineral platelets of hydroxyapatite (HA). The REDOR pulse sequence allows to identify carbon atoms that are spatially close to the phosphorus atoms of the mineral. Thus, the spatial arrangement of the different amino acids and other organic fragments at the organic-inorganic interface can be determined. This approach has been used to study cryogenically powdered equine bone,⁹ dentin²⁶ and mineralized cartilage,²⁷ as well as manually powdered human kidney stones²⁸ and atherosclerotic plaque.²⁹ The $^{13}\text{C}\{^{31}\text{P}\}$ REDOR solid state NMR technique was recently applied to study the organic-mineral interface in *intact* goat bone.²¹ To the best of our knowledge, no $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR studies of intact *human* bone have been conducted. Human bone, especially from clinically relevant old age donors, poses unique challenges related to rapid spinning required in this state of the art NMR technique due to its increased porosity and fragility. Here we present the first study of intact old osteoporotic and young healthy human cortical femoral samples. The experimental requirements for an accurate NMR study are described in detail, including the sample balancing necessary for spinning osteoporotic bone. We then report the organic-mineral interface structure of femoral specimen obtained from 23 year and 77 year old donors, and discuss the results in the context of biochemical analyses of the samples.

2. Materials and Methods

2.1. Bone source and preparation

The bone specimens from two Caucasian female donors age 23 and 77 were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). The death was caused by pneumonia and head trauma for the 23 and 77 year old donor, respectively. The 77 year old donor was diagnosed with osteoporosis. The samples were taken from mid diaphyseal section of the femur. The bones were stored at -80°C prior to use and at -20°C after sample preparation.

The bone samples were machined into cylinders and inserted into 3.2 mm Bruker zirconia NMR rotor. The rotor has a 2 mm inner diameter and accommodates an 8 mm long sample,

which corresponds to approximately 50 mg of bone in the 25 mm³ volume. Machining of the diaphyseal piece into a cylinder to fit in NMR rotor was done on a numerically controlled lathe (Denford Ltd., Brighthouse, England). The machining was done at relatively low speeds because older bone tissue permits only low cutting forces. Excessive heating of the lathe was avoided by continuous supply of a saline solution at room temperature. Three samples were machined in longitudinal and one in transverse orientation. The longitudinal cylinders had axis of symmetry parallel to the long axis of femur and the transverse sample was perpendicular to the long axis of femur (Figure S1 in Supplementary Materials; "S" refers to the supplementary materials).

To determine the effect of site and age, as well as the possible influence of collagen fibril and HA platelet orientations, longitudinal samples from the medial (young, old) and posterior (old) quadrants, and transverse samples from the medial (old) quadrants were studied. Each machined cylinder was marked and partitioned to four 2 mm long segments by slow-cutting diamond saw (Buehler Corp., Lake Bluff, IL). In order to balance out the porosity, the 4 smaller bone cylinders were then inserted in a specific order into the rotor: the first pair of cylinders was mutually 180° turned, and the second pair was turned 180° mutually and 90° from the first pair (Figure S2). One sample (also originating from the medial cortex of the 77 year old donor) was powdered in the form of sub-millimeter size shavings.

2.2. Finite Element Analysis

In order to determine the sample and rotor deflections and stress resulting from rapid rotation, a finite element analysis (FEA) was performed using ABAQUS 6.10 (Simulia, Dassault Systèmes, Paris, France). The 8-node linear brick elements and reduced integration with hourglass control were used. The monolithic bone was represented by 5280 elements and the zirconia NMR rotor by 6748 elements. The model of four bone sub-specimens used 7414 and 1680 elements to represent the rotor and each bone piece, respectively. Loading by rotational body force at 12.5 kHz was applied to the specimen and rotor. The boundary condition was defined as frictionless contact in the case of the 8 mm long monolithic sample, and as rough in the case of four sub-specimens. The bone and zirconia rotor densities were set to 1900 kg·m⁻³ and 6040 kg·m⁻³, respectively. Both materials were modeled as linear elastic isotropic materials, with Moduli of 20 GPa and 207 GPa, and Poisson ratios of 0.26 and 0.32 were used for the bone and commercial ceramic zirconia, respectively.³⁰

2.3. Solid state NMR

All NMR spectra were acquired on a 600 MHz wide bore Bruker NMR spectrometer using a triple resonance 3.2 mm Magic Angle Spinning (MAS) solid state NMR probe (Bruker BioSpin, Karlsruhe, Germany), tuned in ¹H-¹³C-³¹P configuration. The rotors filled with cylindrical bone samples were spun at MAS spinning rate of 12.5 kHz. The temperature was regulated at 4°C throughout all the NMR experiments to counter the frictional heating from spinning. The ¹H-¹³C cross-polarization (¹H-¹³C CP) MAS NMR spectra were acquired with a 2.5 μs 90° ¹H pulse, and contact time (CT) of 2.5 ms. The Spinal-64 ¹H decoupling scheme (100 kHz rf) was applied during acquisition. A total of 2048 scans was accumulated with 2 to 6 s recycle delay between the scans. The peaks were assigned as proposed by Aliev,³¹ based on previous literature.

