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Thermostimulated current study of the dynamic behaviour of a biocomposite: calcified tissue [α]

A. Lamure [a], M. Martin [a], M.F. Harmand [b] and C. Lacabanne [a]

[a] *Laboratoire de Physique des Solides, URA/CNRS 74, Université de Toulouse III, 118 route de Narbonne, 31062 Toulouse Cedex (France)*
[b] *Laboratoire d'Evaluation des Matériaux Implantables, Technopole Montesquieu, 33650 Martillac (France)*

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Abstract

Thermostimulated current (TSC) spectroscopy has been applied to the investigation of molecular mobility in human calcified tissue. A comparative study of extracts and residues, at various stages of demineralization, by gel permeation chromatography and TSC spectroscopy, has shown that collagen is not directly linked to apatite: the response of an organic–mineral complex interphase has been identified.

INTRODUCTION

For physical chemists, calcified tissues are considered as natural composites: the organic matrix, essentially constituted of collagen, is associated with a mineral filler, calcium phosphate. The final macroscopic properties are dependent not only upon the characteristics of the matrix and the filler, but also of the matrix–filler interface/interphase. The aim of this work is to identify the molecular mobility of the mineral–organic interface/interphase in human calcified tissue. We have studied extracts and residues of extraction by EDTA of young adult man femoral diaphysis, at various stages of demineralization. By using a highly dissociative buffer (guanidinium chloride), the collagen was separated from strongly linked non-collagenous proteins of the organic matrix. The various residues were studied by thermostimulated current (TSC) spectroscopy. The analysis of residues, at early stages of demineralization, gave the response of the mineral phase. After a given stage of demineralization, the response of the organic matrix was

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observed. At the last stage of extraction, the molecular mobility of collagen was identified.

EXPERIMENTAL

Sequential extractions have been 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, two 48 h extractions under gentle stirring were followed by two 4 day extractions and two 8 day extractions, the last extraction being performed in the presence of 4 M guanidinium chloride. Supernatants (E_1, E_2, E_3, E_4, E_5, E_6) were separated from the insoluble residues (R_1, R_2, R_3, R_4, R_5, R_6) by centrifugation (15 min, 2000g, 4° C). Proteins were precipitated from the supernatants by ammonium sulphate 50% (v/v) in PBS, all overnight at 4° C. For further analysis, residues were rinsed five times with ultrapure water and freeze dried.

In TSC experiments [1], a potential of 200 V was applied to the sample for 2 min at 25° C. This ordered configuration was quenched and the electric field was cut off at liquid nitrogen temperature (LNT). Then, the return to equilibrium of the sample was induced by a controlled increase of temperature (7 K min^{-1}).

Simultaneously, the TSC was recorded versus temperature giving the "complex TSC spectrum". The experiments were performed on a TSC/RMA spectrometer from Solomat.

TABLE 1

Bone demineralization sequence

Initial products	Extraction time	Middle extraction	Final products
R_0	48 h	EDTA	E_1
			R_2
R_1	48 h	EDTA	E_2
			R_2
R_2	4 days	EDTA	E_3
			R_3
R_3	4 days	EDTA	E_4
			R_4
R_4	8 days	EDTA	E_5
			R_5
R_5	8 days	EDTA + GuHCl	E_6
			R_6

RESULTS

Extract analysis

During the first three extractions (E_1, E_2, E_3) 92% of the extractible proteins were extracted as assessed by the method of Lowry et al. [2]. The extracts were analyzed by size exclusion chromatography and the identification of the remaining proteins was carried out by UV spectroscopy at 280 nm. Moreover, the presence of osteocalcin and osteonectin was checked by ELISA [3], using polyclonal antibodies raised against bovine osteocalcin and bovine osteonectin (Oris Industry, Bagnols-sus-Cèze France). The first group of extracted proteins constituted non-collagenic proteins, seric proteins, osteocalcin and osteonectin. Osteocalcin was found principally in E_2 (56%) and E_3 (35%) whereas osteonectin was found mainly in the first three extracts ($E_1 = 34\%$, $E_2 = 37\%$, $E_3 = 18\%$). Neither osteocalcin nor osteonectin was found in E_5 and E_6. The latter extract principally consisted of phosphoproteins (phosphophorin) and matrix Gla proteins.

TSC study of residues

Data on bone powder (designated as residue 0), will be presented first. The complex TSC spectrum is shown in Fig. 1. Two peaks can be observed: one main peak around 0 °C and another peak around −130 °C. This spectrum was complex and was resolved using fractional polarization [1,4]. The analysis of elementary TSC peaks gives dielectric relaxation times following the Arrhenius equation

$$\tau = \tau_0 \exp \Delta H/kT$$

where τ_0 is the pre-exponential factor and ΔH the activation enthalpy. Moreover, all the relaxation times, isolated in the lower temperature peak,

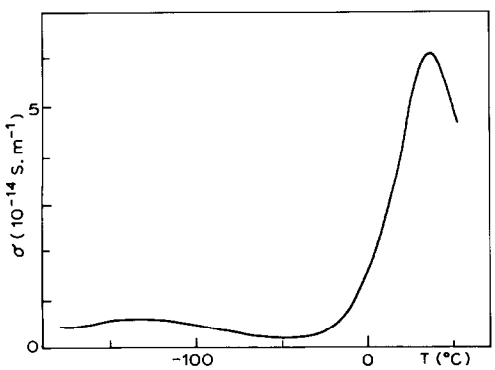

Fig. 1. Complex spectrum of residue 0.

