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Chain dynamic of calcified tissue

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Thermally stimulated current 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 is presented. Results show that:

- the matrix (collagen) is in a glassy state at physiological temperature;
- the filler (apatite) increases the static modulus;
- the interfaces/interphase (non-collagenous proteins and particularly proteoglycans) ensure cohesion and ductability for the composite.

Biomaterials for orthopaedic prostheses require the same morphology in order to phenomenologically reproduce the same dynamic behaviour.

1. Introduction

The development of second generation implants requires a thorough knowledge of the dynamic behaviour of calcified tissue. It is well known that the matrix is constituted of collagen, and reinforced by an apatitic mineral charge; various non-collagenous proteins (proteoglycans or glycoproteins: osteonectin, osteocalcin, phosphoproteins) have also been detected [1–6]. The specific mineral phase confers on the bone not only a mechanical role but also a biological role: bone contains the main ions (calcium, magnesium, sodium, phosphorus) which control certain physiological functions. In a composite, the final properties are not only dependent upon the properties of each component but also of the molecular interfaces between the various constituents. Several studies have been performed on the static modulus of bone but the chain dynamics of calcified tissues is still not understood. The aim of this work is to characterize bone dynamics and to define the nature of the organic/mineral interphase/interface. Extracts and residues of extraction by EDTA, at various stages of demineralization, have been studied by biochemical and biophysical methods (ELISA, radioimmunology, infrared spectroscopy, thermally stimulated current). The relaxation times of residues at various stages of demineralization have been analysed to define the characteristics of the distribution functions and to establish a dynamic model of calcified tissue.

2. Materials and method

2.1. Human bone powder sequential extraction

Non-collagenous proteins from bone, as well as soluble collagen, were extracted from 20 g of human bone powder (radius and cubitus), by sequential extraction using 10 vols EDTA 0.5 M, pH 7.4, supplemented with proteases inhibitors (6-amino hexanoic acid 0.01 M, benzamidinium chloride 0.005 M). As shown in Table I, ten 48 h extractions under agitation (E1 → E10) were followed by two 48 h extractions (EG1, EG2) with EDTA supplemented by 4 M guanidinium chloride. Finally, the two last extractions (EG3, EG4) were performed in the presence of 6 M guanidinium chloride. Supernatants were separated from the insoluble residues (R1 → R10, RG1 → RG4) by centrifugation (15 min, 2000 g, 4 °C). Proteins were precipitated from the supernatant 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.

2.2. Thermally stimulated current (TSC) spectroscopy

In TSC experiments, a potential of 200 V was applied to the sample, placed between the plates of a condensor, for 2 min at 25 °C. This out-of-order configuration was quenched and the electric field 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 depolarization current was recorded versus temperature giving the "complex TSC spectrum".

For bone and bone residues, the TSC spectra were resolved into elementary spectra, i.e. well described by a single relaxation time τ using the fractional polarization method [7]. The fractional polarization experimental procedure was as follows: the field was applied at T_p (the polarization temperature) for 2 min allowing orientation of mobile units with relaxation times $\tau < \tau(T_p)$. Then the temperature was lowered to $T_d = T_p - 10$ °C, when the electric field was cut off

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TABLE I Bone demineralization sequence (48 h extraction time)

Initial products	Extraction buffer	Final products
Bone	EDTA	E_1, R_1
R_1	EDTA	E_2, R_2
R_2	EDTA	E_3, R_3
R_3	EDTA	E_4, R_4
R_4	EDTA	E_5, R_5
R_5	EDTA	E_6, R_6
R_6	EDTA	E_7, R_7
R_7	EDTA	E_8, R_8
R_8	EDTA	E_9, R_9
R_9	EDTA	E_{10}, R_{10}
R_{10}	EDTA + GuHCl 4M	EG_1, RG_1
RG_1	EDTA + GuHCl 4M	EG_2, RG_2
RG_2	EDTA + GuHCl 6M	EG_3, RG_3
RG_3	EDTA + GuHCl 6M	EG_4, RG_4

