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The Correlation between Dielectric Properties and Cortical Bone Tissue Composition: Dispersion Model-Based Analysis using Electrical Impedance

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Abstract

The current study aimed to contribute an understanding of the dielectric properties of various bone constituents. Dielectric properties of the bone tissue were measured under different treatment conditions i.e. dehydration (by lyophilization), fat removal (acetone treatment) and demineralization (using 6% ethylenediamine tetraacetic acid), in the frequency range from 42 Hz to 5 M Hz using Electrical Impedance Spectroscopy (HIOKI 3532 HiTester, Koizumi, Japan). Also, the dispersion model-based analysis has been applied for fitting the electrical data. The results showed variation in the permittivity and conductivity curves as a result of different treatments on bone tissue. Moreover, the dispersion model based fitted parameters were found to reflect the compositional features of bone. The dielectric properties of the bone tissue vary as a result of different treatments thus showing a strong relationship between model fitted parameters and different bone components.

Keywords: Electrical impedance spectroscopy; Demineralization; Dehydration; Rat cortical bone; Femur; Dielectric dispersion model

Introduction

Bone is a composite of an organic matrix (90% Type I collagen) and an inorganic mineralized phase. The integrity of the bone composition and microarchitecture determines the functioning of the bones. Also, bone constitutes a substantial amount of water in the free as well as bound form [1]. Assessment of the close association between these organic and inorganic components or to look into the chemical and structural aspect would provide an early diagnosis of bone-related disorders. Osteoporosis is a metabolic bone disorder that involves a loss of structural stability as a result of changes in bone composition or architecture. This will make the bone more fragile and prone to fractures. Fracture resistance is determined by the strength of the bone, which in turn depends on its geometric properties (size, shape, and connectivity), the activities of the cells in the tissue, and the material properties of the tissue. The material properties of bone include the mineral content, mineral composition, mineral crystal size, and matrix content and composition [2,3]. Information on the bone material properties can prove to be highly beneficial in providing useful information regarding bone health. Therefore, keeping in view the above-mentioned facts, we have tried to explore the potential of Electrical Impedance Spectroscopy (EIS) in determining the bone material properties.

The exploitation of dielectric properties using EIS, for the assessment of bone health may have tremendous applications in diagnostics or therapeutics. For instance, several investigators have measured the electrical and dielectric properties of the cortical and trabecular bone [4-9]. Saha et al. [5], studied the electrical and dielectric properties of wet human cortical bone as a function of frequency and direction. Later on, Sierpowska et al. [10], studied trabecular bone electrical properties and associate water content and dry density with the electrical properties. They had briefly mentioned a close association between dielectric parameters and few of the bone components including fat and collagen. Thereafter, Meaney et al. [9], excised the porcine bone samples from the trabecular region of the femoral head and performed measurements at five mineral density levels. They also showed a strong correlation between both the conductivity and permittivity and bone volume fraction. However, all the above-mentioned investigations restricted themselves to the excised cylindrical sections in which the whole volume of the bone was not taken into consideration. Also, the effects of water loss and mineral changes on bone dielectric properties have not been studied thoroughly. Most importantly, the thorough analysis and interpretation of dielectric data have not been carried out. Recollecting all the above-mentioned studies, an attempt has been made to evaluate and interpret the electrical properties of the bone (extracting each component gradually) using Electrical Impedance Spectroscopy in the frequency range from 40 Hz to 5 MHz. Furthermore, the dispersion model-based analysis has been carried out for the investigation of dielectrical data. Dispersion model-based parameters obtained by the fitting procedure has provided direct evidence of water and mineral loss. These studies have provided direct insight into the bone

compositional changes which may be very supportive in analyzing the pathological bone samples in the near future.

