JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 72–79

Bone composite behaviour: effects of mineral–organic bonding

W. R. WALSH
Department of Orthopaedics, Biomechanics Laboratory, Rhode Island Hospital, Brown University School of Medicine, Division of Engineering, Brown University, 593 Eddy Street SWP-3, Providence, RI 02906, 401-277-8217 USA

M. OHNO
University of Medicine and Dentistry of New Jersey, Department of Pathology/Biomaterials Center, 675 Hoes Lane Piscataway, NJ 08854 USA

N. GUZELSU
University of Medicine and Dentistry of New Jersey – School of Osteopathic Medicine-Biomechanics and Rutgers University Biomedical Engineering, 675 Hoes Lane Piscataway, NJ 08854 USA

The mechanical properties of a composite material rely not only on the volume fraction, orientation and properties of the individual constituents, but upon their bonding interactions as well. This study examines the role of bonding between the mineral and organic constituents of bovine compact bone. Intact and completely demineralized samples were tested in tension following treatment in varying ionic strength sodium chloride or phosphate ion containing buffers to examine the interfacial bonding forces between bone's constituents. Phosphate ion treatment caused a reduction in the mechanical properties of intact samples but not in the demineralized samples. A sodium chloride solution with ionic strength equal to that of the phosphate ion buffer did not alter the mechanical properties of the intact or demineralized samples. Ash weight analysis, calcium probe measurements and SDS-gel electrophoresis indicated intact samples were not demineralized nor were bone structural proteins removed during treatment. Data suggest that the reduction in the mechanical properties of intact samples with phosphate ion treatment was due to an alteration in the interfacial bonding between the mineral and organic constituents of bone. Phosphate ions can compete with the negative domains of organic constituents for calcium binding sites of bone mineral and thereby interrupt or partially debond the interactions between the mineral and organic constituents of bone.

1. Introduction

Cortical bone tissue can be considered a complex hierarchical biological composite [1] composed of an inorganic mineral (hydroxyapatite-like material (HA) [2]) fibre embedded in an organic matrix of type I collagen and non-collagenous proteins [3]. The non-collagenous protein pool are primarily anionic (negatively charged) [4] and bind readily to calcium on the surface of HA [4, 5]. Cortical bone is composed of approximately 70% mineral and 30% organic constituents by weight [6, 7]. Furthermore, cortical bone can be considered a fibre-reinforced composite where the mineral fibres are embedded in the organic matrix [1, 8–10].

A great deal of experimental work has shown that the mechanical properties of bone tissue depend upon the properties, orientation and volume fraction of its individual constituents [11]. The mechanical properties of the primary constituents of bone have also been examined through complete removal of one of the phases [12–14]. The elastic moduli for hydroxyapatite using ultrasound has been reported to be 114 GPa [14]. The elastic moduli for type I collagen has been reported to range from 1.2 GPa [15] to 2.0 GPa [8]. The mechanical properties of cortical bone, however, cannot be predicted based on constituent properties, volume fraction or organization alone.

The mechanical behaviour of all composite materials are influenced by the bonding characteristics at the interface between the constituents [16–21]. A strong interface results in a fibre-reinforced composite with high strength and stiffness [19]. Non-covalent chemical interactions as well as possible covalent interactions between the bone mineral and protein bound organic phosphates have been suggested to exist in bone [22]. A theoretical study has suggested that partial debonding between the mineral and organic phases (collagen and non-collagenous proteins) would result in significant modification in strength and stiffness of bone and may be an important factor

0957–4530

in the mechanical properties of bone in various diseased states [17, 18]. However, no experimental studies have examined the role of interactions between the mineral and organic constituents of bone and their effect on the mechanical properties. Recently, an interface composed of non-collagenous proteins has been identified in cortical bone associated with the mineral and collagen phase [23]. The objective of this investigation was to examine the interfacial bonding interactions between the bone mineral and organic phases on the tensile properties of cortical bone due to potential-determining ion* treatment of phosphate ions at pH 7.5. This work demonstrates the importance of interfacial bonding forces between the organic and inorganic constituents of bone and its effects on the mechanical properties of this calcified tissue.

2. Material and methods

Cortical bone tension samples were prepared from the medial cortex of the diaphysis of 18 adult bovine femurs obtained from a local slaughterhouse. Thin sections of the samples were examined under light microscopy and determined to be Haversian bone. Samples were stored frozen in 0.145 M NaCl soaked paper towels and frozen in heavy duty plastic bags at − 20 °C in 0.145 M NaCl until use. This storage procedure has been shown not to alter the mechanical properties of bone [24]. Intact bone and demineralized bone samples were tested. Sixty-four intact bone samples (parallel to the long bone (z) axis) were machined into a dumbbell shape to serve as intact bone sample. The final dimension in the reduced cross section were 25 mm long, 5 mm wide and 2 mm thick. Twenty-four parallelepipeds were prepared parallel to the z axis to be used in the demineralization phase of this experiment. The final dimensions for these specimens were approximately 60 mm long, 5 mm wide and 2 mm thick.

The intact bone samples were tested in two separate experiments with four groups per experiment (eight samples/group). Intact samples were constantly stirred in 400 ml of 0.1% Nonidet P40 for 24 h at room temperature. Samples were rinsed in distilled water for 5 min prior to placement into the treatment solutions outlined in Table I. Nonidet P40 is a non-ionic alcohol detergent (Sigma Chemical Co., St. Louis MO, No N-3516) with an octylephenol-ethylene oxide condensate containing an average of 9 moles of ethylene oxide per mole of phenol.