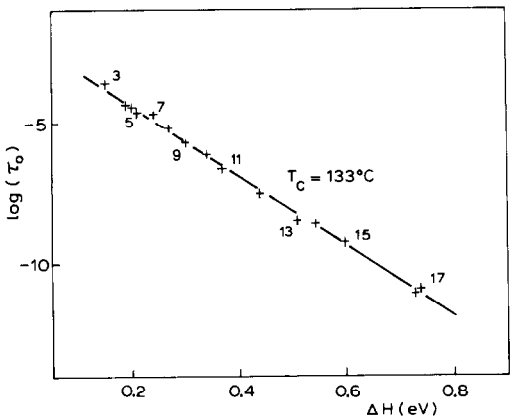

Fig. 2. Compensation diagram τ_0 versus ΔH of residue 0.

take the same τ_c value at the particular temperature $T_c = 133°C$. The corresponding relaxation times obey a compensation law

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

In this case, ln τ_0 varied linearly with ΔH as shown in Fig. 2.

Such a compensation phenomenon has also been observed in synthetic and natural hydroxyapatites [5,6] where it has been attributed to the reorientations of the OH dipoles in the channels responsible for the monoclinic–hexagonal transition. By analogy with hydroxyapatites, the relaxation mode observed in bone has been associated with the dipolar reorientations in apatitic channels. Analogous behaviour has been observed in residues 1–4: all elementary processes, isolated between liquid nitrogen temperature and 0°C, are characterized by relaxation times obeying a single compensation phenomenon with T_c lying in the vicinity of 130°C. Thus at the first

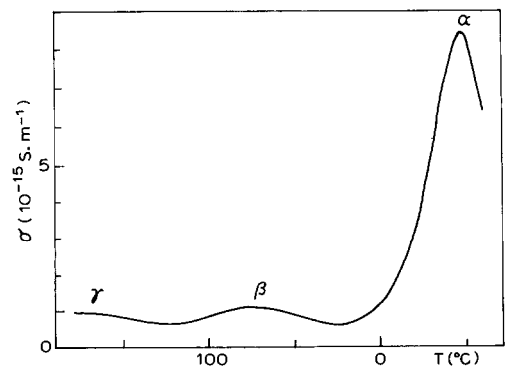

Fig. 3. Complex spectrum of residue 5.

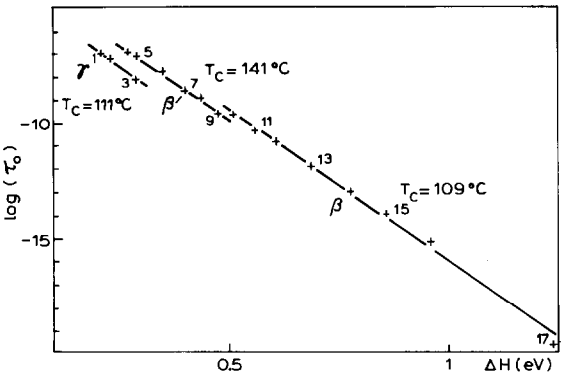

Fig. 4. Compensation diagram τ_0 versus ΔH of residue 5.

stages of demineralization, only the dielectric response of the mineral phase is observed.

At a more advanced stage of demineralization, as shown in Fig. 3, the TSC spectrum of residue 5 is significantly different from that of residue 0 (Fig. 1). Three different peaks, labelled α, β and γ in order of decreasing temperature, are observed. It is also important to note that the intensity of the main peak is significantly lower than that of residue 0. This complex spectrum has been resolved by fractional polarization. As shown in Fig. 4, the elementary processes isolated at temperatures lower than 0 °C follow three compensation phenomena labelled β', β and γ. The compensation parameters defined by eqn. (1) are for $\beta' T_c = 141$ °C and $\tau_c = 1.5 \times 10^{-4}$ s, for $\beta T_c = 109$ °C and $\tau_c = 1.4 \times 10^{-3}$ s and for $\gamma T_c = 111$ °C and $\tau_c = 5.7 \times 10^{-5}$ s.