Materials and Methods

Bone tissue processing

Five male rats at the age of 22 weeks were procured from the central animal house of Panjab University, Chandigarh. The experimental design and procedures were approved by the Ethical Committee on Animal Experiments of the Central Animal House, Panjab University. They were sacrificed by decapitation and their bones (both femora) were excised, cleaned off to remove the muscular tissue and then kept in Phosphate Buffer Saline (PBS, pH 7.4, 37°C). The electrical measurements were then carried out using Electrical Impedance Spectroscopy (EIS) (HIOKI-3532-50, Hi Tester, Japan) [11], and shown in (**Figure 1**) for all the bone samples (extracted from five rats). Then, the control bones were lyophilized in three steps (1 hr, 2 hrs, and 6 hrs) for removing the loosely and tightly bound water and again the electrical measurements were carried out after each step. Further, lyophilized bone samples were treated with acetone (for 72 hrs) to remove fats, followed by buffer saturation (18 hrs). Buffer saturation was done after every treatment so as to preserve the net bone volume. The dielectric measurements were again carried out. Bone tissue weights were recorded after each treatment. Fat removed was analyzed by Sulfo-Phospho-Vanillin (SPV) Colorimetric method [12], from the acetone solution in which the bones were immersed. Lastly, bone samples were kept in 6% of Ethylene Diamine Tetraacetic Acid (EDTA) for the sequential removal of hydroxyapatite (OHAp) in ten different time intervals (4 steps of 15 min each and 6 steps of 1 hr each). The EDTA solution containing extracted hydroxyapatite was analyzed by atomic absorption spectrophotometer (model 3100; PerkinElmer) in order to estimate the calcium removed in each step.

Electrical measurements

Dielectric properties of the bone samples were measured using LCR impedance analyzer (HIOKI 3532 HiTester, Koizumi, Japan) [9], in the frequency range of 50 Hz to 5 MHz (**Figure 1a**). Two pairs of copper strips were used as electrodes in the sample holder assembly (**Figure 1b**). Each pair consisted of two 4 mm wide thin copper strips positioned one atop other. The terminals of each pair of strips were interfaced with the impedance analyzer via coaxial cables. The sample holder assembly was kept inside a rectangular Perspex® chamber (**Figure 1d**). To avoid drying of the sample, which is known to affect the dielectric properties significantly, the inner walls of the chamber were completely covered by absorbent cotton soaked in PBS. Thus the chamber environment becomes humid which prevents loss of moisture from the sample during the measurement procedure. Prior to the measurement, open circuit compensation followed by short circuit compensation were performed that allowed corrections for any stray capacitance, series lead inductance, resistance and shunt conductance arising due to the sample holder assembly. Before the measurement, each bone specimen stored at -40°C was taken out and thawed gradually by keeping on ice for 15 min and then in PBS at 37°C for one hour. The mid-diaphyseal region along the longitudinal axis of the intact bone was selected for the dielectric measurement. On this portion, to ensure good electrical contact between the electrode and the sample, a thin layer of colloidal silver paste was applied to form a 4 mm wide ring of silver on the bone surface. A second such ring at a distance of 3 mm from the first ring was applied in the same manner. After this, the sample was again immersed in PBS for 20 minutes before its affixation to the sample holder. The bone sample was positioned in between the strips of each pair of electrodes and both the terminals of each pair were clamped together so that they form a ring around the bone surface. Care was taken to ascertain uniform contact between the bone surface and the electrode rings without applying excessive pressure on the sample during clamping. In this configuration, the part of the bone subjected to measurement is 3 mm long mid-diaphyseal region with 4 mm wide ring electrodes clamped on both sides (**Figure 1c**). This sample electrode geometry ensures sufficient measuring depth so that the observed dielectric behavior of the measured region is contributed by its entire volume [13].