Demineralized bone samples were prepared by treating the parallelepipeds in 0.12 M ethylenediaminetetraacetic acid (EDTA) for 42 days at 4 °C with daily solution changes [25]. A gauge length of 10 mm was marked and the ends of the parallelepipeds were coated with paraffin wax to isolate the region of bone to be tested and avoid demineralization of the specimens ends where they are gripped during mechanical testing [12]. Demineralization was monitored with X-rays and ashing weight analysis of additional samples.

TABLE I Summary of sample groups and treatment solutions. Treatment time for intact samples was 24 h in Nonidet P40 followed by 72 h in treatment solution. The dry intact samples (*) were wrapped with Kimwipes during handling and testing. The wet intact samples (**) were wrapped with an adsorbent material with a plastic backing during handling and testing. Demineralized samples were treated in 0.12 M EDTA for 42 days at 4 °C with daily changes followed by 72 h in treatment solution (***)

Sample group	Description
Dry intact samples	
1	Control 0.145 M NaCl
2	Nonidet P40 + 0.145 M NaCl
3	Nonidet P40 + 2.0 M NaCl
4	Nonidet P40 + 0.66 M Na_2HPO_4
Wet intact samples	
5	Control 0.145 M NaCl
6	Nonidet P40 + 0.145 M NaCl
7	Nonidet P40 + 2.0 M NaCl
8	Nonidet P40 + 0.66 M Na_2HPO_4
Demineralized samples	
9	EDTA + 0.145 M NaCl
10	EDTA + 2.0 M NaCl
11	EDTA + 0.66 M Na_2HPO_4

The treatment solutions (500 ml of solution per 35 g of bone) used were 0.145 M NaCl, 2.0 M NaCl and 0.66 M Na_2HPO_4 all adjusted to pH 7.5 (Table I). Total treatment solution time was 96 h at room temperature (24 h NP40 + 72 h treatment solution). Intact and demineralized samples were tested in uniaxial tension at a strain rate of $2.65 \times 10^{-3}\ s^{-1}$. Intact specimen deformation was recorded with a dynamic strain gauge extensometer (12.5 mm gauge length) while actuator displacement (10 mm gauge length between the grips) was used for the demineralized samples. The applied load was measured directly from the testing equipment.

Two different wrapping materials were used to avoid drying during installation into the grips and testing. Fluid saturated Kimwipe tissues were used for groups 1–4 in an attempt to keep samples wet during the testing procedures. These samples, however, were not completely wet since the tissues had a tendency to dry out very quickly. Groups 1–4 are classified as "dry-intact bone specimens" (Table III). A wet adsorbent material with a plastic backing was used for all other groups. This material kept samples completely saturated with fluid and completely wet during testing. Groups 5–8 are classified as "wet-intact bone specimens" (Table III).

Ultimate stress, yield stress and elastic modulus were determined for all intact bone samples (groups 1–8). Ultimate stress, strain to failure and elastic modulus were determined for all demineralized samples (groups 9–11). The ash fraction (mineral content) was determined for all samples after mechanical testing (group 2). Samples were dehydrated in acetone for three days and ashed in a muffle furnace at 650 °C for 24 h [26]. The ash fraction was calculated by dividing the ashed weight by the dry weight.

* Potential-determining ions of bone mineral (hydroxyapatite) are PO_4^{-3}, Ca^{+2} and OH^{-1} [2]. Potential-determining ions preferentially adsorb to the mineral surface and can exchange for lattice ions and thereby exert a fundamental control on the surface charge of the mineral.

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out on the extractions from the concentrated buffer treatment solutions (Table I) following equilibration with intact bone samples according to the method of Laemmli [27]. This was done to determine if any organic constituents were removed from the intact specimens during treatment with solutions shown in Table I. Polyacrylimide gel concentrations of 5% and 10% were used to cover high and low molecular weight components respectively. Molecular weight standards used were myosine (200 000), b-galactosidases (116 250), phosphorylase β (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400). Following the 72 h treatment period, the treatment solutions were lyophilized to reduce the volume, dialyzed continuously for 1 week for the NaCl and Na_2HPO_4 and 3 weeks for Nonidet solution with a daily change of distilled water. The samples were dissolved in 1% SDS-urea sample-buffer. Low power (50 V, 75 mA) was used to bring samples to the end of the stacking gel. High power (150 V, 125 mA) was applied at the onset of the separation gel and ran for 4 h and 30 min. The gels were stained by 0.25% Coomasie Blue dye for 3 h then destained in 10% acetic acid for 20 h.

A calcium electrode (Orion) (sensitivity $> 10^{-5}$ M) was used on treatment solutions from the intact bone group to determine if calcium ions were lost and the bone samples were demineralizing during equilibration with the treatment solutions outlined in Table I. Treatment solutions from the demineralized samples were not subjected to calcium probe analysis since the mineral phase was removed prior to treatment. Infrared (IR) spectra were obtained for the groups 1–4. IR spectra were obtained on a Perkin-Elmer 1725 Infrared Spectroscopic System. Bone wafers were ground with a mortar and pestle. Approximately 0.6 mg of sample was mixed with 300 mg KBr (Sigma Chemical Co., St. Louis MO, IR grade) and made into a pellet. IR spectra were obtained between 4000 cm^{-1} and 400 cm^{-1} at a resolution of 2 cm^{-1}.

Statistical differences between the groups were determined using statistical analysis software (SAS) (Cary, NC). Analysis of variance (ANOVA) and multiple comparison post-hoc analyses (Duncan's multiple range test, least significant difference (LSD) and Bonferroni t-tests, Tukey's and Scheffe's to control for type I and type II errors) were carried out to determine the differences and levels of significance between mechanical parameters between groups. A coanalysis of variance (COANOVA) was performed using ash fraction as a covariate to account for any biological variation between groups based on mineral content.