Analysis of ¹H-¹³C CPMAS NMR spectra acquired before and after each long ¹³C{³¹P} REDOR NMR experiment (Figure S3) shows that alanine peaks at ~17.5 and ~49 ppm and citrate/GAG peak at ~76 ppm maintain their linewidth, as opposed to significant line broadening which occurs upon bone dehydration.²⁰ The samples therefore do not exhibit

any signs of dehydration. The CO/COO⁻ region at 165–185 ppm was not resolved into three peaks, unlike in previous reports.^{20,21} Since our specimens were hydrated at all times, the differences in the CO/COO⁻ region may be sample dependant. The ¹H-¹³C CPMAS NMR spectra of all samples are displayed in Figure S4.

The ¹³C{³¹P} REDOR NMR pulse sequence used the same CP conditions as for ¹H-¹³C CPMAS, with a contact time of 2.5 ms. In the ¹³C{³¹P} REDOR experiment, a total of 10.2 ms dephasing (and for some samples 15.4 ms dephasing) was applied using rotor-synchronized π pulses on ³¹P. Spinal 64 ¹H decoupling (100 kHz RF) was used during the dephasing and acquisition periods. The total number of scans ranged from 16800 to 19456. Each ¹³C{³¹P} REDOR NMR acquisition was preceded and followed by a ¹H-¹³C CP NMR experiments to monitor the integrity of the organic phase (and notably its hydration state).²⁰

The ¹³C{³¹P} REDOR NMR pulse sequence^{23,24} allows the study of the organic-mineral interface. With ¹³C{³¹P} REDOR, we acquire and compare two ¹³C NMR spectra: one corresponds to a normal “spin echo” ¹³C NMR spectrum while the other has additional ³¹P recoupling π pulses (Figure S5). The series of rotor-synchronized π pulses on ³¹P allowed us to re-introduce dipolar coupling to the nearest carbons, causing them to “dephase” and their signal to decrease in intensity. When we compare spectra acquired with and without the ³¹P pulses, we can identify carbon atoms that are in the organic-mineral interface region. The spatial range which is probed during the ¹³C{³¹P} dephasing is governed by the duration of the dephasing period. The dephasing is stronger when a ¹³C atom is spatially closer to the ³¹P in the mineral, or when it is more frequently located by the mineral surface.

Despite extensive acquisition times, ¹³C{³¹P} REDOR NMR can have poor signal to noise ratio. The noise originates from multiple sources, including low sensitivity of the NMR-active isotope ¹³C (natural abundance ~1.1%). Increased specimen size could increase the signal; however, the small rotor size is necessary to permit rapid spinning and imposes a limit on the specimen size. Decrease in signal to noise ratio is also caused by the transverse relaxation of ¹³C during the dephasing period. In order to ensure better interpretation of data it is therefore desirable to employ strategies for decreasing the level of noise.

Denoising was done on all ¹³C{³¹P} REDOR spectra using a freeware application (“nmrsvd2” by Pascal P. Man).³² The program applies mathematical singular value decomposition (SVD)³³ on the free induction decay (FID, time domain data), reduces the decomposed FID by a user-specified noise threshold, and mathematically reconstructs the FID solely from the significant (above-threshold) signal. The SVD noise suppression is controlled by two parameters: the fraction of the original FID that is dismissed, and the noise threshold specified by user in the transform domain. Although SVD is a well established denoising technique which can be used to improve the processing of 1D and 2D NMR spectra,^{34,35} it is not yet a routine method for data processing in NMR. Therefore, details on how to best apply this SVD program to the denoising of the REDOR spectra are discussed in the supplementary materials (Figures S6 and S7). All SVD denoised ¹³C{³¹P} REDOR FIDs were then Fourier transformed without any line broadening. A comparison of SVD-denoised and conventionally processed NMR spectra (by exponential multiplication with a line-broadening of 40 Hz) is also given in the supplementary materials (Figure S8).

To quantify the dephasing, the ¹³C{³¹P} REDOR NMR spectra were fitted by sets of Gaussian/Lorentzian peaks in freeware program DMfit.³⁶ The relative decrease in each peak’s intensity between “¹³C{³¹P} dephased” and “¹³C{³¹P} non-dephased” state ($\Delta S/S_0$) was used to quantify the dephasing. To minimize the subjectivity, each spectrum was fitted three times by a new set of peaks and the extracted dephasing values were averaged. The error bars in the dephasing $\Delta S/S_0$ of individual peaks originate from two factors. The first

type of error originates in parameterization of the spectral peaks by fitting, and its contribution to the total error can be readily determined from the differences in the dephasing values between the 3 fits. A second type of error originates from non-perfect description of lines by the SVD fits, and from small instabilities of the hardware over long experimental times, which may result in very small increases of intensity in the dephased spectrum when decrease is actually expected, as observed in previously reported $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR experiments (see Figure 6 of reference 37).³⁷ For our spectra, this error was determined to affect the $\Delta S/S_0$ by $\sim 3\%$, and this value represents our detectable dephasing limit. It should be noted that the overall error bars we determined here for the $\Delta S/S_0$ values are consistent with those reported in the literature for $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR spectra of bone recorded at similar dephasing times.²¹ A comparison of the dephasing results (and associated errors) of spectra processed using conventional exponential multiplication (with 40 Hz linebroadening) and SVD processing was also carried out (Figure S9).