Measurements were started after a delay of 30 minutes from mounting of the sample to allow its acclimatization with the chamber environment. The dielectric measurements were carried out at the operating voltage of 1 volt. For each sample, the impedance (Z) and the phase angle (θ) were measured at ninety-eight different frequencies in the range of 50 Hz to 5 MHz. Uncertainties in the Z and θ values were evaluated according to the procedure described in the user manual of the LCR meter (Instruction manual 2006) [9]. From Z and θ the relative permittivity ε and conductivity σ were evaluated assuming parallel plate capacitor model for the sample.

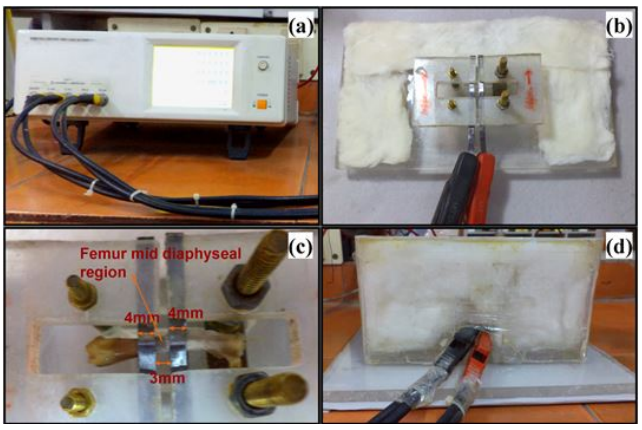

Figure 1: Electrical impedance analyzer and sample holder used for data acquisition (a): Set up of electrical impedance analyzer; (b): Sample holder assembly; (c): Bone sample placed in the sample holder prior to the measurement; (d): Sample holder assembly inside the Perspex chamber

Fitting procedure

Fitting of the whole dielectric data (frequency range 42 Hz to 5 MHz) has been carried out using the parametric expression used by Raicu [14].

$$\varepsilon^* = \varepsilon' - i\varepsilon'' = \varepsilon_\infty + \frac{\Delta\varepsilon}{\left[(i\omega\tau)^\lambda + (i\omega\tau)^{1-\mu}\right]^\nu} + \frac{\sigma_i}{i\omega\varepsilon_0} \quad (1)$$

Where and ε" are the real and imaginary parts of the complex permittivity; ε_∞ denotes the high frequency limit of the permittivity; ε_s is the static permittivity; Δε is the dielectric increment (ε_s-ε_∞); σ_i is the ionic conductivity; ω is the angular frequency of the applied electric field; τ is the relaxation time characteristic to each polarization mechanism and; λ, μ and ν are real constants whose values lie between 0 and 1. The dielectric loss factor (ε") is maximum when ωτ=1, and the corresponding ω is known as the characteristic relaxation frequency ($\omega_c=2\pi f_c$).

The fitting procedure has been carried out in accordance with the methodology followed in one of our recent research paper [15]. Initially, two frequency regions of the dielectric spectrum were taken for fitting the data. The first frequency region was from 42 Hz to 10 kHz and the second region was from 10 kHz-5 MHz. The values obtained from these two independent fittings were taken as initial values for the final fit. Finally, fitting was carried out for the whole frequency region (42 Hz-5 MHz). In each fitting step, iterations were performed until convergence of the best fit occurred. The lowest possible chi-square values were obtained. The whole data fitted after 20,000 iterations.

Results

In the current study, each component of the bone tissue was extracted in a number of steps and analyzed electrically using electrical impedance spectroscopy.

Table 1 depicts the percentage of calcium removed gradually at each step and was found to be around 2% to 3%. Moreover, the total calcium removed at the end of the demineralization steps was around 20%. In OHAp $\{Ca_{10}(PO_4)_6(OH)_2\}$ calcium contributes about 40% by wt. which confirmed that on 20% removal of calcium, about 50% OHAp might have removed.

Table 1: Assessment of calcium removed at each step of demineralization using Atomic Absorption Spectrophotometer (AAS).