3. Results

Sample preparation consisted of wet grinding of a portion of the diaphysis to 2 mm in thickness under a continuous stream of distilled water followed by cutting with a low-speed saw under distilled water. This procedure no doubt removed a large portion of cells and cellular debris present in the Haversian systems and debris from grinding at this stage of preparation. Gel electrophoresis experiments confirmed that the total amount of proteins extracted from the intact bone samples following Nonidet P40 and the treatment solutions were relatively small compared with the total organic phase. The results indicate no protein equivalents to collagen bands α1, α2 and β (dimer). Protein bands larger than 100 000 daltons were not detected. The major protein bands detected were 60 000–70 000 daltons equivalent to bovine serum albumin in all extracts (Fig. 1). Overloaded samples revealed 5–6 low molecular weight components ranging from 45 000 to 21 000 daltons in the phosphate and Nonidet solutions (Fig. 2).

Biochemical analysis of the extracted material confirms that Nonidet primarily removed organic material associated with the vascular channel system and that collagen and other bone structural proteins were not removed from the samples by the treatments. It is likely that the majority of the organic material present in the Haversian system (blood vessels, nerve, red blood cells and lining osteoblasts [11, 28] had been removed along with their enzymes during earlier sample preparation. Calcium probe experiments on the treatment solutions indicates intact samples did not demineralize during the 72 h equilibration time within the detection limits of the probe. Ash weight analysis confirmed that all the intact bone samples (groups 1–8) had similar mineral content and were not

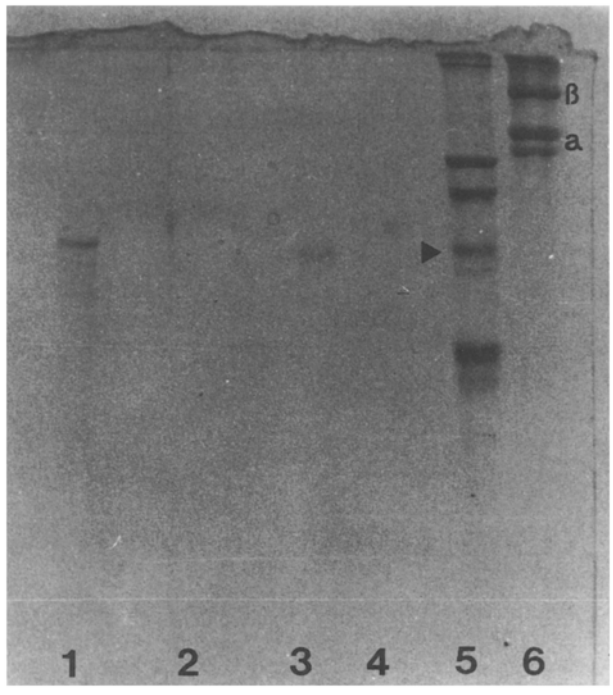

Figure 1 SDS gel electrophoresis results: Lane 1: Nonidet extract; Lane 2: NaCl extract—0.145 M; Lane 3: Na_2HPO_4—0.66 M; Lane 4: NaCl extract—2.0 M; Lane 5: molecular weight standard, from top (a) myosin (200 000), (b) galactosidase (116 250), (c) phosphorylase (92 500), (d) bovine serum albumin (BSA) (68 200) which is marked with a triangle, (e) ovalbumin (45 000); Lane 6: Calf skin collagen standard β (dimer) (200 000), α-chain (100 000) (α_1 and α_2). The samples for lanes 1–4 were obtained from 400 ml of treatment solution. Concentration of total protein in the samples for SDS gel electrophoresis was 0.83 μg per ml. Concentration of the standards were 0.5 μg per ml.

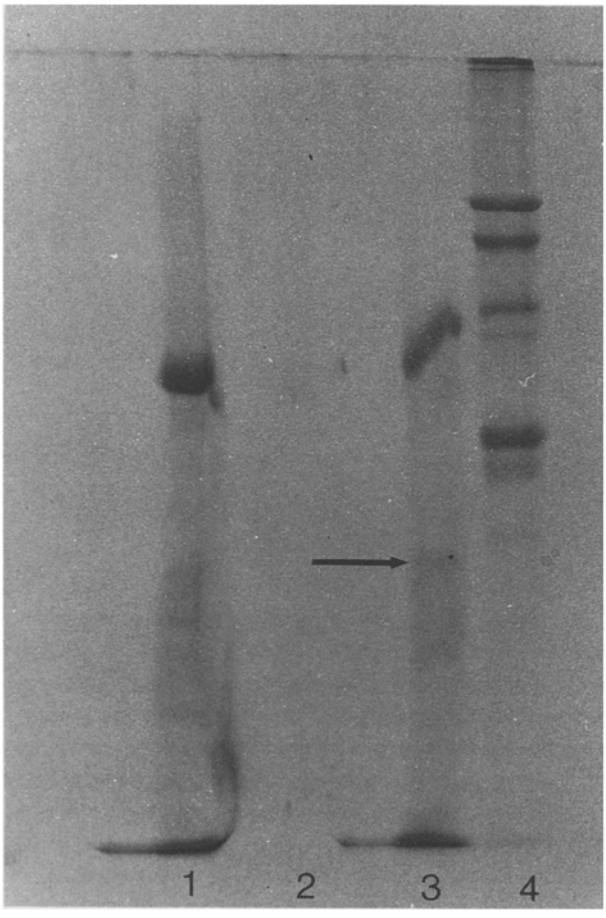

Figure 2 Overloaded SDS gel electrophoresis results: Lane 1: Na_2HPO_4—0.66 M; Lane 2: NaCl extract, combined total of 0.145 M and 2.0 M; Lane 3: Nonidet extract; Lane 4: Molecular weight standard from top (a) myosin (200 000), (b) galactosidase (116 250), (c) phosphorylase β(92 500), (d) bovine serum albumin (BSA) (68 200), (e) ovalbumin (45 000). The maximum amount of samples were loaded in order to see the minute amount of constituents to such an extent where the protein migration patterns were distorted. Small amounts of low molecular weight components became barely visible indicated by the white arrow (Lane 1 and Lane 3 at the level of the arrow). Samples were concentrated 10 times as compared to the first measurements (8.3 μg per ml). Also the volume of the samples were doubled in the SDS gel electrophoresis.