2.4. Analysis of Advanced Glycation Endproducts (AGEs)

AGEs content was determined in the old medial and posterior, and in the young medial bone quadrant of femoral mid diaphysis. For each quadrant, two parts were taken from tissue adjacent to the samples studied by NMR. Adipose tissue was removed from the bone samples by successive washings in isopropyl ether. Approximately 40 mg of each sample was lyophilized and then hydrolyzed in 6 M HCl.³⁸ The AGEs content was determined using fluorometric assay, where quinine sulphate serves as a standard.³⁹ Fluorescence intensity of the samples was measured using microplate reader (Synergy HT Microplate Reader, BioTek, Winooski, VT, USA) at 370 nm excitation and 440 nm emission wavelengths. AGEs content was expressed as a relative fluorescence of the quinine sulphate standard per the total collagen, which was determined for each sample using hydroxyproline assay.^{40,41} Commercial hydroxyproline was used as a standard. Measurement of hydroxyproline concentration was performed at 570 nm using the same microplate reader as for fluorescence measurement. To calculate the content of AGEs per collagen, we used 9.5% of hydroxyproline per collagen (Table 1).

3. Results

3.1. Rapid spinning of intact bone

The NMR experiment requires the sample to rotate at the rate of 12.5 kHz, *i.e.* 750 000 RPM. In the case of bone, this rapid spinning has been most commonly achieved by powdering the sample. Indeed, with exception of a few recent reports,^{20,21,42} the solid state NMR work on biological solids (bone, dentin, kidney stones, calcified plaque and calcified cartilage) was done on powdered samples.^{5,6,9,11,25,26,28,29} However, there is evidence that powdering the sample causes undesired structural changes.²⁰ Therefore, we chose to study organic-mineral interface in *intact*, unprocessed bone. However, to spin intact bone, we needed to address issues related to loading of the 0.6 mm thick shell of the ceramic rotor. To be able to spin osteoporotic bone with significantly inhomogeneous density, we also had to improve the balancing of the osteoporotic intact samples, which are more fragile and difficult to handle.

The rotational body force at 12.5 kHz spinning is considerable. Expansion of the bone specimen against the far stiffer ceramic rotor wall could cause a failure, resulting in costly damages to the equipment. To determine whether this condition occurs, we carried out finite element analysis (FEA). It confirmed that rotor loading from centrifugal force by intact bone specimen is lower than in bone powder. Indeed, while grains of powdered bone move towards the walls of the rotor during spinning, the intact bone supports its own mass. In the FEA model, the maximum radial displacements for zirconia rotor and intact bone were 0.29

and 0.11 μm , respectively. The maximum stress was 58.5 MPa in the zirconia rotor and 3.9 MPa in the intact bone, which presents approximately 24 % and 4 % of typical yield strength for zirconia and bone, respectively. Thus, the intact bone does not expand against the wall of the ceramic rotor during the rapid rotation, and the loading of the rotor by intact bone therefore does not present a risk of fracture upon spinning.

Following this FEA, we decided to perform experiments on intact (non-powdered) bone samples. However, the 8 mm-long monolithic specimen from osteoporotic cortical bone did not reach rotation above 1 kHz. This problem of rotational imbalance was caused by the strong porosity gradient in the specimen, and it was overcome by the following measures. The sample was separated into four 2 mm long cylinders which were inserted manually into the rotor following a specific order (Figure S2). The off-center mass was balanced out by axial symmetry in the 180° turns, and the static balance condition was satisfied. The dynamic balancing condition was not perfectly satisfied, but separating the sample into two counter balanced pairs decreased the dynamic imbalance. According to the analytical solution of the dynamics of this system, arranging the 4 unbalanced sub-specimens yielded approximately 80% reduction of bearing reaction forces (see Figure S10, and associated explanations), which was sufficient to achieve and maintain the rapid rotation.

Separating the monolithic bone into four sub-specimens presents a different loading scenario. The stress and displacement in rotor and sub-specimens were therefore evaluated by FEA (Figure 1). In the model, we considered that the bone specimens are 50 μm smaller in diameter than the inner rotor diameter, and that they each contact the rotor in one small region. The inner surface of the rotor wall was loaded irregularly by contacts with the specimens. The stress ranged from 54.8 MPa to 61.8 MPa (Figure 1A, 1C), a value well comparable to the stress of 58.5 MPa determined previously for the 8 mm-long monolithic specimen loading scenario. It should be noted that 61.8 MPa corresponds to only ~25 % of yield strength of commercially available zirconia ceramic. The stress at the outer surface of the rotor ranged from 41.4 MPa to 51.6 MPa, which is lower than in the inner surface. The highest stress occurred in the regions of contact with the four different bone pieces. The radial displacement of the bone specimens is shown (exaggerated by factor of 1000) in the Figure 1B. Their critical expansion can be ruled out based on the previous loading scenario. In summary, the FEA study shows that the stress both in the bone pieces and in the rotor are well below yield strength of zirconia, and that the radial deflection of the rotor does not risk damaging the NMR probe.

The gap between the specimen cylinders and the rotor wall was filled with saline to ensure bone hydration. However, the saline also functions as a ballast. It has approximately half the density of bone and this compensates better than an air gap for potential imperfections of sample shape. According to the analytical solution (see Figure S11 and associated explanations), the saline becomes highly pressurized (up to 3.08 MPa) by the rotational body force. The saline pressure buoys the bone, acting to center the bone cylinder inside the rotor, and thereby decreases (without cancelling) the force at the contact with the rotor wall. Due to all these different balancing precautions, the automated air bearing and drive system of the spectrometer could repeatedly spin rotors containing highly porous intact bone pieces at 12.5 kHz, without damaging any of the equipment.

