

DETERMINING THE PERMEABILITY OF CORTICAL BONE AT MULTIPLE LENGTH SCALES USING FLUORESCENCE RECOVERY AFTER PHOTBLEACHING TECHNIQUES

*‡Patel, R B; *O'Leary, J M; *Bhatt, S J; ‡Vasanja, A; +*‡Knothe Tate, M L
*Dept. of Biomedical Engineering, Case Western Reserve University, Cleveland, OH
‡Dept. of Mechanical & Aerospace Engineering, Case Western Reserve University, Cleveland, OH
‡Orthopaedic Research Center, Cleveland Clinic Foundation, Cleveland, OH
knothetate@case.edu

INTRODUCTION

Orthopaedic surgeons, endocrinologists, tissue engineers and pharmaceutical developers are directing increased attention to decipher the permeability and molecular transport characteristics of bone tissue. Osteocytes, embedded within the mineralized bone matrix, can be at distances up to 250 μm away from the nearest blood supply, which necessitates molecular transport through the porous network of cortical bone. This transport is not only vital for cell survival but also for cellular signaling and drug delivery between the blood supply and cells. Bone's hierarchically organized transport network includes haversian capillaries (50-70 μm in diameter), canaliculi channels (0.10-1 μm in diameter), pericellular, proteoglycan meshwork-filled fluid spaces (15-50 nm), and matrix microporosity (5-12 nm) through which molecules pass as they are transported between the blood supply and osteocytes [1,2]. It is the nanoporous structure of cortical bone that confers size exclusion properties to the tissue, restricting free diffusion of bioactive molecules and drugs between cells and to/from the blood supply. The ultimate goal of this study was to define the upper permeability limit of large molecular weight molecules in cortical bone. Fluorescence Recovery After Photobleaching (FRAP) was applied to measure diffusive permeability of 300-70,000 Dalton (Da) molecular weight fluorescent probes in fresh, bovine cortical bone. The specific aims of this study were threefold, (i) to measure the currently unknown diffusion constants of various size macromolecules through the lacunocanaliculi network, (ii) to compare differences in permeability in the transverse and longitudinal planes in cortical bone, and (iii) to compare permeability across length scales including the matrix microporosity, cellular syncytium, and "bulk tissue" permeability.

MATERIALS AND METHODS

Diffusion constants were calculated based on FRAP experiments conducted on a laser scanning confocal microscope (SP2 AOBS, Leica Microsystems, Mannheim Germany).

Sample Preparation

Longitudinal and transverse sections were obtained from the mid-diaphysis of a fresh bovine femur cortex. After polishing to 100 μm , samples were soaked in free dye solutions (smallest molecules) for 4 hours or fluorescent probe solutions (larger molecules) for 4 days, at 4 $^{\circ}\text{C}$, to allow tracer fluorescent molecules to fully permeate the bone samples. Acid Yellow Fluorescein free dye (Sigma) and fluorescein conjugated dextran molecules of 3, 10, 40, and 70 kDa (Molecular Probes, Eugene, OR) were suspended in PBS with 2 mM sodium azide at concentrations of 0.05 mM, 0.13 nM, 0.023 mM, 0.032 mM, respectively, and then wet-mounted on a glass slide with dye/tracer solution used as the mounting medium.

Bleaching Methods and FRAP Techniques

Prior to bleaching, images were obtained using the laser scanning confocal microscope. To measure diffusion at the level of the matrix microporosity, bleach spots were placed on an area of matrix between canaliculi and recovery was measured post bleaching. To measure cell-level diffusion, bleach spots were placed on lacunar regions. Tissue level diffusion was measured by bleaching a tissue region consisting of matrix, canaliculi, and lacunae and measuring recovery of the fluorescent probes. Samples were bleached by exposing the sample to a high intensity laser for 400 ms. Recovery was measured by calculating the mean intensity of the bleached region in images collected post bleaching.

Quantification

Ten measurements were made per sample ($n = 3$); for each sample, diffusion constants were reported as the mean (\pm standard error) of these ten measurements. Using a least squares method and a custom written Matlab program, experimental data was fit to a curve and diffusion constants were calculated based on the slope of the curve for all samples.

RESULTS

Permeability versus Molecular Weight

The measured diffusion coefficient decreased exponentially with increasing molecular weight (Fig. L). Using FRAP, it was possible to measure diffusion coefficients for free dye and the 3, 10, and 40 kDa dextrans, respectively. It was not possible to obtain diffusion curves for the 70 kDa dextran molecules, because the recovery was too slow to measure.

Longitudinal versus Transverse Diffusion

Significant differences were observed in longitudinal and transverse diffusion constants (Fig. R) for small molecular weight free dye (300 Da). These differences were not observed for the 3kDa or larger molecular weight tracers.

Tissue, Cellular, and Canalicular Permeability

The permeability of cortical bone is highly dependent on length scale measured. The highest permeability was measured at the tissue level ($D = 1.57 \text{ E-}02 \pm 8.71 \text{ E-}04$), followed by the cellular syncytium level ($D = 6.2143 \text{ E-}06 \pm 6.6915 \text{ E-}07$) and matrix microporosity ($D = 1.0187 \text{ E-}09 \pm 3.9427 \text{ E-}11$), respectively.

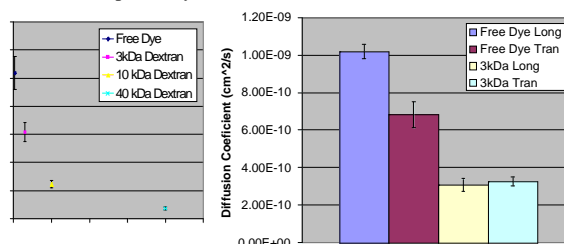


Figure Left: Permeability (Diffusion Coefficients) as a function of molecular weight. **Right:** Longitudinal vs. transverse diffusion for the 300 Da and 3 kDa tracers.

DISCUSSION

These studies show, for the first time to our knowledge, that the permeability of cortical bone is not only dependent on the size of the molecule being transported but also on the direction of transport, and the length scale of the system through which transport occurs. These studies show that diffusive transport of the 70 kDa tracer is so slow in cortical bone to prevent measurement of a diffusion constant using FRAP, corroborating *in vivo* tracer studies in which convective transport was necessary for movement of biomolecules including albumin (67 kDa) through cortical bone tissue. Of particular interest was the observation that the low pass filter function of bone's molecular sieve shows anisotropy, whereby small molecules (300 Da) are transported more rapidly in the longitudinal plane than in the transverse plane. This anisotropy, not observed in molecules larger than 3 kDa, has not been reported previously and may confer an additional means by which low molecular weight substances are transported preferentially, *e.g.* in the direction of growth during bone modeling. In conclusion, elucidation of the diffusive permeability and molecular transport pathways at different length scales in cortical bone provide a foundation for new strategies to deliver nutrients and growth factors to promote bone regeneration *in situ* and/or *in vitro* and develop new treatment modalities for bone disease.

REFERENCES [1] Knothe Tate M.L. *et al.*, *Bone* 22(2): 107-117, 1998. [2] Tami A. *et al.*, *Biorheology*, 40(6): 577-590, 2003.

ACKNOWLEDGEMENTS: This study was supported in part by a grant from the NASA John Glenn Bioscience and Engineering Consortium (NCC3-1008). Dr. Judy Drazba, Director of the CCF Imaging Core gave invaluable advice in design and implementation of this study.