demineralized during the 72 h equilibration time (Table II). Ashing of the demineralized samples (groups 9–11) did not reveal any residual mineral. Infrared (IR) spectroscopy *did not* reveal any significant differences between any groups. The spectra between 1200 and 900 cm^{-1} (v_1 and v_3 stretching mode PO_4 regions), and 700 and 500 cm^{-1} (v_4 bending modes PO_4 region) *did not* vary with respect to treatment solutions (Fig. 5).

Analysis of variance (ANOVA) demonstrated the ultimate stress, yield stress and elastic modulus of the dry intact samples to be significantly greater than the wet sample counterparts ($p < 0.0001$) (Table III). The increase in the mechanical properties for the dry specimens demonstrates the effect of drying artefact on the mechanical properties of bone. ANOVA analysis within the dry and wet groups, respectively, revealed no statistically significant differences between the 0.145 M NaCl, Nonidet + 0.145 M and Nonidet + 2.0 M NaCl (dry groups 1 versus 2 versus 3; wet groups 5 versus 6 versus 7). A statistically significant

TABLE II Ash content. All sample groups were taken from the same region of bone samples. Ash weight analysis confirms that all samples had similar mineral content and were not demineralized by the treatments

Sample group	Description	Ash fraction
Dry intact samples		
1	Control 0.145 M NaCl	0.70
2	Nonidet 0.145 M NaCl	0.70
3	Nonidet 0.66 M Na_2HPO_4	0.69
4	Nonidet 2.0 M NaCl	0.69
Wet samples		
5	Control 0.145 M NaCl	0.71
6	Nonidet 0.145 M NaCl	0.70
7	Nonidet 0.66 M Na_2HPO_4	0.69
8	Nonidet 2.0 M NaCl	0.70

difference was found for the phosphate treated groups (groups 4, 8) compared with their sodium chloride ion solution controls ($p < 0.05$ for yield stress and ultimate stress; $p < 0.001$ for elastic modulus). ANOVA analysis did not reveal any significant differences amongst the demineralized groups (groups 9–11) or the ash fraction (mineral content) for the intact bone samples. Finally, the analysis of covariance (COANOVA) using ash fraction as the covariate, did not reveal any new findings between the groups.

Stress–strain curves for intact samples were typical of bone tissue having no toe region, a linear region followed by a minimal plastic deformation (Fig. 3). Phosphate ion treatment resulted in a significant alteration in the stress–strain behaviour of intact bone samples (Fig. 3). The stress–strain curves of the demineralized parallelepiped samples were characteristic of type I collagenous soft tissues (Fig. 4). A significant toe region followed by a linear region and a strain greater than 10% was found for all demineralized samples. Ion treatment solutions did not alter the

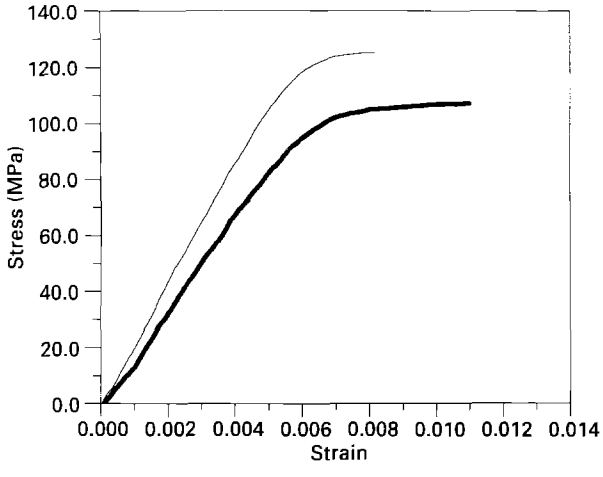

Figure 3 Typical stress–strain behaviour for intact control and phosphate samples treated with Nonidet P40 for 24 h followed by 0.145 M NaCl or 0.66 M Na_2HPO_4 at pH 7.5 for 72 h. This figure demonstrates the classic stress–strain behaviour of intact cortical bone tested in tension. Phosphate ion treatment lowers the yield and ultimate stress as well as the elastic modulus compared with control samples. The reduction in mechanical properties is attributed to an alteration in the interfacial bonding between the mineral and organic constituents of bone (— Phosphate; — control).

TABLE III Summary of mechanical properties. All solution pH were 7.5 and did not vary during equilibration. Groups 1–4 (Kimwipe specimens) were kept wet during milling with a squirt bottle filled with testing solution and during alignment and testing with kimwipes. Groups 5–8 were completely submerged during milling and kept wet during alignment and testing with a special adsorbent material with a plastic backing. Comparison of these groups indicates that the drying artefact present in groups 1–4 has been removed in groups 5–8. Nonidet detergent treatment does not alter the mechanical properties of bone tissue, but removes enough organic material in the vascular channel system to allow phosphate ions access to the mineral organic interface [31]. Nonidet plus phosphate ion treatment causes a statistically significant decrease in mechanical properties compared to respective controls. Phosphate ions compete with anionic groups of proteins for positively charged calcium sites of the bone mineral. In addition, the adsorption of phosphate ions to bone mineral at pH 7.5 causes the mineral to obtain a negative charge. The organic constituents will also have a negative charge at pH 7.5. This creates an electrostatically unfavourable condition for bonding between constituents and weakens the interfacial bonding causing a decrease in the mechanical properties. The action of phosphate holds for both the drying artefact and wet samples. Demineralization results in significant reduction in the mechanical properties compared to intact bone samples. No reduction in the mechanical properties was seen in the demineralized samples with phosphate or high sodium chloride concentration. This indicates that phosphate ions or high ionic strength do not alter the interactions among the organic constituents of bone, (* = $p < 0.05$, # = $p < 0.001$)

