Quantum dots as mineral- and matrix-specific strain gages for bone biomechanical studies

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ABSTRACT

We report the use of quantum dots (Qdots) as strain gages in the study of bone biomechanics using solid state nuclear magnetic resonance (NMR) spectroscopy. We have developed solid state NMR sample cells for investigation of deformations of bone tissue components at loads up to several Mega Pascal. The size constraints of the NMR instrumentation limit the bone specimen diameter and length to be no greater than 2-3 mm and 30 mm respectively. Further, magic angle spinning (MAS) solid state NMR experiments require the use of non-metallic apparatus that can be rotated at kilohertz rates. These experimental constraints preclude the use of standard biomechanical measurement systems. In this paper we explore the use of quantum dot center of gravity measurement as a strain gage technology consistent with the constraints of solid state NMR. We use Qdots that bind calcium (625 nm emission) and collagen (705 nm emission) for measurement of strain in these components. Compressive loads are applied to a specimen in a cell through a fine pitch screw turned with a mini-torque wrench. Displacement is measured as changes in the positions of arrays of quantum dots on the surface of a specimen. Arrays are created by spotting the specimen with dilute suspensions of Qdots. Mineral labeling is achieved with 705 nm carboxylated dots and matrix labeling with 565 nm quantum dots conjugated to collagen I antibodies. After each load increment the new positions of the quantum dots are measured by fluorescence microscopy. Changes in Qdot center of gravity as a function of applied load can be measured with submicron accuracy.

Keywords: Quantum dots, NMR, fluorescence imaging, bone, biomechanics

1. INTRODUCTION

Mechanical properties of bone have been well studied by a variety of mechanical and analytical testing techniques [1-7]. However, most common mechanical testing techniques measure mechanical properties at a higher level of hierarchy and therefore studies at an ultrastructural or chemical level have been difficult. As a result of these limitations, it is not known how individual collagen fibrils or mineral crystallites react to mechanical load in either the elastic or plastic deformation regimes. High-pressure NMR spectroscopy has been useful in the study of biomaterials. Combined with other techniques, it has been utilized to investigate the pressure-induced reversible unfolding and pressure-assisted cold denaturation of some proteins like Arc repressor monomers [8], apomyglobin [9] and ubiquitin [10] and the immersion depth of a peptide into a lipid bilayer model membrane [11]. Using a combined experimental and computational approach, we can apply solid state magic angle spinning nuclear magnetic resonance spectroscopy (MAS-NMR) to measure changes in the chemical structure of bone tissue under mechanical load. MAS-NMR will allow us to examine high-resolution structural contributions to bone quality that have been so far unobtainable using traditional imaging techniques or other spectroscopies. The enabling advance is a special NMR cell that allows compressive loading and strain measurements.

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Recent advancements in photonics technology and improvements in single particle tracking methods allow the use of fluorescence microscopy within nanometer resolution [12, 13]. In the present study, we report the use of quantum dots (QDs) as fluorescent probes to quantify small deformations of mineral and matrix constituents of bone under mechanical load at the submicron level. Further, we correlate the high-resolution structural information obtained from MAS-NMR to mechanical strain measurements from quantum dot fluorescence microscopy as the bone specimens are deformed elastically under constant applied pressure.

2. MATERIALS AND METHODS

2.1 Sample preparation

Bovine cortical bone was harvested from freshly slaughtered animals (2–4 years old) at a local slaughterhouse. Cortical bone specimens were prepared from central diaphyseal sections. After dissection and removal of soft tissue, each bone specimen was machined to a rectangular shape 2mm by 2mm in cross section and 10mm in length. For collagen-specific labeling, biotinylated goat anti-type I collagen antibodies (a.b.) (Southern Biotech., Birmigham, AL) were first conjugated with steptavidincoated Qdots (λem = 625 nm) (Invitrogen, Carlsbad, CA). Carboxylated Qdots (λem = 705 nm) (Invitrogen) were used for calcium (mineral) measurement. The bovine cortical bone specimens were incubated with solutions containing 1 nM each of the carboxylated Qdots for 20 min, and then washed with PBS buffer three times. The specimens were transferred to 1 nM solution of antibody-conjugated Qdots and incubated for 30 min. Specimens were then washed with PBS buffer three times.

