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THERMALLY STIMULATED CURRENT STUDY OF THE MOLECULAR MOVEMENTS IN BONE

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Thermally Stimulated Current (TSC) has been used for investigating the interface and interphase in a natural composite, bone. Young adult male femoral diaphysis has been studied at different stages of demineralization by EDTA. Analysis of the fine structure of complex TSC spectra has shown that in the early stages of demineralization, dielectric relaxations are due to the hydroxyl reorientations in the mineral apatite structure. After a certain stage of demineralization, the mineral response disappears and organic matrix mobility is observed. A dissociative buffer has been used to identify collagen relaxations. The contribution of the mineral–organic interface/interphase has been isolated.

Keywords: mineral–organic interface/interphase, thermally stimulated current

Introduction

It is well known that bone is a natural composite: the organic matrix, composed essentially of collagen, is associated with a mineral filler, calcium phosphate (apatite). In previous reports we have shown that the Thermally Stimulated Current (TSC) technique can be used to characterize either dipolar reorientations of stoichiometric [1] and non-stoichiometric [2] apatites or molecular movements of collagen [3, 4]. Hence, this technique has been used to study the complex structure of calcified tissues. The aim of this investigation was to identify the molecular mobility of the mineral–organic interface/interphase.

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Experimental

Sequential extractions were performed on human bone powder. Non-collagenous proteins, as well as soluble collagen, were extracted from 100 g human bone powder (femoral diaphysis), using 10 vols 0.5 *M* EDTA at *pH* 7.4 supplemented with protease inhibitors (6–aminohexanoic acid, 0.01 *M*, and benzamidinium chloride, 0.005 *M*). As shown in Table 1, ten two-day extractions under gentle stirring were followed by a two-day extraction in the presence of 4 *M* guanidinium chloride and a one-day extraction in the presence of 6 *M* guanidi-nium chloride. Supernatants (E_1 to E_{10} and E_{G1} to E_{G2}) were separated from the insoluble residues (R_1 to R_{10} and R_{G1} to R_{G2}) by centrifugation (15 min, 2000 **g**, 4°C). Proteins were precipitated from the supernatants by ammonium sulphate 50% (v/v) in PBS, overnight at 4°C. For further analysis, residues were rinsed five times with ultrapure water and freeze dried.

Table 1 Bone demineralization sequence

Initial products	Extraction time	Extraction middle	Final products
Bone	48 h	EDTA	E_1, R_1
R_1	48 h	EDTA	E_2, R_2
R_2	48 h	EDTA	E_3, R_3
R_3	48 h	EDTA	E_4, R_4
R_4	48 h	EDTA	E_5, R_5
R_5	48 h	EDTA	E_6, R_6
R_6	48 h	EDTA	E_7, R_7
R_7	48 h	EDTA	E_8, R_8
R_8	48 h	EDTA	E_9, R_9
R_9	48 h	EDTA	E_{10}, R_{10}
R_{10}	48 h	EDTA + GuHCl 4M	E_{G1}, R_{G1}
R_{G1}	48 h	EDTA + GuHCl 4M	E_{G2}, R_{G2}

In TSC experiments, a potential of 200 V was applied to the sample for 2 min at 25°C. This ordered configuration was quenched to liquid nitrogen temperature and the electric field was cut off. Return to equilibrium of the sample was induced by a controlled increase of temperature (7 deg·min^{-1}). Simultaneously, the depolarization current was recorded *vs.* temperature to give the 'complex TSC spectrum'.

Results and discussion

TSC spectra of mineral phase

The complex TSC spectrum of bone is shown in Fig. 1. The first spectrum (solid line) corresponds to the air-dried sample and the second spectrum (dashed line) to the dehydrated state obtained after pumping for 12 h under 10^{-5} Torr vacuum. Two peaks can be observed on both spectra: one main peak around 0°C and another peak around −130°C. Since the temperature position of the two peaks does not vary between the two runs, it can be concluded that both relaxation modes are independant of free water. In order to define precisely the origin of these peaks, the complex TSC spectrum was resolved in elementary TSC spectra using fractional polarizations [5]. Analysis of elementary TSC peaks shows that all dielectric relaxation times τ follow an Arrhenius equation:

$$\tau = \tau_o \exp(\Delta H / kT) \quad (1)$$

where τ_o is the pre-exponential factor, ΔH is the activation enthalpy and k is the Boltzmann constant. Moreover, all relaxation times isolated in the lower temperature peak take the same value τ_c at the particular temperature $T_c = 144$°C. The corresponding relaxation times obey a compensation law:

$$\tau = \tau_c \exp(\Delta H / k)(T^{-1} - T_c^{-1})) \quad (2)$$

As shown in Fig. 2 (cross), lg τ_o varies linearly with ΔH.