Sample group	Description	Modulus (GPa)	Yield stress (MPa)	Ultimate stress (MPa)
Dry artefact samples				
1	Control 0.145 M NaCl	27.3 (0.9)	161.0 (12.7)	161.9 (12.5)
2	Nonidet 0.145 M NaCl	28.3 (1.5)	173.1 (13.3)	173.7 (13.7)
3	Nonidet 0.66 M Na_2HPO_4	23.1 (1.9)#	150.3 (7.3)*	154.1 (11.1)*
4	Nonidet 2.0 M NaCl	29.9 (1.0)	175.1 (14.8)	178.5 (16.9)
Wet samples				
5	Control 0.145 M NaCl	19.8 (1.6)	113.0 (4.9)	116.1 (6.9)
6	Nonidet 0.145 M NaCl	20.4 (1.4)	119.4 (6.5)	121.5 (5.1)
7	Nonidet 0.66 M Na_2HPO_4	16.5 (1.6)#	107.5 (10.5)*	109.8 (10.9)*
8	Nonidet 2.0 M NaCl	20.8 (1.2)	118.1 (3.9)	120.1 (4.1)
Demineralized samples				
9	EDTA 0.145 M NaCl	54.2 (21.2)	14.6 (1.3)	16.9 (1.3)
10	EDTA 2.0 M NaCl	53.6 (26.9)	15.3 (11.6)	17.8 (1.4)
11	EDTA 0.66 M Na_2HPO_4	57.9 (20.7)	15.7 (3.5)	16.6 (1.0)

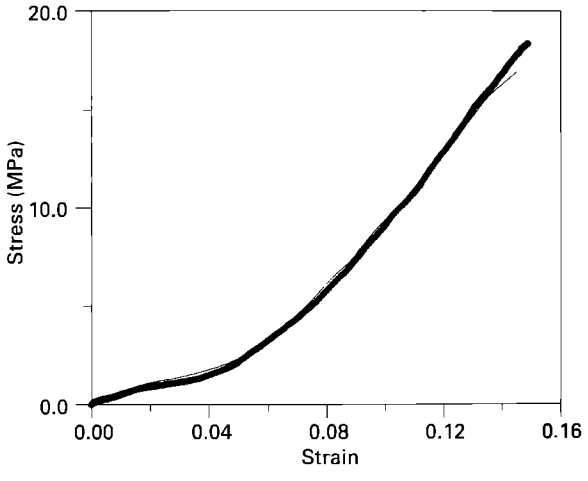

Figure 4 Typical stress–strain behaviour for demineralized bone samples (EDTA-treated) control 0.145 M NaCl and 0.66 M Na_2HPO_4 at pH 7.5 for 72 h. The stress–strain behaviour is typical of type I collagenous soft tissues with a toe region followed by a linear elastic region. Treatment with phosphate ions does not alter the stress–strain behaviour of completely demineralized bone samples. This indicates that the organic–organic interactions are not susceptible to phosphate ion treatment (— phosphate; — control).

stress–strain behaviour of demineralized bone samples (Fig. 4).

4. Discussion

Most of the variations in the mechanical properties of compact bone have been accounted for statistically by a few variables. The most important of these are porosity, orientation and amount of mineralization [29]. Bone is a composite tissue and as in all composites the bonding between the constituents plays an important role in determining the strength and stiffness. The goal of this study was to examine mineral–organic interfacial bonding and determine its influence on the mechanical properties of compact bone in tension. In order to alter the interfacial bonding forces between the mineral and organic constituents of bone and change the mechanical properties, treatment ions must be able to reach this interface. Preliminary mechanical tests on intact bone samples (data not reported) treated with a phosphate ion containing solution without a prior Nonidet detergent treatment did not alter the properties compared with control samples. This suggested that phosphate ions were not able to access the mineral–organic interface to alter the mineral–organic bonding relationship and affect the mechanical properties.

The presence of an ion-containing fluid phase in contact with a charged solid surface establishes an electrical double layer [30]. We have previously characterized the electrical double layer of cortical bone using a streaming potential experiment where an ion-carrying fluid is forced through a bone sample [31]. The flow of fluid generates electrical currents and at steady state an electrical potential difference called the streaming potential can be measured. Streaming potential measurements allow the charge in the electrical double layer to be characterized through the calculation of the electrokinetic or zeta potential. The charge and sign of the zeta potential is related to the charge in the electrical double layer and the species that comprise it. Streaming potentials can be used to

determine whether an ion is adsorbing or able to reach the interface and alter the zeta potential. Streaming potential experiments of control intact cortical bone plug samples indicated that phosphate ions did not alter the electrokinetic potential on intact bone samples for similar treatment times [31]. This confirmed that phosphate ions cannot access the mineral–organic interface in control intact bone samples without a prior treatment to remove the diffusional barriers present in the vascular channel system [31]. Streaming potential experiments support a compartmental model for bone fluid spaces [31]. The organic linings present in the vascular channel system shields the mineralized bone matrix and therefore the mineral–organic interactions from the vascular channel fluid space [31]. The organic linings of the vascular channel system act as a diffusional barrier to ions accessing the calcified matrix where the mineral–organic relationship occurs. This barrier must be removed or stripped away to some degree to allow ions access to the mineral–organic interface in order to alter the bonding forces between constituents [31].

