Bone 59 (2014) 199–206

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Water residing in small ultrastructural spaces plays a critical role in the mechanical behavior of bone

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ARTICLE INFO

Article history:
Received 28 June 2013
Revised 23 October 2013
Accepted 19 November 2013
Available online 27 November 2013

Edited by: David Fyhrie

Keywords:
Water
Bone
Toughness
Solvents
Ultrastructural spaces

ABSTRACT

Water may affect the mechanical behavior of bone by interacting with the mineral and organic phases through two major pathways: *i.e.* hydrogen bonding and polar interactions. In this study, dehydrated bone was soaked in several solvents (*i.e.* water, heavy water (D_2O), ethylene glycol (EG), dimethylformamide (DMF), and carbon tetrachloride(CCl_4)) that are chemically harmless to bone and different in polarity, hydrogen bonding capability and molecular size. The objective was to examine how replacing the original matrix water with the solvents would affect the mechanical behavior of bone. The mechanical properties of bone specimens soaked in these solvents were measured in tension in a progressive loading scheme. In addition, bone specimens without any treatments were tested as the baseline control whereas the dehydrated bone specimens served as the negative control. The experimental results indicated that 22.3 ± 5.17 vol% of original matrix water in bone could be replaced by CCl_4, 71.8 ± 3.77 vol% by DMF, 85.5 ± 5.15 vol% by EG, and nearly 100% by D_2O and H_2O, respectively. CCl_4 soaked specimens showed similar mechanical properties with the dehydrated ones. Despite of great differences in replacing water, only slight differences were observed in the mechanical behavior of EG and DMF soaked specimens compared with dehydrated bone samples. In contrast, D_2O preserved the mechanical properties of bone comparable to water. The results of this study suggest that a limited portion of water (<15 vol% of the original matrix water) plays a pivotal role in the mechanical behavior of bone and it most likely resides in small matrix spaces, into which the solvent molecules larger than 4.0 Å cannot infiltrate.

Introduction

Bone is a natural composite material with a highly hierarchical structure and consists of three major constituents: *i.e.* mineral, organic matrix, and water, respectively. It has been known for decades that removal of water (dehydration) may lead to a marked decrease in the toughness and an increase in the stiffness of bone, suggesting that water plays an important role in both pre- and post-yield behavior of bone [1,2].

Previous NMR studies reveal that water in bone is present in three different conformations: namely *freely mobile water* in pores, such as Haversian canals, canaliculi, and lacunae spaces; *bound water* at surfaces and/or within the mineral and collagen phases; and *structural water* as part of collagen and mineral molecules [3–5]. Water may reside in the gap between the mineral–collagen interface in an order of several angstroms [6]. On the other hand, such matrix water may be replaced by minerals during continuous mineralization process [7]. Moreover, removal of water was speculated to alter the behavior of the collagen phase, thus reducing its capacity to dissipate energy in bone [8]. Furthermore, dynamic mechanical analyses indicate that bone viscoelastic behavior is most likely related to water in bone rather than the collagen phase itself [9,10]. However, the respective contribution of these three types of matrix water to the mechanical properties of bone is still poorly understood.

In this study, we hypothesized that the bulk mechanical properties of bone are significantly related to the water molecules that reside in extremely small (*i.e.* angstrom level) spaces of bone matrix, into which only water or a solvent akin to water can infiltrate. To test the hypothesis, we proposed to replace water in bone matrix with several solvents that are harmless to the structural integrity of bone constituents (*i.e.* mineral and collagen) and have different molecular size (*i.e.* kinetic diameter) and/or chemical characteristics (*i.e.* polarity and hydrogen bonding ability). Then, the correlation of molecular size, polarity, and hydrogen bonding ability with the soaking ability of the solvents into bone matrix and its effect on the mechanical behavior of bone were investigated.

Materials & methods

Specimen preparation

Six human cadaveric tibiae of male donors ($N = 6$) were procured from a Willed Body Program (UT Southwestern Medical Center at

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http://dx.doi.org/10.1016/j.bone.2013.11.018

Dallas, TX) with the stipulation that the donors had no known bone diseases. The donor ages were 51, 52, 54, 56, 58 and 76 years, respectively. Seven (7) dog-bone-shaped tensile test specimens were prepared from the mid diaphysis of each tibia using a CNC machine and randomly divided into seven (7) groups, including four (4) test groups (Table 1) in addition to a control (dehydrated and rehydrated), a baseline control (wet bone without any treatment), and a negative control (dehydrated) group. The specimens had a gauge length of 10 mm and a gauge cross-section of 2.0 mm × 2.0 mm. The prepared specimens were preserved in a phosphate buffered saline (PBS) solution and stored in a freezer at −20 °C prior to the treatments.

Selection of solvents

Since water molecules interact with the mineral and organic phases in bone through two major pathways: *i.e.* hydrogen bonding and polar interactions, the following solvents were selected from a pool of potential solvents that have different polarity, hydrogen bonding capability and molecular size (Table 1).