Steps of calcium extraction	Calcium removed (%)
1st	1.6
2nd	3.1
3rd	5
4th	6.5
5th	8.3
6th	10.4
7th	12.2
8th	15.7
9th	17.6
10th	19.7

Dielectric measurements were conducted for all the treated bone samples over the frequency range from 42 Hz-5 MHz. The effect of each component of the bone was evaluated in terms of permittivity and conductivity. For all the treatments at lower frequencies (42 Hz-200 kHz), the decrease in permittivity was found to be strongly frequency dependent, whereas at higher frequencies (200 kHz-5 MHz) the response was almost frequency independent. The permittivity and conductivity values got reduced for acetone treated bones in comparison to the control ones. With each step of demineralization (EDTA1-7), both the permittivity and conductivity showed a decreasing trend until the third last step (**Figure 2**). However, in the last three steps (EDTA8-10), enhancement in the permittivity values was observed.

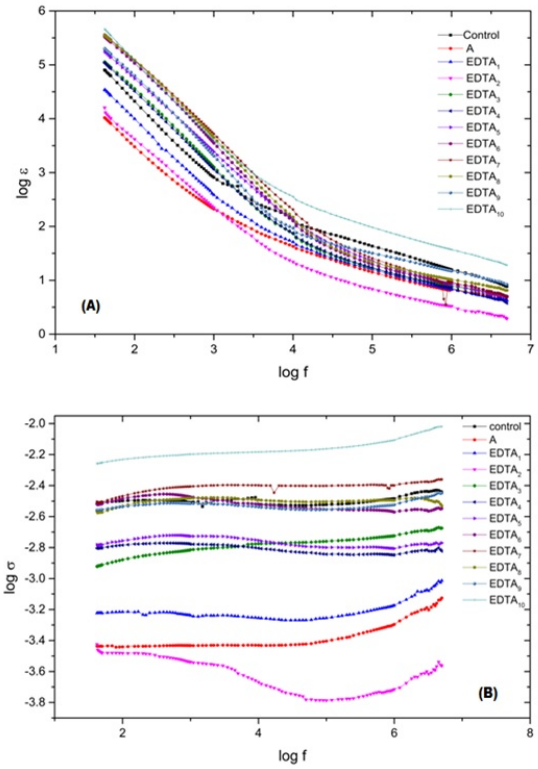

Figure 2: The scatter plot of, A: permittivity and; B: conductivity of bone in different treatment conditions (control, acetone and 10 steps of EDTA) as a function of frequency.

Figure 3 shows the variation in conductivity and permittivity values for dehydrated bone in comparison to the control bone sample. After lyophilization of the bone samples, the impedance values become so high that it was beyond the measurement limit of the LCR i.e. 200 kΩ. These points in the graphs can be

seen as discontinuity. As a result of lyophilization, conductivity reduces sharply with respect to the control bones.

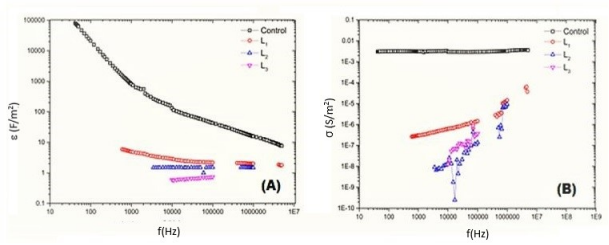

Figure 3: Measured dielectric spectrum (A): for permittivity and; (B): for the conductivity of dehydrated bone.

After obtaining the conductivity and permittivity values for all bone samples, the fitting procedure was performed on the dielectric data using Raicu model [14]. As discussed earlier, the fitting of dielectric data for lyophilized bone could not be carried out due to non-availability of data at many frequencies. Experimental dielectric spectra of the rat bone of the acetone-treated and demineralized bones are shown in **Figures 4 and 5** along with the model based best fit. The best-fit parameters of the dispersion model for different treated bones are compiled and tabulated in **Table 2**.