2.2. Fluorescence and NMR experiments

For these experiments the strain measurement were performed in a metal apparatus, while the NMR experiments were performed in a ceramic apparatus of similar design. Figure 1 shows a schematic of the mechanical test system used for each type of experiment. In these initial experiments the ceramic system did not have slits for viewing Qdot fluorescence. The specimen was loaded in compression with a machine screw tightened with a min-torque wrench. Load was transferred to the specimen through a ceramic disk. Parts were lubricated with Teflon powder to prevent twisting the specimens. The load and displacement were recorded at each step, and fluorescence images of the Qdots were obtained simultaneously. The fluorescence images were acquired using a Nikon E800 microscope, equipped with a 60X/1.0 NA objective (Nikon, USA) and a Quad view split field optical system with filters for viewing simultaneously emission at 525 nm, 565 nm, 625nm and 705 nm. Epi-illumination was obtained with a 405 nm laser (CrystalLaser, Reno, NV). An Andor Ixon EMCCD camera (Andor Tech, South Windsor, CT) with a 512x512 pixel array and 14 bit resolution was used to record the images with 0.1 s acquisition time. This process was repeated for each specimen and for several locations on each bone. All measurements were performed at room temperature.

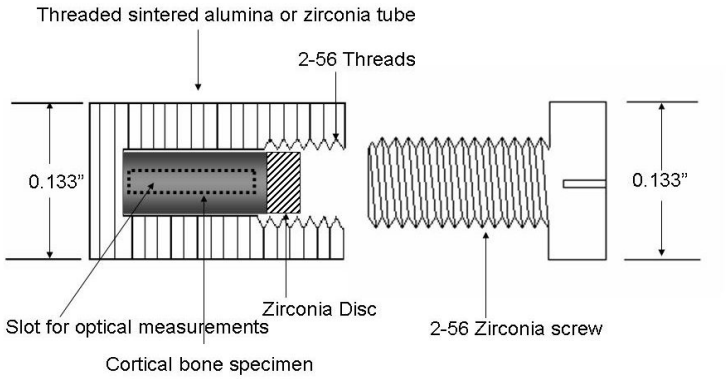

Figure 1. Cutaway view of the NMR cell for high pressure measurements. The cell is a hollow ceramic (currently zirconia) cylinder with slots (dotted line) milled to view the specimen. Load is applied with a torque wrench and transferred to the bone specimen through a zirconia disk. The cell fits into a standard NMR rotor.

The ^{31}P NMR experiments were peformed under MAS on a Varian 400 MHz solid-state NMR spectrometer using a double-resonance ^{1}H-X MAS probe and the spin-echo pulse sequence outlined in figure 2.

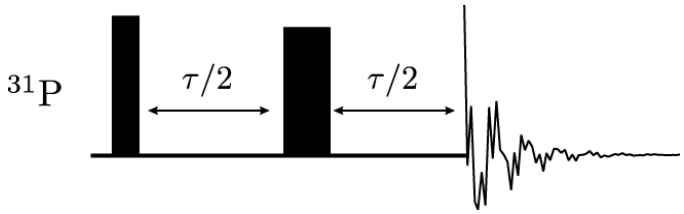

Figure 2. Spin-echo pulse sequence to measure ^{31}P isotropic chemical shifts from bone specimens under MAS. The first and second solid blocks are 90° and 180° RF pulses.

3. RESULTS AND DISCUSSION

By measuring changes in the center-of-gravity of Qdots, we have measured submicron compression or tension of bone using a conventional wide-field fluorescence microscope. The diameter of the diffraction-limited fluorescence spot from a Qdot emitting at 625 nm is about 250 nm, but the spot is very bright. So, the center of gravity can be measured to a small fraction of this diameter, ideally about 1-2 nm. Accordingly, center of gravity changes can also be measured to a small fraction of this diameter. In practice, the smallest displacement measurable is about 20 nm, because the Qdot is bound to the bone surface by a linker.