3.2. $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR: Denoising spectra by Singular Value Decomposition

The REDOR NMR spectra recorded for medial femoral specimen of 77 year old donor at 10.2 ms dephasing time are shown in Figure 2A. We used Singular Value Decomposition (SVD)³³ to suppress noise (“denoise”) from these $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR spectra (Figure 2C), and improve the quantification of the carbon-phosphorus proximity information. Indeed, conventional noise suppression by exponential multiplication (Figure 2B) permits

noise reduction at the cost of loss of signal intensity and resolution, due to the peaks broadening. It was thus preferable to use SVD for the noise suppression without compromising the peak widths. It should be noted that in the literature, little information is given as to how the REDOR NMR spectra of bone or teeth tissues have been processed,^{21,26} but it can be assumed that conventional exponential multiplication was applied.

A convenient feature of the SVD denoising is that the noise is practically eliminated and the “real” intensity of the peaks is preserved (Figure 2C). The denoised spectra truly represent the original spectra. In particular, the shouldering of the peak at ~47 ppm is reproduced properly (Figure 2D). This result was further confirmed by comparing the REDOR spectra of all *intact* bone samples that were transformed using either the conventional exponential multiplication (with 40 Hz line broadening) or SVD treatment (with the same SVD processing parameters) prior to Fourier transform (Figure S8). Following the simulation of conventionally processed and SVD processed spectra, we also verified that fairly similar values of dephasing ($\Delta S/S_0$) were observed between the REDOR spectra recorded with or without ³¹P recoupling pulses (Figure S9). Indeed, the trends in *relative* dephasing for a given peak from one sample to another were globally maintained (for example for the hydroxyproline C-4 signal). Only few peaks were found to have a significantly different $\Delta S/S_0$ value for the two types of processing, notably for the peak at ~25 ppm (assigned to the overlapping of proline C4 and glutamic acid C3 signals). This discrepancy could be due to the overlap with the neighboring ¹³C signals of C4 of arginine and lysine (at ~23 ppm) and C4 of glutamic acid and C-5 of lysine (at ~28 ppm).³¹

One of the advantages of SVD over conventional processing is that as a result of the noise suppression, the simulations (and thus $\Delta S/S_0$ values) can be slightly more reliable, which is important when trying to compare very small differences from one REDOR spectrum to another. This is particularly true in cases when it may be difficult to determine the exact position of the baseline within the noise. Small errors in the vertical positioning of the baseline may indeed result in significantly different ($\Delta S/S_0$) values (Figure S12), and compromise the quantification of the REDOR data.

Despite these advantages, there are some limitations to the SVD approach. When considering samples for which the noise level becomes significantly high, it becomes difficult for the SVD to reconstruct the signals. This is the case for the signal at ~76 ppm in the powdered bone sample (Figure S13). Indeed, while the SVD denoising can suppress noise, it does not increase the signal. If the intensity of a signal of interest is approximately equal to the noise, it becomes impossible to extract it correctly from the baseline. Furthermore, SVD processing can start yielding artifacts over spinning sidebands (SSB), weak signals or shoulders (Figures S6 and S7). Thus, for such peaks, ($\Delta S/S_0$) dephasing values were not considered to be reliable, and we mainly focused on the other, better-resolved signals.

3.3. Choice of length of REDOR dephasing period

In REDOR, the spatial range probed during the dephasing is controlled by the length of the dephasing period during which recoupling pulses are applied.^{25,43–45} In general, for a given sample, the REDOR NMR experiment is carried out using different dephasing times, and the analysis of the REDOR “build-up” curve (plot of $\Delta S/S_0$ as a function of the dephasing time) then allows estimations of ¹³C-³¹P distances to be derived.^{21,25} However, the distances obtained may depend on the model used to simulate the build-up curve,²¹ and such a complete analysis would be very time-consuming to carry out on several samples. In previous studies, dephasing periods ranging from 1 to 40 ms have been used.^{11,21,26} In most cases, the main goal was to look at the dephasing of the peak at ~76 ppm (which was initially assigned to GAGs,⁹ and more recently to citrate).¹¹ This peak corresponds to carbon

atoms of the organic species closest to the mineral surface. This signal starts dephasing at ~1 ms, and is completely dephased after ~10 ms. Much less attention has been given to the other ^{13}C signals on the spectra. Recently, Rai *et al.* demonstrated that for some of the amino acid signals, a very weak dephasing can start to appear after ~10 ms recoupling period.²¹ In order to gain *preliminary* insight into the changes in the organic-mineral interface with site and age, and more specifically on how some of the amino acid peaks of collagen are arranged with respect to the mineral surface, we decided to chose a single $^{13}\text{C}\{^{31}\text{P}\}$ REDOR dephasing period, such that only some of the amino acid signals dephase.