and the temperature kept constant for $t = 2$ min, allowing the return to equilibrium of mobile units with relaxation times $\tau < \tau(T_d)$. Hence, the cycle used to obtain TSC "elementary" spectra permits recording of an isolated process with relaxation times τ such that $\tau(T_d) < \tau < \tau(T_p)$. By shifting T_p along the temperature axis, the whole TSC spectrum can be explored. The narrow temperature window $T_p - T_d$ effectively selects out oriented dipoles with practically the same activation enthalpy ΔH and the same characteristic relaxation times τ_0 so that each elementary TSC spectrum may be analysed, as it arises from a single Debye relaxation process. The relaxation time depends on temperature according to the Arrhenius equation:

$$\tau = \tau_0 \exp \frac{\Delta H}{kT} \quad (1)$$

where k is the Boltzmann constant, and

$$\tau_0 = \frac{h}{kT} \exp - \frac{\Delta S}{k} \quad (2)$$

where h is Planck's constant and ΔS is the activation entropy. In apatites and collagen, some elementary processes have a particular behaviour: a linear relationship exists between the Arrhenius factors $\log(\tau_0)$ and ΔH. So the corresponding relaxation times are linked by a compensation law:

$$\tau = \tau_c \exp \frac{\Delta H}{k}\left(\frac{1}{T} - \frac{1}{T_c}\right) \quad (3)$$

with

$$\tau_c = \tau_0 \exp \frac{\Delta H}{kT_c} \quad (4)$$

This compensation effect between the activation enthalpy and entropy can be explained on the basis of the two sites model proposed by Hoffman *et al.* [8]. Molecular movements are hierarchically correlated. This dependence of activation parameters, widely observed in polymers [9], is characteristic of the dynamics of a transition.

3. Results

3.1. Biochemical analysis of extracts

During the first three 48 h extractions, respectively 48%, 14% and 5% of the EDTA-soluble proteins were extracted as assessed by the method of Lowry *et al.* [10]. As for the calcium determination by atomic absorption, it was shown that practically all the calcium disappears during the three first extractions. These results show that 3×48 h are not sufficient to extract all of the EDTA-soluble proteins, although the mineral phase is dissolved. The three first extracts ($E_1 \rightarrow E_3$) contain proteins which are probably bound to the mineral phase whereas the seven last extracts ($E_4 \rightarrow E_{10}$) are certainly constituted by proteins linked to the collagen.

Moreover, to define the origin of non-collagenic proteins, the extracts were analysed by size exclusion chromatography and the identification of the remaining proteins was carried out by u.v. spectroscopy at 280 nm. The presence of osteonectin was checked by ELISA [11], using polyclonal antibodies raised against bovine osteonectin (Cis Bioindustry, Gif sur Yvette/France). Finally, the presence of osteocalcin was measured by radioimmunology. Osteocalcin was found principally in E1 and E2 (57 and 29%), confirming its high affinity for hydroxyapatite observed *in vivo* [12]. In addition the last extracts with guanidinium chloride consisted principally of phosphoproteins (phosphorin) and matrix-Gla-proteins [13].

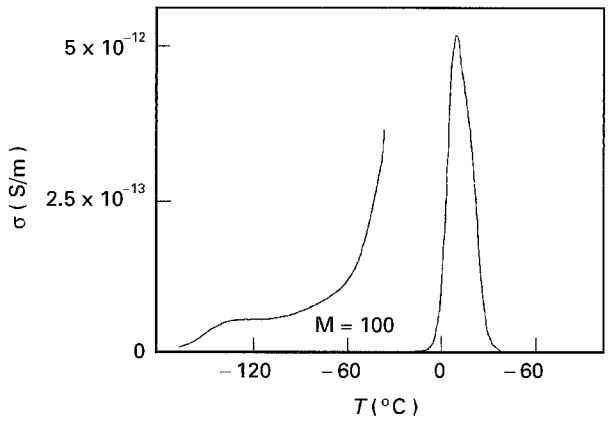

Figure 1 Complex spectrum of bone.

3.2. Biophysical analysis of residues

Three groups of spectra can be distinguished, corresponding to:

- bone
- residues obtained after demineralization with EDTA
- residues obtained after demineralization with EDTA + Glu HCl.