The fine structure of residue 5 indicates that, at advancing stages of demineralization, the dielectric response of the apatite has disappeared. The observed dipolar reorientations are due to the organic matrix and the

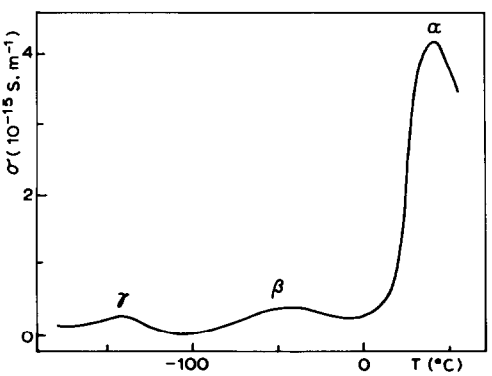

Fig. 5. Complex spectrum of residue 6.

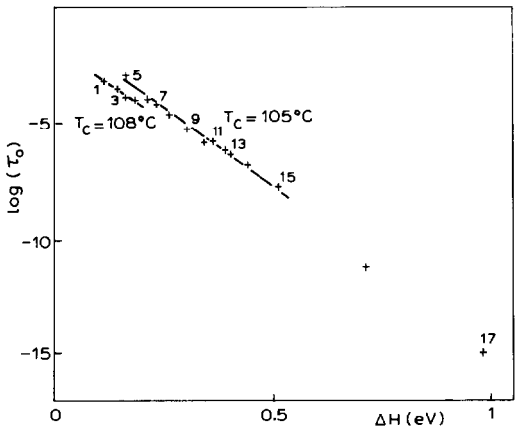

Fig. 6. Compensation diagram τ_0 versus ΔH of residue 6.

existence of three compensation phenomena indicates that this residue is a triphasic material.

For the last stage of extraction, the complex TSC spectrum of residue 6 (Fig. 5) is quite analogous to that of residue 5 (Fig. 3), but as shown in Fig. 6, the relaxation time diagram is characterized by only two compensation phenomena β and γ in order of decreasing temperature. The compensation parameters, defined by eqn. (1), are as follows: for $\beta T_c = 105\,^\circ C$ and $\tau_c = 2.1 \times 10^{-2}$ s, and for $\gamma T_c = 108\,^\circ C$ and $\tau_c = 7.6 \times 10^{-2}$ s.

This behaviour is analogous with that observed for type I collagen [7]. From previous work [4,7], the γ and β lines, associated with the γ and β relaxation modes, have been respectively attributed to the intramolecular mobility of "apolar" and "polar" sequences of tropocollagen molecules. Since the β' mode has disappeared in the last residue, it has been assigned to the interface between collagen and non-collagenous proteins.

DISCUSSION

The results obtained on human calcified tissue, by gel permeation chromatography on extracts and by TSC on residues, show that proteins which disappear during the first stages by demineralization (non-collagenous proteins, osteocalcin, osteonectin) do not modify the mobility of the mineral phase. As the dipolar reorientations in the mineral phase have been attributed to ionic movements inside channels, these proteins cannot be located in the vicinity of the apatitic domains. It is also important to note that only osteonectin is found in extract E_1 and is hence less strongly linked to the mineral phase than osteocalcin which appears principally in E_2.

The fine structure of the TSC spectra of residues 0–4 shows the existence of a single compensation phenomenon with a compensation temperature

approximately equal to 130 °C, while in stoichiometric hydroxyapatites, the compensation temperature was T_c 210 °C. This drastic decrease of T_c in the mineral phase of calcified tissue can be attributed to foreign ions (carbonate, fluoride, chloride, etc.) or molecules (water) in the channels; the organic matrix might also play a significant role.

Previous study [8] has shown that phosphoproteins appear only after several days of demineralization. Simultaneously, the pattern of the mineral phase disappears. Hence phosphoproteins must be strongly linked to the apatitic domains and the interaction may be ensured by the polar orthophosphate groups. This result confirms the strong Ca^{2+} ion affinity with proteins attributed to residues of γ-carboxyglutamic acid [9,10].

As collagen consists of "apolar" and "polar" sequences, it can be considered as a block-copolymer. The fine structure of collagen shows that relaxations of both "apolar" and "polar" sequences are cooperative: these movements are the dielectric manifestation of a double glass transition around 100 °C. This high transition temperature shows that, at room temperature (and even at physiological temperature), the collagen is a stiffened matrix.

In an organic matrix, analysis of TSC experiments shows that the γ and β modes, associated respectively with the "apolar" and "polar" movements, have the same characteristics as those found in collagen. Hence, the intramolecular mobility of collagen is not modified by the presence of proteins. By contrast in a preceding study, we have shown that strongly bound proteins modified the intermolecular mobility of collagen; these proteins are probably located in the gaps of the quarter-stagger structure of the collagen.

CONCLUSION

This study has shown the similarity between composite and calcified tissue: collagen can be considered as the organic matrix and the apatite as the mineral filler. A first group of proteins including osteonectin, osteocalcin and phosphoproteins, is bound to the apatite. By contrast at the last stage of demineralization, interactions of collagen with remaining proteins have been found. Thus the interface/interphase of the biocomposite is ensured by multiple proteins.

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