As shown in Figure 3, we determined that a dephasing period of ~10 ms is satisfactory to compare the interface structure in different bone samples from a single REDOR NMR experiment (Figure 3A). Although it yields relatively small decreases in intensity of some of the amino acid ^{13}C NMR peaks in the 10 to 75 ppm region, it is better suited than the longer dephasing periods. Indeed, when the dephasing period was increased from 10.2 to 15.4 ms, almost *all* amino-acid peaks were dephased (Figure 3B), in agreement with previous observations.²¹ Indeed, we observed at 15.4 ms dephasing the additional decrease in intensity of the peaks at ~30 ppm (assigned to proline/arginine/lysine carbon atoms), ~38 ppm (hydroxyproline/aspartic acid/lysine), ~43 ppm (glycine/arginine) and ~47 ppm (proline). Thus, at 15.4 ms, *all* the significantly dephased amino acid ^{13}C NMR peaks correspond to carbon atoms which are distributed *throughout* the collagen structure and the experiment is thus less selective of the species closest to the interface. Consequently, a 10.2 ms dephasing period was chosen in order to compare the interface structure in different samples from a single REDOR experiment.

3.4. Powdering disrupts the organic-mineral interface

Powdering of solid materials for NMR spectrometry sometimes causes narrow spectral peaks to broaden, presumably because of structural changes at the molecular level. Powdering, commonly done in earlier NMR studies of bone,^{5,6,9,11,25,29} has been suspected to cause structural differences. For example, increased lipid mobility was observed in ^1H NMR spectrum of powdered bovine bone as compared to spectrum of intact bone.²⁰ Here, we show that powdering also caused structural disorder in the organic-mineral interface. This was evidenced by changes in dephasing in some of the ^{13}C amino acids peaks in REDOR NMR spectra of powdered bone, compared to intact bone. Notably, we saw a stronger dephasing of some ^{13}C signals of the powdered sample (spectrum “OMP” in Figure S8) at ~71 ppm (C-4 of hydroxyproline) and ~43 ppm (C-2 of glycine and C-5 of arginine) compared to a parent intact bone sample (“OML” spectrum at 10.2 ms in Figure S8). Similarly, dephasing of a large number of amino acid signal had been observed in previous REDOR studies of *powdered* bone samples at similar dephasing times.²⁶ Such an effect can be attributed to the increased structural disorder created at the mineral-organic interfaces upon powdering, which results in a larger variety of amino acid carbons approaching the HA surface.

Another disadvantage related to the powdering of bone is that the citrate/GAG peak at ~76 ppm becomes weaker and broader, making the quantification of the dephasing more difficult, and the SVD analysis less efficient (Figure S13). Despite its low intensity, this peak is still present (spectrum “OMP” in Figure S8, and Figure S13). The increase in linewidth may indicate a wider distribution of local environments of citrate/GAG due to the overall increased structural disorder at the interface. The lower peak intensity in the REDOR spectra can be related to the smaller signal-to-noise ratio obtained in powdered bone, the powder being introduced in the rotor with a lower packing density than intact bone. The decrease in intensity may also reflect changes in the transverse relaxation of ^{13}C citrate/GAG signals because of the powdering.

Thus, in order to help preserve the interface structure (i.e. the mode of association of the *proteins* and *collagen* with respect to the HA mineral platelets), and to maximize the signal to noise ratio, we recommend to use intact bone.

3.5. Interface organic moieties in young and old bone

The $\Delta S/S_0$ dephasing was quantified in all the spectra acquired at 10.2 ms dephasing conditions (i.e. for the 23 year old medial longitudinal intact bone, and the 77 year old medial longitudinal, transverse and posterior longitudinal intact bone samples). In the following sections, we discuss the dephasing of the peaks at ~76 ppm (assigned to citrate/GAGs), in the 165–185 ppm-region (associated with CO, COO⁻ function of organic molecules), and in the 10–75 ppm region (other amino acid peaks). For all four samples, only dephasing values $\Delta S/S_0$ above 3% were considered significant.

3.5.1. Presence of citrate/glucosaminoglycans in the interface—For all samples, the strongest dephasing was observed at ~76 ppm (peak assigned to GAGs/citrate).^{9,11} The total dephasing observed for this peak at 10.2 ms dephasing time did not reveal any clear differences between the samples. Nevertheless, from the spectra recorded, we show, for the first time, that some citrate/GAG is likely to remain intimately adsorbed on the mineral platelet surface even in older human bone. Dedicated studies at shorter dephasing times¹¹ would be thus worth carrying out to try to determine changes in citrate proximity or concentration at the mineral surface with age.

The dephasing of carbonyl and carboxyl carbon peaks between 165 and 185 ppm was found to be in agreement with previous studies of bone,⁹ mineralized cartilage,²⁷ and dentin.²⁶ This dephasing can be partly ascribed to the presence of citrates and GAGs at the mineral surface, although other amino acid carbons may also contribute to it. Given that peaks are not well resolved in this region, our deconvolution process treated them as a single composite peak. The total dephasing in this region ranged from 7 to 10 %, but showed no apparent trend with respect to age.

3.5.2. Dephasing in amino acid carbon atoms—In addition to the CO/COO⁻ and citrate/GAG dephasings, which have been extensively studied in the literature,^{9,11,21,25} we quantified the weaker dephasings of some of the carbon atoms of individual amino acids (Figure 4). As collagen is by far the most abundant protein in bone (Table 1), further analysis of the amino acid region dephasing may allow us to gain information on the arrangement of the collagen protein with respect to the HA platelets.