In the graphs, the open squares and open circles represent the permittivity and conductivity values (**Figure 4**). The parameters De and t of the first dispersion are represented by subscript 1 and second dispersion by subscript 2 (**Table 2**). For the acetone-treated bones, the value ofdecreases and conductivityincreases. Values for,, and decreases in comparison to the control bone.

With demineralization the values ofdecreases till the 7th step and thereafter it increases. Conductivityvalues were found to increase with the demineralization process. In the first dispersion region,decreases and increases. Similarly, in the second dispersion region increases and decreases (**Table 2**).

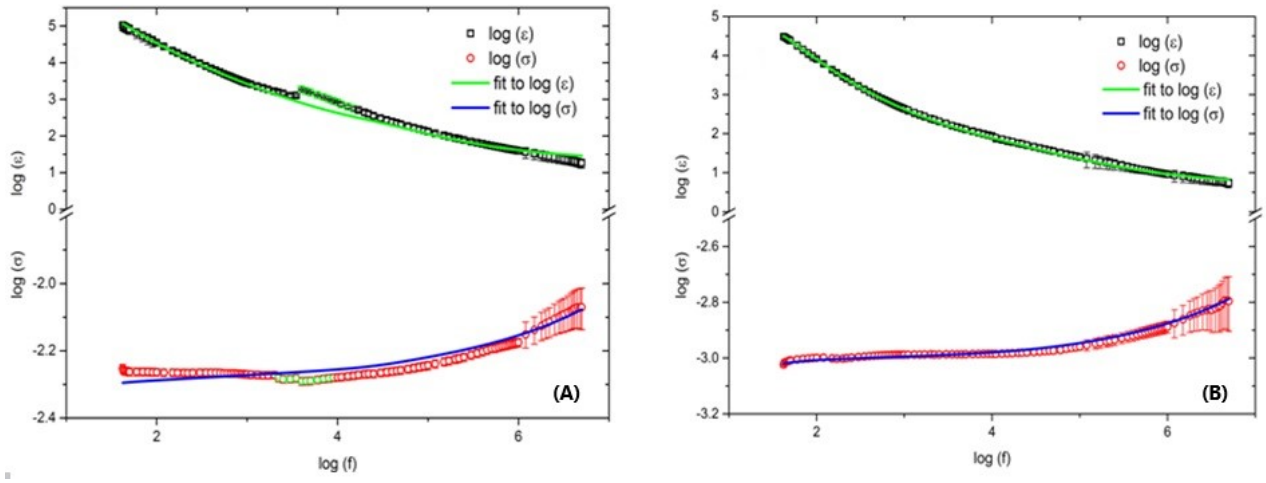

Figure 4: Dielectric spectrum of (a): control rat femur bone; (b): acetone treated, representing permittivity (open squares) and conductivity (open circles) with increase in frequency (f) range. Green and blue line represents the fitted curve of permittivity and conductivity using Raicu model.

The best-fit parameters of the model are tabulated in **Table 2**.

Table 2: Best fitted dielectric parameters of control, defatted and demineralized bone.

Parameters	ϵ_f	σ_i	$\Delta\epsilon_1$	τ_1	$\Delta\epsilon_2$	τ_2
Control	7.3	2.2E-3	1.17E6	8.01E-3	169.65	4.8E-6
Acetone	2.9	0.27E-3	0.92E6	27.5E-3	21.03	11.1E-6
EDTA1	0.4	0.03E-3	0.26E6	16.7E-3	5.52	2.1E-66
EDTA 2	3	0.56E-3	0.15E6	3.7E-3	1.25	0.25E-66
EDTA 3	3.4	0.26E-3	0.44E6	13.03E-3	3.11	0.65E-6
EDTA 4	2.1	0.06E-3	0.72E6	26E-3	10.29	0.15E-6
EDTA 5	4.5	0.45E-3	0.18E6	5.3E-3	0.14	2.8E-6