Water (H_2O) — Water is a good polar solvent [11,12] and has an estimated intermolecular hydrogen bond energy of 20.5 kJ/mol [13]. Water has the smallest molecular size (2.4–2.6 Å) compared with the other solvents [14,15].

Heavy water (D_2O) — Heavy water molecules contain two deuterium (hydrogen isotope) atoms in lieu of the hydrogen atoms in water molecules. It has similar polarity, chemical structure and molecular size (2.6 Å) [16] compared to water, with a slightly higher hydrogen bond energy (~8%).

Ethylene glycol (EG) — Ethylene glycol molecules have two hydrogen atoms attached to two separate oxygen atoms, thus allowing it to form strong hydrogen bonds readily with other molecules and high polarity akin to water. In addition, EG is the smallest organic molecule that can form hydrogen bond network like H_2O [17]. However, it has a much larger molecular size (4.0 Å) [18] compared with H_2O and is the greatest among all other solvents in viscosity. Since it is harmless to biological systems, the polymerized ethylene glycol (polyethylene glycol) have been used as cell culture scaffolds in tissue engineering studies [19,20].

Dimethylformamide (DMF) — DMF has a polarity comparable to EG, but lacks the ability to serve as a donor of hydrogen bonding like EG. DMF has a similar viscosity, but a larger molecular size (5.50 Å) compared to EG [15]. DMF also has been safely used as a co-solvent in cell culture [21,22] and does not cause denaturation of collagen molecules [17].

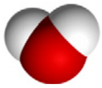
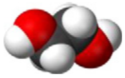
Carbon tetrachloride (CCl_4) — CCl_4 possesses neither the hydrogen bonding ability nor the polarity due to its tetrahedral symmetry and has a molecular size (4.65–5.90 Å) comparable to that of MDF [14]. Its viscosity (0.84–0.95 cP at 20 °C) is slightly lower than that of H_2O (1.00 cP at 20 °C) even though its density is much higher. CCl_4 has also been used as co-solvent in cell culture studies [23].

In this study, the kinetic diameter was used to estimate the molecular size of the solvents. In addition, the hydrogen bonding energy was used as the measure of the ability of solvent molecules to form hydrogen bonds with others. Moreover, the dielectric constant and dipole moment were used to define the polarity of the solvents. Finally, the viscosity of the solvents was also listed in the table in comparison between the solvents for viscous flow in bone.

Although it is hard, if not impossible, to adjust only one variable (*i.e.* polarity, hydrogen bonding ability, and molecular size) while keeping all others exactly the same, it is still possible to markedly vary one parameter while keeping the others relatively similar between two solvents. In this study, we managed to select the solvents that could be compared in this manner. For instance, water (H_2O) is very similar to heavy water (D_2O) except for a slight difference in hydrogen bonding energy. In addition, the major difference between water (H_2O) and ethylene glycols (EG) is the molecular size (2.6 Å vs. 4.0 Å) while the other chemical characteristics are very similar. Comparing EG and DMF, their major difference is reflected in the hydrogen bonding ability. Comparing DMF and CCl_4, their major difference is in polarity.

In theory, these solvents are considered to be chemically inert to the mineral (mainly hydroxylapatite) phase of bone. Also unlikely is the negative effect of the selected solvents on the structural integrity of collagen as they are often used as co-solvents in biological studies [19,20,23–26]. To further verify this, a pilot study was performed by treating demineralized bone samples ($N = 2$) in each of the selected solvents for three days at ambient temperature and then having them tested in tension. During the entire soaking process, we did not observe any visual damage and dissolved residues in the solvents. The mechanical tests indicated that the failure strain of all samples was between 0.21 and 0.25 irrespective of the solvents, which is very consistent with that (0.21–0.22) of controls (soaked only in PBS). By ruling out the negative effect of the selected solvents on the structural integrity

Table 1
Chemical and physical properties of the selected solvents ($N = 6$) [11–16,18,32–42].

Solvents	Molecular structure	Kinetic diameter d_k (Å)	Molecular weight M (g/mol)	Density @25 °C ρ (g/cm^3)	Relative permittivity ε_r (-)	Dipole moment p (D)	H–H bond energy (kJ/mol)	Viscosity @20 °C η (cP)
H_2O		2.4–2.6	18.015	0.997	78.30	1.85	20.5	~1.000
D_2O		2.6	20.04	1.104	77.94	1.85	8% higher than H_2O	1.251
EG $HO(CH_2)_2OH$[28]		~4.0	62.07	1.11	42.0	2.33	25.1	16.10
DMF $(CH_3)_2NC(O)H$		5.5	73.09	0.944	36.7	3.80	No donors, but acceptors	0.920
CCl_4		4.65–5.9	153.8	1.584	2.238	N/A	N/A	0.84–0.95

H_2O: Water; D_2O: Heavy water; EG: Ethylene Glycol; DMF: Dimethylformamide; CCl_4: Carbon Tetrachloride.