The most reliable dephasing values are obtained from peaks that are well resolved and assigned to a *single* amino acid carbon. The hydroxyproline C-4 signal at ~71 ppm (Peak #9 in Figure 4A) was the best resolved peak on the spectra. A similar dephasing of about 7 % is observed for the young medial cortex and the old posterior cortex of femur. In contrast, the dephasing for the medial cortex of the old bone was below the limit of detection. Another well resolved peak, at ~17.5 ppm, can be assigned to the carbon of a *single* amino acid, i.e. alanine C-3 (Peak #3 Figure 4A). Here, the young medial and old posterior longitudinal bone samples do not dephase significantly, while the two old medial samples dephase by ~4.5 to 5 %.

Although the other well resolved peaks correspond to overlapping ¹³C signals from different amino acids, they also reveal differences between samples. For example, for the peak at ~43 ppm assigned to overlapping glycine C-2 and arginine C-5 carbon atoms (Peak #1 in Figure 4A), only the sample from the posterior site of old bone did not dephase significantly. The well resolved peak at ~59 ppm (peak #4 in Figure 4A), corresponding to collagen backbone C-2 carbon atoms of proline, hydroxyproline and lysine, is associated with a small decrease

in dephasing at all three anatomic sites of the old bone compared to young bone. For the peak at ~25 ppm (Peak #6 in Figure 4A), which corresponds to ^{13}C signal proline C-4 overlapping with glutamic acid C-3, an overall dephasing trend similar to the one described for hydroxyproline C-4 is observed. Indeed, the two old medial cortical bone specimens hardly dephase ($\Delta S/S_0$ below the detection limit), while the young medial bone specimen dephases by ~4% and old posterior bone specimens by ~7%. However, as mentioned previously, peak #6 is shouldered by weaker signals at ~23 ppm (C-4 of arginine and lysine, and C-5 of leucine) and overlaps with peaks at ~28 ppm (C-4 of glutamic acid and isoleucine and C-5 of lysine). This implies a larger error in its parameterization, making the dephasing values less reliable for this signal.

Besides the observations made on two uniquely-assigned peaks (#3 and 9), two well resolved peaks (#1 and 4) and one shouldered peak (#6), all other amino acid peaks (#2, 5, 7, 8 and 10, Figure 4A) are not considered in detail. Indeed, the peaks of alanine C-2 and proline C-5 (#2 and 7, respectively) overlap and when quantified by Gaussian/Lorentzian peak fit, their parameters suffer from mutual dependence, resulting in a large error of the dephasing value. Peaks #5, 8 and 10 are associated with signals from multiple amino acids and mutually overlap or are shouldered. Their deconvolution can thus yield unacceptable variation and it was not possible to recover objective dephasing information.

4. Discussion

From the analysis of the available REDOR data, obtained using a single REDOR experiment at 10.2 ms dephasing time, we make two main observations. First, samples of different site or age show differences. Second, in this particular study, we observed some dephasing similarities for posterior femoral cortex of old and medial cortex of young femur, as opposed to 2 samples from the medial femoral cortex of the old bone.

Regarding the first point, the comparison of the REDOR data of the four samples points to differences in dephasing for several amino acid peaks (Figure 4), as detailed in the previous subsection. This suggests that the overall positioning of the collagen with respect to the apatite crystallites varies between samples. Unfortunately, we cannot determine in detail how all individual amino acids rearrange with respect to the surface in native human bone, notably because of the significant overlapping between carbon signals of different amino acids for some of the peaks, and because a single dephasing time was used. Nevertheless, we can conclude that it is possible to shed light on differences between samples of varying age and anatomic sites, using a single REDOR experiment. Thus, the analysis of REDOR dephasing in individual carbon species may potentially inform on a unique aspect of bone quality – the overall arrangement of collagen at the mineral surface.

Regarding the second point, in contrast with the two old medial specimens, the comparison of the dephasings observed for *well-resolved and non-overlapping* ^{13}C resonances showed dephasing similarities for posterior cortex of old and medial cortex of young femur (Peaks #9 and #3 in Figure 4, which correspond to the C-4 of hydroxyproline and C-3 of alanine, respectively). According to REDOR, hydroxyproline C-4 would be more commonly present at the mineral surface of these two samples, in contrast to alanine C-3. Hydroxyproline represents approximately 9.5 % of collagen (Table 1) and its C-4 carbon is one of the outermost atoms of the tropocollagen and collagen fibril (Figure 5A, C, E). The C-4 carries an OH group, which makes it a strong candidate for hydrogen bonding, but not for covalent crosslinking of collagen (which typically involves ammonium groups of arginine or lysine or hydroxylysine). Hydrogen bonds from hydroxyproline are known to stabilize collagen triple helices, and the hydroxyproline OH groups are found on the surface of the fibrils. This arrangement allows interactions not only between the triple helices but also with the

inorganic platelets of HA (Figure 5C, E). Alanine is equally abundant (10.5 % in collagen, Table 1) but, in contrast to hydroxyproline, it carries no charged function nor OH group (Figure 5A) and has a shorter sidechain. The position of its sidechain (C-3 carbon) with respect to the axis of the helix cannot be more central than glycine carbon atoms (Figure 5B,D), but it is also not as peripheral as the C-4 of hydroxyproline, making it more complex to derive conclusions on its proximity to the interface. All in all, given the ionic nature of the surface of HA platelets, the increased presence of C-4 of hydroxyproline in proximity to the mineral would allow more hydrogen bonding with HA (or with the OH group of molecular ions like citrate which are tightly absorbed on the HA surface),¹¹ and may increase the overall strength of organic-mineral interaction.