A non-ionic detergent Nonidet P40 (NP40) was chosen to remove the diffusional barriers because it is known to solubilize organic membranes and proteins [32, 33]. A non-ionic detergent also bears no apparent ionic charge and does not generally adsorb onto a surface and affect its surface charge significantly [31, 34]. Furthermore, treatment with 0.1% Nonidet P40 detergent has been shown to remove some of the organic linings (lining cells (resting osteoblasts), endothelial cells, basement membrane) present in the vascular channel system allowing phosphate ions access to the calcified matrix to alter the electrokinetic properties of 0.5 mm thick cortical bone plugs after 24 h in phosphate buffer [31]. Long-term equilibration studies of 0.5 mm cortical bone plugs in phosphate buffer revealed no additional change in the electrokinetic properties after the initial 24 h equilibration [31]. Considering the thickness of our samples was three to four times as thick as our electrokinetic samples and our ionic strength increased from 0.145 to 2.0 ionic strength, a treatment time of 72 h was chosen.

The use of Nonidet P40 detergent treatment, however, increased the samples' susceptibility to drying. NP40 treatment clears the vascular channels of cellular debris and solubilizes some of the organic linings of the vascular channels [31]. This allows water in the matrix to communicate with the external environment and makes the samples more susceptible to a drying artefact in open air. This accounts for the increase in mechanical properties for the 0.145 M NaCl and the NP40-treated 0.145 M NaCl groups (groups 1 and 2; groups 5 and 6). Drying of bone samples is known to increase the yield stress, ultimate stress and elastic modulus of bone approximately 30% [35]. The ratio of wet to dry sample reveals approximately a 70% reduction and confirms the effect of drying on the properties (Table IV). The wet Kimwipe wrap was not efficient enough to keep the samples wet during testing. The second wrap material was able to keep the samples wet during handling and testing.

TABLE IV The ratio of wet samples (groups 5–8) to drying artefact samples (groups 1–4). The increase in mechanical properties in the drying artefact samples in groups 1–4 is consistent with literature on effects of drying [35].

Sample description	Modulus (GPa)	Yield stress (MPa)	Ultimate stress (MPa)
Control 0.145 M NaCl	0.73	0.70	0.72
Nonidet 0.145 M NaCl	0.72	0.70	0.70
Nonidet 0.66 M Na_2HPO_4	0.71	0.72	0.71
Nonidet 2.0 M NaCl	0.70	0.67	0.67

Treatment with NP40 does not alter the mechanical properties of bone tissue compared to controls as long as the samples are handled in the same manner (groups 1 versus 2 and 5 versus 6). NP40 did not remove any organic material which had a mechanical load-bearing function in bone tissue. In addition, the values of our control samples and NP40-treated controls were not statistically different and fit well with the published data for cortical bone in tension at a similar strain rate (8). This indicates that our samples were not destroyed by enzymatic release during the treatment time used in our experiments.

Treatment with phosphate ions caused a significant reduction in the tensile properties of intact bone samples compared with samples treated in NP40 + 0.145 M NaCl (Fig. 2). The modification can be attributed to adsorption of the potential determining phosphate ions to the bone mineral surface and competition with the negative binding sites of the proteins (carboxy or phosphate groups) for positively charged calcium sites on the bone mineral [36, 37]. Phosphate ion bonding and an alteration in the interfacial bonding between constituents results in a decrease in mechanical properties. The effect of phosphate ions on mineral–organic interfacial bonding may be similar to the elution of proteins from hydroxyapatite columns by phosphate ions. Phosphate ions compete with anionic groups of the proteins for positively charged calcium sites of hydroxyapatite and debond and removed organic material [38–40]. Our data supports Bundy's hypothesis on the effect of partial debonding between the constituents of bone and the effect on mechanical properties.

Electrokinetic experiments have also shown that the adsorption of phosphate ions to the bone mineral will also cause a charge reversal for the mineral phase (hydroxyapatite) from positive to negative at pH 7.5 [41–45]. The organic constituents of bone, which have low isoelectric points, are negatively charged at pH 7.5 [41, 42]. Therefore, the electrostatically favourable condition (positively charged mineral and negatively charged organic phase) that had existed prior to phosphate ion adsorption at pH 7.5, no longer holds since both species are negatively charged. This creates an electrostatically unfavourable condition between the mineral and organic constituents. The introduction of phosphate ions to the interface between the bone mineral and organic constituents may weaken the interfacial bonding (based on electrostatic forces) and thereby reduces the mechanical properties of the

tissue. The properties of the "dry" Kimwipe-wrapped phosphate samples were also statistically lower than their respective controls. This suggests that the action of phosphate ions holds for both cases of wet and dry samples.

A 2.0 M NaCl group was tested in order to determine that the action of phosphate ions was not due to ionic strength of the solution. Ionic strength (I) of a solution is defined as $(1/2)\ \Sigma\ (C_{mi}Z_i^2)$ where C_{mi} is molar concentration and Z_i is valence of the ion. Ionic strength considers not only the bulk molar concentration but the charge of the particular ion [32, 46]. The ionic strength of 0.66 M Na_2HPO_4 at pH 7.5 is 2.0 which is equal to the ionic strength of 2.0 M NaCl. A high ionic strength solution has more charged species present that can compete for spaces on the solid matrix, thus displacing the original occupants [32, 45, 46]. In addition, as the ionic strength increases the energy of interaction between two charges decreases as predicted by the Debye–Huckel theory [44].

High ionic strength NaCl treatment did not alter the mechanical properties (groups 3 and 7, Table I) compared with controls. Biochemical analysis on the treatment solutions equilibrated with the intact bone samples indicated proteins were not removed due to high ionic strength solution treatment (Fig. 1). This suggests that the effect on the mechanical properties of the phosphate-treated samples was by phosphate ions and not attributed to ionic strength amplification and the removal of organic material through a salting out mechanism.