EDTA 6	2.8	0.92E-3	0.09E6	2.25E-3	2.42	2.11E-16
EDTA 7	3.3	0.87E-3	0.18E6	4.01E-3	2.64	0.09E-6
EDTA 8	7.1	1.57E-3	0.42E6	3.18E-3	0.89	0.56E-6
EDTA 9	5.6	2.7E-3	0.56E6	1.95E-3	5.57	1
EDTA 10	7.1	1.87E-3	0.76E6	2.03E-3	14.43	0.06372

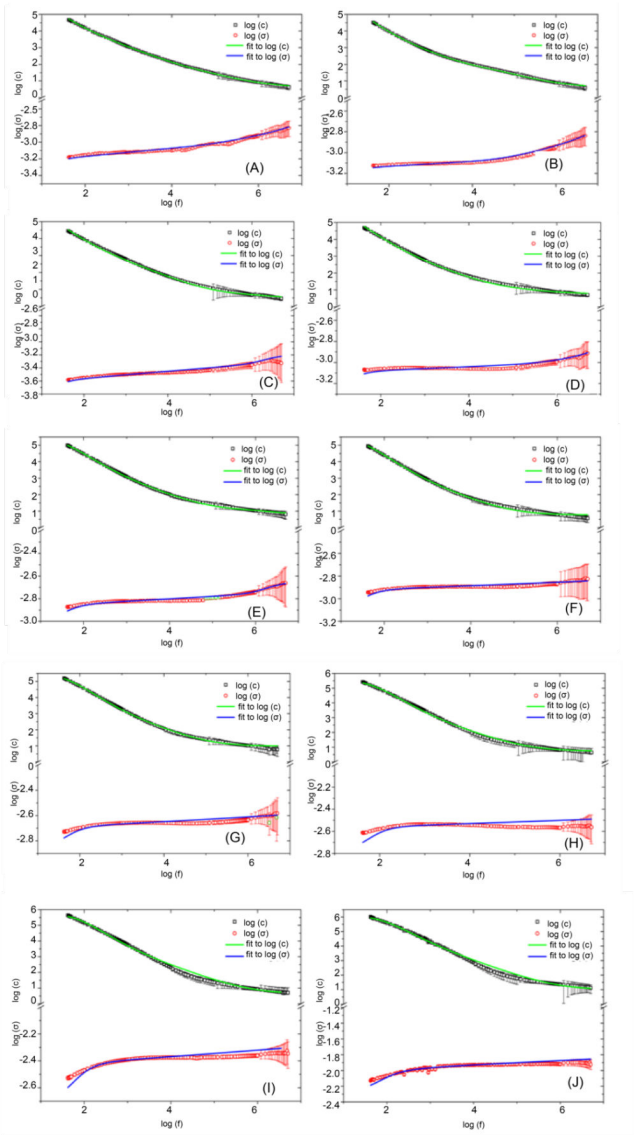

Figure 5: Experimental relative permittivity (open squares) and conductivity (open circles) spectra of the bone in each extraction step [step 1 (A)-10 (J)].

Discussion

In the normal bone, where all the bone constituents were intact, the relative permittivity value ranges from 10^4-10^5 (at frequency 42 Hz) to 10^1 (at 5 MHz) (**Figure 2**).

The frequency independent permittivity (ε_f) value was found to be 7.3 for the control bone (**Table 2**). ε_f represents the instantaneous polarization mechanism. When the initial pulse was given, small molecules (water and electrolytes) get polarized and relax at a higher frequency thereby giving rise to instantaneous polarization. Ionic conductivity (σ_i) value was found to be 2.2E-3 which may arise due to the electrolytic phase of the bone. This phase will be prominent in the bone marrow, Haversian canal and the surface of the moistened bone. Value of $\Delta\varepsilon_1$ and τ_1 was found to be 1.1E6 and 8.01E-3respectively (**Table 2**). These parameters signify alpha dispersion which is due to the bulk effect of all the components of bone in the presence of water. Value of $\Delta\varepsilon_2$ and τ_2 was found to be 169.65 and 4.8E-6 respectively (**Table 2**). These parameters represent the fitted parameters of the beta dispersion region. $\Delta\varepsilon_2$ is due to the organized collagen-mineral matrix or the arrangement and integrity of collagen and hydroxyapatite and it also represents fat content present in the bone marrow and various bone cells. Thus, $\Delta\varepsilon_2$ signifies the tight packing of the mineral component to that of the collagen.