Analogous behaviour was observed in residues R_1 to R_5 : all elementary processes isolated between liquid nitrogen temperature and 0°C are characterized by relaxation times obeying a single compensation phenomenon with the T_c

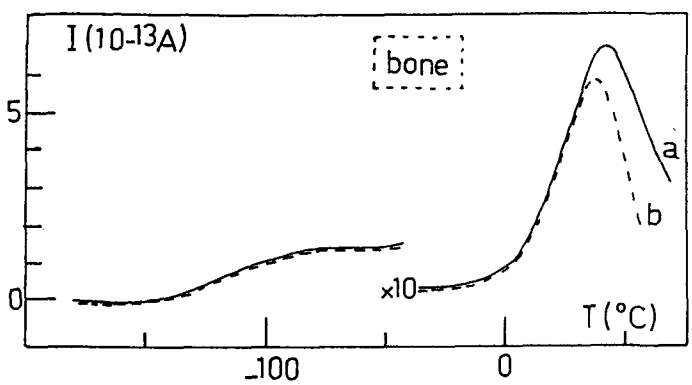

Fig. 1 Complex spectra of bone

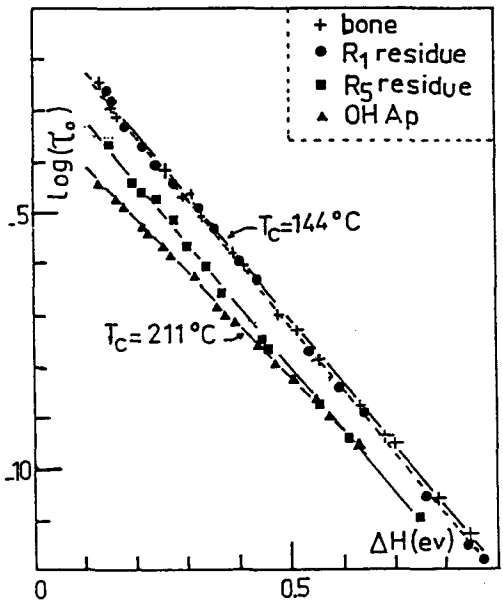

Fig. 2 Relaxation map analysis of bone (+), R_1 residue (•), R_4 residue (■), and hydroxyapatite (▲)

temperature lying in the vicinity of 130°–140°C. As an example, results for R_1 and R_5 residues are reported in Fig. 2 (circles and squares). Such a compensation phenomenon has also been observed in synthetic apatites [1]. In stoichiometric hydroxyapatites the compensation temperature $T_c = 211$°C corresponds to the monoclinic-hexagonal transition. Dielectric energy losses are associated with OH dipole reorientations inside apatite channels.

Comparison between the two compensation temperatures shows that the monoclinic-hexagonal transition is lower in calcified tissues. Complementary studies on synthetic, non-stoichiometric hydroxyapatites [2] have shown that hydroxyl reorientations are facilitated by the presence of foreign ions (carbonates, fluorides, chlorides, ...) and molecules (water) inside channels. Thus the decrease of T_c in bone might be explained by its non-stoichiometric structure. However, comparison between bone, bone residues and hydroxyapatite (cf. Fig. 2 triangles) shows that compensation lines in calcified tissues are shifted towards higher τ_o. The pre-exponential factor τ_o can be linked to the activation entropy by the Eyring relation:

$$\tau_o = h / kT \exp(-\Delta S / k) \tag{3}$$

where h is the Planck constant. The compensation line position indicates that the activation entropy in calcified tissues is lower. The number of accessible sites would be lower in bone than in synthetic stoichiometric hydroxyapatite. This mobility restriction has been associated with the mineral–organic interface/interphase.

TSC spectra of organic phase

After 6 days of demineralization, as shown on Fig. 3, the complex TSC spectrum of bone residue is significantly different from that of bone (Fig. 1). Four different peaks, labelled α', α, β and γ in order of decreasing temperature, are observed. It is important to note first that the intensity of the main peak is significantly lower than that of bone. Second, the β peak shifts towards higher temperature on dehydration. Consequently, after 6 days of demineralization the complex TSC spectrum of the mineral phase vanishes and the dielectric response is due to molecular movements in the organic matrix. Comparison of spectra of mineral and organic phases shows that movements are more complex and weaker in the organic phase. In order to determine the origin of these peaks, they were resolved using fractional polarizations. As shown on Fig. 4 (cross), the elementary processes isolated at temperatures lower than 0°C follow three compensation phenomena labelled β', β and γ. The compensation temperatures deduced from analysis of these lines are respectively 110°, 100° and 110°C. This fine structure of residue 6 confirms that, at advancing stages of demineralization, the dielectric response of the apatite has disappeared. The existence of three compensation phenomena in this residue indicates that this sample is a triphasic material.