Regarding the effects of our ion treatment protocol on the bone mineral phase, the IR spectra data indicate that the mineral phase is not being significantly altered by phosphate ion treatment (Fig. 5). The IR spectra support the finding that the mineral phase is not changing its crystallinity or altering its crystal structure significantly due to our treatments. Had the mineral phase become an amorphous phase due to our treatments, we would have expected a change in the 1200–900 cm^{-1} and 700–500 cm^{-1} regions. However, the similar spectra for all treatments supports the view that the bulk properties of the bone mineral did not significantly change [47, 48].

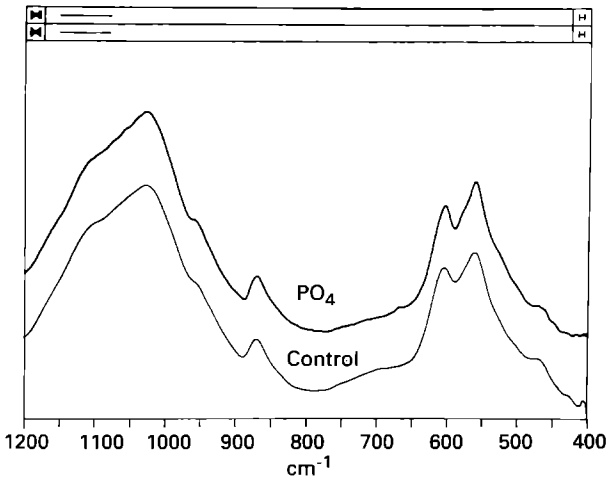

Figure 5 Infrared (IR) spectrum of a NP40 + 0.145 M NaCl and NP40 + 0.66 M Na_2HPO_4. The IR patterns of the two do not differ. This suggests that the phosphate ion treatment does not have a significant effect on the mineral phase size or crystallinity.

The question arose whether or not the ionic strength and ion treatments may also influence organic–organic interactions in bone leading to a change in the mechanical properties. Demineralized samples were tested with the same treatment solutions in order to confirm that phosphate ions or high ionic strength were not altering any organic–organic interactions resulting in a change in the mechanical properties of the organic phase. The mechanical properties of the demineralized groups did not differ (Table III). Bone particle electrophoresis of EDTA-treated samples tested in phosphate buffer showed phosphate ions had no effect on the electrokinetic potential [46]. This suggests that phosphate ions do not adsorb or alter the surface charge and zeta potential characteristics of EDTA-treated demineralized bone. Therefore, phosphate ions presumably do not alter the mechanical properties of the type I collagen of bone remaining after EDTA demineralization.

EDTA treatment for 42 days completely removed the inorganic bone mineral leaving type I collagen and its associated organic phase behind. The stress–strain behaviour for EDTA-treated samples was consistent with a type I collagenous soft tissue (Fig. 4). Histological analysis with hematoxylin and eosin staining revealed a normal-appearing type I collagen phase. Demineralization with EDTA does not alter the mechanical properties of bone type I collagen [25]. The treatment solutions used in the present study did not alter the mechanical properties of the demineralized samples (Table II). This confirms that the organic–organic interactions were not influenced by either phosphate ions or high ionic strength to alter the mechanical properties of demineralized bone at pH 7.5. Demineralized sample deformation was monitored using the testing machine LVDT. An extensometer was not used since only soft tissue (type I collagen) remained after demineralization. However, all demineralized specimens were tested in the same manner and no differences were found following treatments.

The experimental results found in the present study suggests that phosphate ions are capable of altering the mineral–organic interfacial bonding resulting in a reduction in the tensile properties of cortical bone. Our data supports the theoretical debonding hypothesis proposed by Bundy [17, 18]. Interfacial bonding forces between the mineral and organic constituents gives bone its unique composite behaviour and may be an additional parameter to account for the variation in the properties.

The technique of a detergent treatment to remove some of the organic linings present in the vascular channels and potential-determining ion treatment may be useful to investigate the mechanical properties of bone tissue and mineral–organic interfacial bonding in normal, aged and diseased states. The sample preparation technique of wet grinding and cutting most likely washes away the majority of the cellular material and enzymes released in the vascular channels that may have a detrimental effect on the mechan-

ical properties. The sample preparation technique also accounts for the small amounts of protein material extracted with Nonidet P40 and the even smaller amounts removed by the treatment solutions. Finally, had enzymatic release and degradation occurred during the time period, we would not expect the samples treated in 0.145 M NaCl to be similar to literature-reported values.

Changes in bone quality (bonding between the mineral and organic constituents) may be important in addition to changes in bone quantity in aged and diseased bone (osteoporosis) [17, 18]. The work presented here suggests that the mineral and organic phases of bone are linked through adsorption forces via electrostatic bonding (van der Waals', hydrogen bonding and hydrophobic interactions) and other possible adsorption forces [48]. This results in load transfer through the mineral and organic phases where the bone mineral acts a short fibre to reinforce the organic matrix. Phosphate ion access to the mineral–organic interface can interrupt or partially debond this interaction and weakens the interfacial bonding causing a decrease in the tensile mechanical properties.

Acknowledgement

This work was supported in part by NIH-National Institute of Health, Arthritis, Musculoskeletal and Skin Disease, Ortho., grant R23-AR36951.