The permittivity and conductivity of the dehydrated bone decrease with each step of lyophilization (**Figure 3**). Similar observations were found by Pethig [16], and Grimness et al. [13]. The decrease in the conductivity and permittivity values could be attributed to the gradual loss of loosely and tightly bound water molecules from the bone tissue. The ions present in the bone tissue are free to move within the water phase which contributes to the electrical and dielectric properties. With bone dehydration, bony matrix acts as a barrier for current flow resulting in lesser conductivity value. The discontinuity in the curves obtained for dehydrated bone has been observed in the frequency regions in which impedance values exceeds the detection limit of the instrument. These frequency regions were from 42 Hz-3 kHz, 120 kHz-400 kHz and 1.2 MHz-5 MHz (**Figure 3**). As a result of discontinuity in the curves, the fitting procedure could not be carried out for dehydrated bone samples.

EDTA extraction method was carried out to dissolve the mineral portion of the bone tissue in a gradual manner. In the final step of extraction protocol, almost all the hydroxyapatite would have been dissolved and the bone collagen portion was left behind. At this time point, the bone tissue becomes very soft because of the absence of the mineral component. In an electric field, the collagen component of the demineralized bone may behave in an almost similar manner as that of any other soft tissue. With demineralization, ionic conductivity increases almost till the last step of EDTA extraction (**Table 2**) and thus could be the outcome of the formation of vacant spaces in the bone tissue as a result of mineral dissolution. These spaces

might be occupied by the water molecules (in the form of buffer) which would have increased the observed conductivity of the bone tissue. However, instantaneous polarization decreases initially until the third last step and then increases till the last step of extraction. This unexpected increase in the polarization could be due to the gradual increase in the flexibility of bone tissue. This flexibility of the bone gives rise to the Electrode Polarization Impedance (EPI). This EPI arises due to the presence of water molecules between the tissue and electrode. The effect was almost negligible in the initial steps as the bone tissue was quite rigid.

The decrease in the dielectric increment $\Delta\varepsilon_1$ (**Table 2**) with demineralization signifies reduced counter ion diffusion due to the decrease in the surface area of the mineralized collagen fibers. Li et al. [17], observed that after mineralization the diameter of the collagen microfibril increases from 2 nm to 7.5 nm to form the mineralized subfibril. The increase in relaxation frequency (τ_1) could be due to the presence of smaller subunits of collagen. These free units have different properties in comparison to the intact collagen. These collagen subunits relax at a very high frequency. $\Delta\varepsilon_2$ parameter also decreases with demineralization which could be attributed to the interfacial polarization. Interfacial polarization is between the interfaces of collagen and hydroxyapatite portion. With demineralization, the interfaces get reduced thereby causing a significant decrease in the $\Delta\varepsilon_2$. Foster et al. [18], also suggested a decrease in interfacial polarization as the tissue integrity gets impaired.

Conclusion

The results of this study revealed that the dielectric parameters of the bone tissue correlated well with its composition. The knowledge of bone electrical properties would enable us to understand the solid state properties of bone constitutes. Thus, Electrical impedance spectroscopy could provide a foundation for subsequent *in vivo* experiments on animal bones. Also, the fitting method described in the current study may be employed in the quantitative evaluation of bone grafts used during bone transplantations. A future study should extend these measurements to *in vivo* bones to determine the structural and compositional changes.

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