Figure 3 shows the Quad view fluorescence image of Qdots on the surface of a bovine cortical bone. Using the NMR load cell, we measured displacement in bovine cortical bone tissue by tracking the center of gravity of Qdot arrays spotted onto the specimen surface. The bone mineral was labeled with 705-nm carboxylated Qdots, and the bone matrix was labeled with 625-nm Qdots conjugated to collagen type I antibodies. The bone was loaded in compression incrementally, and the positions of the Qdots were measured by fluorescence microscopy after each load increment. Young's modulus was calculated from the measured strain and stress. The strain was measured directly from the displacements of the quantum dots from the fluorescence images. The stress was calculated based on the torque applied

using the equation $P = T / r$, where T is torque, P is compressive load and r is the radius of the bone specimen. Stress and strain were plotted (Figure 4), and the elastic modulus of the bone tissue was computed as 18.7 GPa and 16.9 GPa using data from the 705-nm and 625-nm channels of the fluorescence microscope respectively. The Young's modulus of bovine cortical bone by split Hopkinson pressure bar is typically in the range of 11.1-19.4GPa [1, 16].

The resonance frequency of phosphate was independent of load (Figure 5). Experiments were performed at a low spinning frequency, resulting in the spinning side bands shown in the figure. The intensity of spinning side bands increases linearly with the load (Figure 6). The reason for this observation is that homonuclear and heteronuclear dipolar coupling constants, which measure the interaction between adjacent atoms or ions, increase linearly with the inverse cube of ion spacing Therefore the slope of the curve is directly proportional to the decrease in mineral volume accompanying the load. The same linear change with loade will be observed for any effect that has the same dependence on the dipolar coupling constants.

That there are no observed changes in the isotropic and anisotropic chemical shift values of ^{31}P nuclei under the compressive load suggest that the chemical environment of the phosphate group in the bone sample is unaltered or very slightly altered. However, bone samples under pressure showed an increase in the intensity of spinning sidebands, suggesting that dipolar couplings are not completely suppressed by the MAS speed. This observation is most likely due to the pressure-induced decrease in the distance between dipolar coupled nuclei. As with most minerals, loads in the MPa range change ion spacings, but does not deform covalent bonds. Most importantly, these results demonstrate that a variety of biomechanical NMR studies of bone are possible. In particular, any effect that results in a linear dependence of dipolar coupling constants will be amenable to relatively straightforward interpretation.

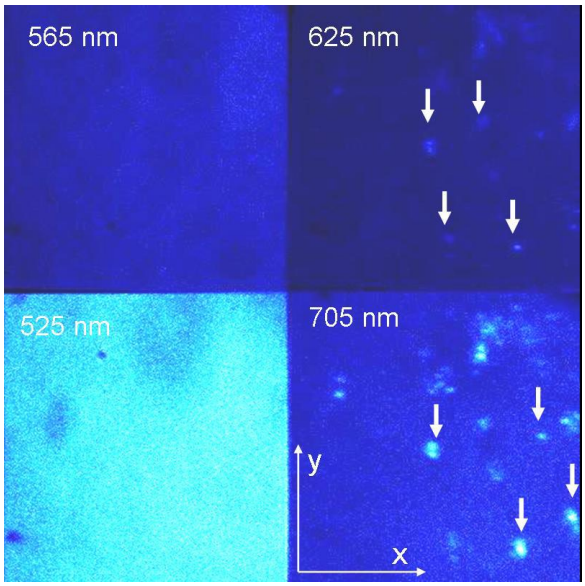

Figure 3. Fluorescence images of Qdots (arrows) on a bovine bone specimen, captured using four emission wavelengths (background at 525, 565 nm; matrix at 625 nm; mineral at 705 nm). Bone strain was measured during loading by tracking the positions of the Qdots over time.