Analogous behaviour was observed in residues R_5 to R_{10} : all relaxation processes isolated between liquid nitrogen temperature and 0°C are characterized

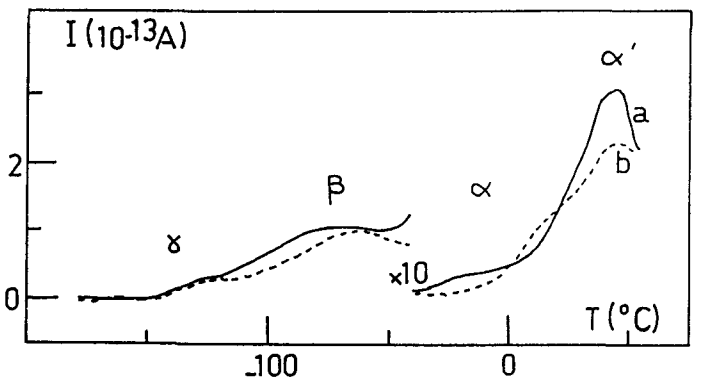

Fig. 3 Complex spectra of organic matrix

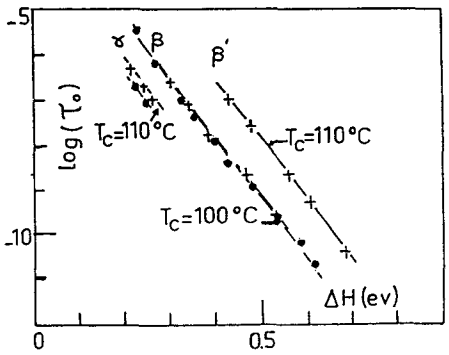

Fig. 4 Relaxation map analysis of organic matrix (+) and bone collagen (•)

by three compensation phenomena with T_c temperatures around 100°–110°C. Conversely, as shown in Fig. 4 (circles), the R_{G1} residue presents only two compensation phenomena. As it is well known that guanidinium chloride isolates collagen from other non-collagenous proteins, the R_{G1} residue can be considered to consist of pure collagen. Since the β' mode has disappeared in this residue, it has been assigned to the non-collagenous proteins present in the organic matrix (proteoglycans, glycoproteins ..). These latter proteins probably ensure the interface between organic collagen and mineral apatite. As for the two other compensation phenomena, previous investigations [3, 4] have shown that they can be associated with the intramolecular mobility of, respectively, 'apolar' and 'polar' sequences of tropocollagen molecules. Since these intramolecular movements are not modified by the presence of other proteins, it seems logical to think that these proteins are located in the gaps of the quarter stagger structure of the collagen.

Conclusion

This study has shown the similarity between synthetic composite and calcified tissue. Bone is composed principally of a collagenic organic phase, reinforced by an apatite mineral filler. Interphase between organic and mineral phase would be ensured on one hand by multiple non-collagenous proteins linked to collagen and on the other hand by defects inside apatite channels. These organic or mineral defects would explain why non-stoichiometric bone apatites have an activation entropy lower than stoichiometric synthetic hydroxyapatite.

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Zusammenfassung — Thermisch erregter Strom (TSC) wurde zur Untersuchung von Grenzflächen und Grenzphasen in der natürlichen Substanz Knochen verwendet. Mittels EDTA wurde die Femoraldiaphyse von jungen erwachsenen männlichen Individuen in verschiedenen Stadien der Demineralisierung untersucht. Eine Analyse der Feinstruktur von TSC-Spektren zeigte, daß in den frühen Stadien der Demineralisierung dielektrische Relaxationen in Verbindung mit der Hydroxyl-Reorientierung in der Apatitstruktur stehen. Nach einem gewissen Demineralisierungsstadium verschwindet die Mineral-Antwort und die Mobilität der organischen Matrix wird beobachtet. Zur Identifizierung von Kollagen-Relaxationen wurde ein dissoziativer Puffer eingesetzt. Der Beitrag von mineral–organischer Grenzfläche/Grenzphase wurde voneinander getrennt.