References

1. J. L. KATZ, H. S. YOON, S. LIPSON, R. MUHARIDGE, A, MEUNIER and P. CHRISTEL, *Calcif. Tiss. Int.* **36** (1984) S31.
2. A. S. POSNER and F. BETTS, *Acct. Chem. Res.* **8** (1975) 273.
3. S. SKINNER, in "The scientific basis of orthopaedics", edited by J. Albright and R. A. Brand (Appleton-Century Crofts, New York, 1979).
4. S. T. LI and E. KATZ, *New England J. Medicine* **22** (1990) 275.
5. R. B. MARTIN and J. ISHIDA, *J. Biomechanics* **5** (1989) 419.
6. S. C. COWIN, in "Bone Mechanics", edited by S. C. Cowin (CRC Press, Boca Raton, FL, 1989) p. 97.
7. S. C. COWIN, W. C. VAN BURSKIRK and R. B. ASHAMN, in "Handbook of Bioengineering", edited by R. Skalak and S. Chien (McGraw Hill, New York, 1987) p. 2.1.
8. J. D. CURREY, "The mechanical adaptations of bones" (Princeton University Press, Princeton, NJ, 1984).
9. K. PIEKARSKI, *ASME Technical Report* No. T9-2.2 1969.
10. Idem, *Int. J. Eng. Sci.* **11** (1973) 557.
11. R. B. MARTIN and D.B. BURR, "Structure, function and adaptation of compact bone" (Raven Press, New York, 1989).
12. A. H. BURSTEIN, J. M. ZIKA, K. G. HEIPLE and L. K. KLEIN, *JBJS* **57-A** (1975) 956.
13. S. SAHA, *J. Mater. Sci.* **12** (1977) 1798.
14. S. S. GILMORE and J. L. KATZ, *J. Mater. Sci.* **17** (1982) 1131.
15. J. D. CURREY, *Biorheology* **2** (1964) 1.
16. Y.-C. CHEN and C.-Y. HUI, *Mech. Mater.* **10** (1990) 161.
17. K. J. BUNDY, in "Bone mechanics", edited by S. C. Cowin (CRC Press, Boca Raton, FL, 1989) p. 197.
18. Idem, *Ann. Biomed. Engng.* **13** (1985) 119.
19. C. C. CHAMIS, *NASA Technical Note* TND-6588, Washington, DC 1972.
20. D. HULL, "An introduction to composite materials" (Cambridge University Press, Cambridge, 1981).
21. Y. WANG, S. BACKER and V. C. LI, *J. Mater. Sci.* **22** (1987) 4281.
22. M. J. GLIMCHER and S. M. KRANE, in "Treatise on collagen", Vol. 2B, edited by G. N. Ramachandran and B. S. Gould (Academic Press, New York, 1968) p. 68.
23. M. MARTIN, A. LAMURE, C. LACABANNE, C. BETIN and M. F. HARMARD, *Biomaterials* **11** (1990) 11.
24. E. D. SEDLIN and C. HIRSCH, *Acta Orthop. Scand.* **37** (1966) 29.
25. C. C. DANIELSEN, T. T. ANDREASSEN and L. MOSEKILDE, *Calcif. Tiss. Int.* **39** (1986) 69.
26. A. C. ABRAMS, T. S. KELLER and D. M. SPENGLER, *J. Biomechanics* **21** (1988) 755.
27. U. K. LAEMMLI, *Nature* **266** (1970) 680.
28. S. C. MILLER and W. S. S. JEE, *Calcif. Tiss. Int.* **41** (1987) 1.
29. J. D. CURREY and K. BREAR, *Biomimetics* **1** (1992) 103.
30. R. J. HUNTER, "Zeta potentials in colloid science (Academic Press, New York, 1981).
31. W. R. WALSH and N. GUZELSU, *J. Orthop. Res.* **9** (1990) 683.
32. A. HELENIUS and K. SIMONS, *Biochim. Biophys. Acta* **415** (1975) 29.
33. D. TRAGNER and A. CSORDAS, *Biochem. J.* **244** (1987) 605.
34. M. J. ROSEN, "Surfactants and interfacial phenomena" (New York, Wiley 1989).
35. F. G. EVANS, "Mechanical properties of bone" (C. C. Thomas, Springfield, IL, 1973).
36. G. BERNARDI and T. KAWASAKI, *Biochim. Biophys. Acta* **160** (1968) 301.
37. G. BERNARDI, M. GIRO and C. GAILLARD, *Biochim. Biophys. Acta* **278** (1972) 409.
38. E. GLUECKOUF and L. PATTERSON, *Biochim. Biophys. Acta* **315** (1974) 57.
39. E. C. MORENO, M. KRESAK and D. I. HAY, *Arch. Oral Biol.* **23** (1978) 525.
40. Idem, *Calcif. Tiss. Int.* **36** (1984) 48.
41. N. GUZELSU and R. REGIMBAL, *J. Biomechanics* **23** (1990) 661.
42. N. GUZELSU and W. R. WALSH, *J. Biomechanics* **23** (1990) 673.
43. D. N. MISRA, in "Methods of calcified tissue preparation", edited by G. R. Dickson (Elsevier, New York, 1984) p. 435.
44. R. GABLER, "Electrical interactions in molecular biophysics" (Academic Press, New York, 1978) p. 316.
45. Z. SALEEB and P. L. DEBRUYN, *J. Electroanalytical Chem.* **37** (1972) 99.
46. R. Z. LEGEROS and S. SUGA, *Calcif. Tiss. Int.* **32** (1980) 169.
47. R. Z. LEGEROS, L. SINGER, R. H. OPHAUG, G. QUIROLGICO, G. A. THEIN and J. P. LEGEROS, in "Osteoporosis", edited by J. Menczel, G. C. Robin and R. Steinberg (J. Wiley & Sons, New York, 1982) p. 327.
48. E. I. F. PEARCE, *Calcif. Tiss. Int.* **33** (1981) 95.

Received 20 August 1992
and accepted 30 April 1993