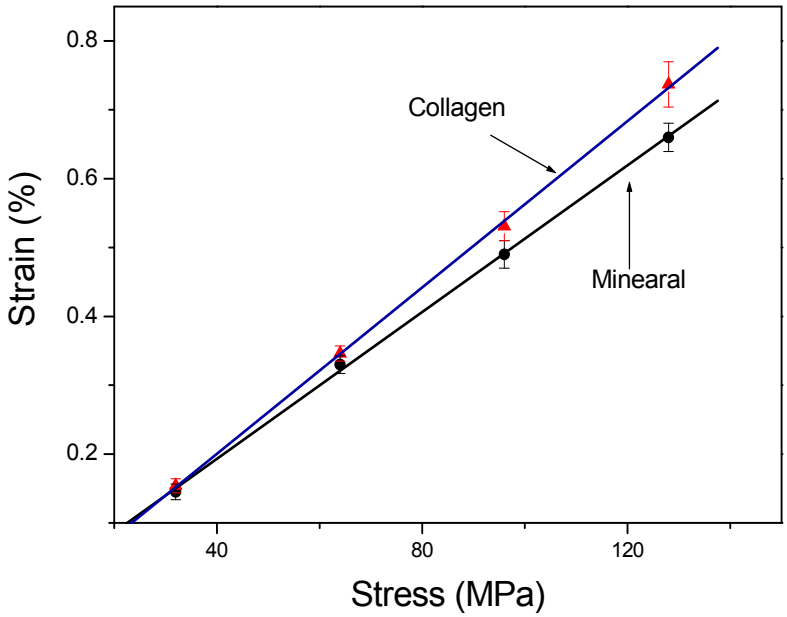

Figure 4. Stress-strain curve of a bovine bone specimen loaded in compression in the load cell. Stress was computed from the applied torque, and strain was computed as the average motion of four Qdots.

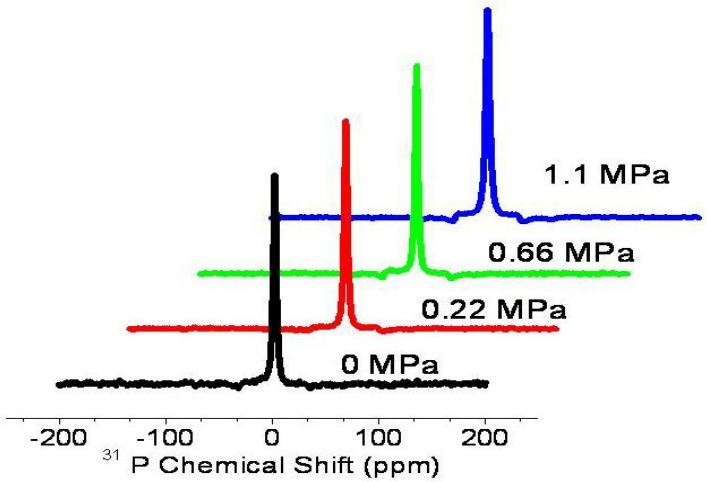

Figure 5. ^{31}P NMR spectra of bone mineral as a function of compressive load. Intensity of spinning side bands increase with pressure.

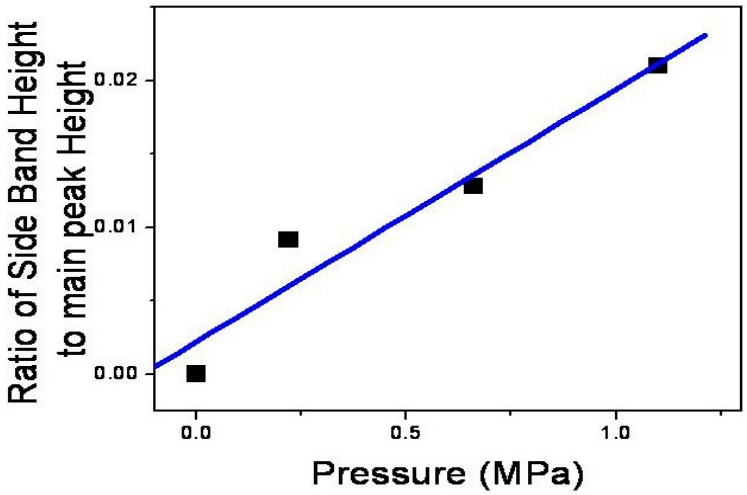

Figure 6. Magic angle spinning side band intensity of bone mineral ^{31}P NMR resonance scales linearly (R^2=0.97) with the compressive load.

4. CONCLUSIONS

Through measuring changes in the center-of-gravity of light spot of Qdots on the specimen surface, we have measured submicron compression of bovine cortical bone in a mock-up of an NMR load cell using a conventional wide-field fluorescence microscope and a split-field image viewing system. With addition of viewing slots to the ceramic cell, which must be performed before it is hardened by sintering, the apparatus will comprise a complete NMR-compatible bone biomechanical test system. Of course, loading and strain measurement must be performed outside of the NMR probe itself. These techniques are extendable to NMR biomechanical measurements on other mineralized tissues, such as tooth specimens or even the shells of invertebrates.

Solid-state MAS-NMR can now be used to measure atomic-level changes in the chemical structure of bone tissue caused by mechanical load. If NMR techniques that use dipolar coupling are employed, linear dependences on pressure are expected and spacing between ions in matrix or between nuclei in bone collagen can be measured directly.

ACKNOWLEDGEMENTS

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