Differences in the organic-mineral interface arrangement in bone may originate from a wide range of factors, including gender, diet, or anatomic site. In this study, only 4 samples from 2 donors were analyzed, meaning that many more samples would need to be studied to evaluate the respective role of these different factors on the organic-mineral association.

As a preliminary illustration of the kind of study which can be performed, we decided to confront the dephasing similarities observed here between young medial and old posterior tissues to a biochemical analysis of AGE content. Indeed, more bone remodeling is known to occur in posterior cortex compared to medial cortex. Thus, it may contain a larger proportion of biologically younger bone, and some degree of similarity with young bone could be expected. Among the different biochemical changes which occur in bone upon ageing, it has been shown that the AGE content increases, and that it affects remodeling. An increase in AGE content reflects an increase in the number of covalent bonds between collagen molecules, and it is therefore plausible that the organic-mineral interface in more cross-linked tissue reflects the presence of these covalent links. AGE contents of the three samples analyzed are shown in Table 2. Contrary to our expectation, the old medial sample had lower AGE content than both the old posterior and the young medial samples. At present, we have no explanation on the presence of lower AGE content in the old medial sample, but this interesting observation will be investigated in more detail in order to elucidate this phenomenon. Nevertheless, in context of the NMR work, the AGE analysis showed that the old medial sample was significantly different from the old posterior and young medial samples. This pattern of AGE distribution is in agreement with the similarity observed in REDOR NMR for hydroxyproline and alanine (Peaks #9 and #3 in Figure 4). These results suggest that collagen cross-linking may affect the organic-mineral interface structure, and that in more glycosylated bone, the collagen periphery (C-4 of hydroxyproline) is found closer to the HA surface than in less glycosylated bone. It should be noted that in this study, only 4 samples from 2 donors were analyzed, meaning that *many* more samples would thus need to be analyzed to determine if this relationship between REDOR dephasing and AGE concentration can be generalized. Additionally, it would be desirable to also include several other biochemical measures of bone quality such as concentration of non-collagenous proteins, in order to fully rationalize the differences in dephasing in REDOR.

The $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR technique can be considered complementary to biochemical analyses in determining the bone quality. Indeed, modern highly localized biochemical analyses of content of AGEs or concentrations of non-collagenous bone matrix proteins use as little as 0.00075 mm^3 , and have been used to demonstrate heterogeneities at the micrometer scale.^{13,14} In contrast to these methods, NMR analyses deal with larger volumes of sample (here 25 mm^3), and thus provide a more averaged measure of the tissue state. However, more importantly, NMR informs on the average structure of the organic-mineral interface at the atomic scale. Recent NMR developments may help address the averaging effect. Indeed, it should be possible to perform such REDOR experiments on much smaller specimens by using smaller volume rotors (such as those of 1.3 mm diameter, which can

host biological samples as small as a mouse tooth)⁵¹ or by techniques like MACS (magic angle coil spinning) which require as little as 100 µg of sample,^{52–55} and by working at ultra-high magnetic fields.

Our results suggest that the NMR analysis of the organic-mineral interface structure is a valuable method to probe bone quality. Indeed, using $^{13}\text{C}\{^{31}\text{P}\}$ REDOR (in combination with the SVD-denoising of the NMR spectra), we show that information on the arrangement of collagen amino acids at the surface of apatite crystallites can be accessed. Additionally, we also demonstrate that GAGs/citrate remain the most intimately bound organic species with the mineral in bone. Differences in concentration of GAGs/citrate at the surface of HA crystallites may occur, but these were not observed here with age or anatomical site, due to the length of the REDOR dephasing time chosen. Analyzing changes in GAG/citrate concentration would offer additional means of probing bone quality and help improve the diagnostic and treatment modalities for osteoporosis. For example, concerning citrates, it has been shown that they adsorb at the surface of bone mineral and help prevent bone resorption. Additionally, calcium citrate is commonly administered in osteoporosis intervention.⁵⁶ Estimation of citrate content at the surface of the mineral platelets in osteoporotic bone may therefore help to determine the most appropriate treatment (dose) necessary to prevent bone resorption.

5. Conclusion

In conclusion, we used the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR technique to probe organic-mineral interface in intact young healthy and old osteoporotic human bone with a goal to characterize the bone quality changes. First, we developed a method to balance the porous intact bone for safe fast rotation in NMR spectrometer, and implemented SVD denoising technique to overcome the poor signal to noise ratio. We demonstrated that powdering by milling causes structural changes at the organic-mineral interface and must thus be avoided for NMR studies of the interface structure. We determined that ~10 ms REDOR dephasing period is well suited for comparing the interface structure in different samples on the basis of a single REDOR experiment. Citrate/GAGs were found to remain present in the interface as the most intimately bound species with no regard to age. In contrast, changes in dephasing of the different amino acid peaks were observed, pointing to variations in the arrangement of collagen with respect to the mineral surface, which could be due to differences in site, age, and/or glycation between the four samples studied here. By detecting spectrally small but structurally significant differences in dephasing, the $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR technique could thus be used to determine the organic-mineral interface arrangement as a novel aspect of bone quality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FEA analysis of stress and displacements of the rotor and bone pieces, resulting from loading by rotational body force

The stress in the inner surface of rotor (A) varies and is the largest at the points of contact with the specimen. The stress in the bone (B) is also the greatest in the points of contact with the rotor. Displacements of the individual bone pieces is exaggerated (by factor of 1000) in (B) for clarity. The histograms summarize and compare the values of Von Mises stress (C) in monolithic and separated specimen scenarios. On these histograms, the dotted lines represent the range of variation of stress in the separated specimen scenario.

Figure 2. Comparison of conventional denoising and singular value decomposition denoising methods

Noise suppression is demonstrated on a pair of $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR spectra of 77 year old human bone recorded with 10.2 ms dephasing, and processed by (A) simple Fourier Transform, (B) conventional exponential multiplication (40 Hz line broadening) followed by Fourier Transform, and (C) singular value decomposition (SVD) denoising followed by Fourier Transform (with no line broadening). The small grey spectrum below each pair represents a subtraction of the two spectra to help visualize the difference in intensity due to $^{13}\text{C}\{^{31}\text{P}\}$ dephasing. The inset (D) demonstrates the fidelity of the SVD denoised spectrum (blue) to the original $^{13}\text{C}\{^{31}\text{P}\}$ REDOR spectrum (black) recorded without ^{31}P recoupling pulses.

Figure 3. Comparison of $^{13}\text{C}\{^{31}\text{P}\}$ REDOR NMR spectra acquired with 10.2 and 15.4 ms dephasing periods

The presented spectra were acquired on medial quadrant of femoral mid-diaphyseal cortex of 77 year old osteoporotic human bone (the carboxylate/carbonyl signals are not shown). The presented spectra were SVD denoised and Fourier transformed without any line broadening. The pair of “dephasing on” (red) and “dephasing off” (black) spectra is displayed for each dephasing condition. Shorter 10.2 ms dephasing (A) causes weaker dephasing effect relative to the longer 15.4 ms dephasing (B), which has longer spatial range and reaches amino acids in all parts of collagen. The corresponding spectra processed using conventional exponential multiplication prior to Fourier transform are shown in Figure S8.

Figure 4. Amino acid region in the ^1H - ^{13}C CP MAS NMR spectrum of human bone with table of assignments

All data presented was obtained from SVD denoised spectra and the spectral region with the carboxylate and carbonyl groups is not shown here for clarity. The asterisk marks the spinning sideband of carbonyl/carboxylate peaks at 165–185 ppm. Only peaks #2, 3, 7 and 9 are assigned to a single amino acid. Amino acid carbon peaks which occur at the same chemical shifts are in the same column, and chemically similar amino acids are grouped to adjacent lines of the assignment table. Error bars are sum of the hardware error and error originating in parameterization (fitting) of the spectra. For clarity, the minor peaks and shoulders are not highlighted. For complete assignments see reference 31. The dashed horizontal line represents the limit of detection.

Figure 5. Structures of abundant organic species in bone and spatial distances at the bone's organic-mineral interface

The chemical structures of the most abundant amino acids and citrate are shown in (A). The individual carbon atoms are numbered. The distance between amino acid carbons and HA was measured as approximately 9 Å.²¹ Glycine is at the core (B) and hydroxyproline C-4 is at the periphery (C) (highlighted by ball and stick scheme) of the tropocollagen triple helix (PDB structure 1BKV).⁴⁹ When considering a model collagen fiber (PDB structure 4CLG),⁵⁰ glycine is found approximately 2 Å deeper in the collagen fibril (D) than hydroxyproline (E). The ball and stick scheme uses red color for oxygen, grey for carbon and blue for nitrogen. The grey rectangles represent parts of hydroxyapatite platelet.

Table 1

The most abundant amino acids in collagen and in the non-collagenous proteins osteocalcin and osteopontin.

Amino acid	Collagen ~90%			Osteocalcin ~2%			Osteopontin ~2%		
	Per protein [*] (%)	Total in Bone (%)	Per protein ^{**} (%)	Per protein ^{***} (%)	Total in Bone (%)	Per protein ^{***} (%)	Per protein ^{***} (%)	Total in Bone (%)	Total in Bone (%)
Glycine	33.2	29.9	5.4	5.4	0.11	2.3	2.3	0.05	
Alanine	10.5	10.1	8.1	8.1	0.16	5.0	5.0	0.10	
Proline	12.0	11.5	10.8	10.8	0.22	9.0	9.0	0.18	
Hydroxyproline	9.5	9.1	0.0	0.0	0.00	0.0	0.0	0.00	
Arginine	4.9	4.7	10.8	10.8	0.22	3.3	3.3	0.07	
Lysine	3.0	2.9	0.0	0.0	0.00	6.1	6.1	0.12	
Glutamic acid	4.5	4.3	5.9	5.9	0.12	8.0	8.0	0.16	
Aspartic acid	2.8	2.7	8.8	8.8	0.18	15.7	15.7	0.31	

* Structure PDB ID 3HQV, 49
 ** Structure PDB ID 1Q3M, 50
 *** GenBank entry AAA59974.1, 51

Table 2

AGE concentration in one young and two old donor femoral quadrants.

Total AGEs (mg quinine FLR/mmol collagen)		
Young Medial	50.8	± 3.9
Oldss Medial	31.0	± 7.9
Old Posterior	61.2	± 5.4