3.2.1. Bone

TSC spectra of bone contain two broad peaks: one main peak around room temperature and another peak around $-130\,^{\circ}C$ (Fig. 1). In order to determine their origin, the complex TSC spectrum was resolved into elementary TSC spectra using fractional polarization. All TSC spectra were resolved into elementary TSC spectra using fractional polarization. All elementary processes isolated between nitrogen temperature

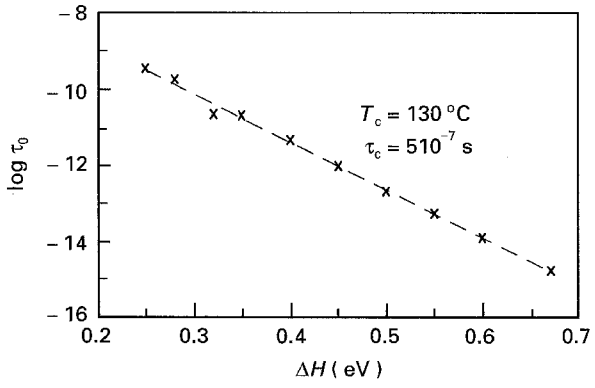

Figure 2 Compensation diagram of bone.

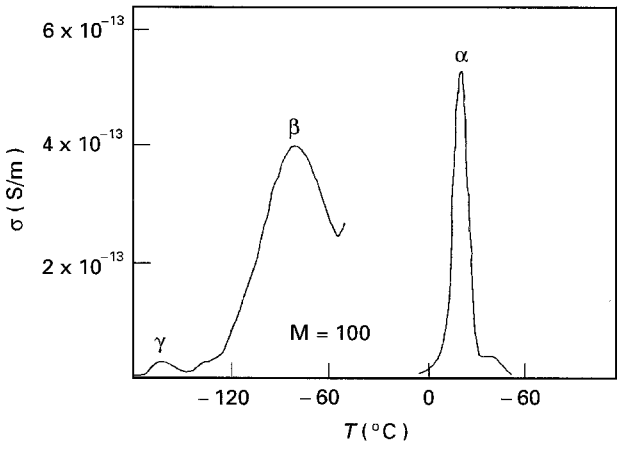

Figure 3 Complex spectrum of R1 residue.

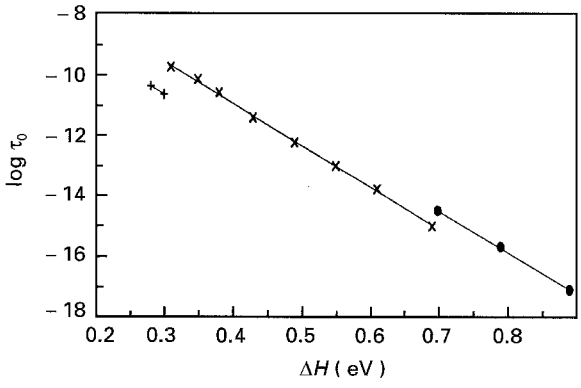

Figure 4 Compensation diagram of R1 residue: + γ(T_c = 100 °C; $\tau_c = 1 \times 10^{-7}$ s); × β′ (T_c = 90 °C; $\tau_c = 5 \times 10^{-6}$ s); ● β(T_c = 100 °C; $\tau_c = 1 \times 10^{-5}$ s).

and 0 °C are characterized by relaxation times obeying a single compensation phenomenon with a T_c compensation temperature lying in the vicinity of 130 °C and a compensation time $\tau_c = 5 \times 10^{-7}$ s (Fig. 2). Such a compensation phenomenon has also been observed in synthetic apatites [14]. In stoichiometric hydroxyapatites the value of the compensation temperature T_c = 211 °C corresponds to the monoclinic–hexagonal transition temperature. Hence, dielectric energy losses have been associated with OH dipole reorientations inside apatite channels.

Comparison between the bone and hydroxyapatite compensation temperatures shows that the monoclinic–hexagonal transition is lower in calcified tissues. Complementary studies on synthetic, non-stoichiometric hydroxyapatites [15] 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. Moreover, comparison between bone and hydroxyapatite compensation times (7×10^{-4} s in hydroxyapatite) shows that in calcified tissues the Arrhenius pre-exponential factors are shifted towards higher τ_0. As the pre-exponential factor τ_0 can be linked to the activation entropy by the Eyring relation (Eqn. 2) the compensation time values indicate that the activation entropy in calcified tissues is decreased. The number of accessible sites would be lower in bone than in synthetic stoichiometric hydroxyapatite. This mobility restriction can be associated with the mineral–organic interface.

3.2.2. Demineralization with EDTA

TSC spectra obtained at the early stages of demineralization are significantly different from those of bone. It is important to note first that the magnitude of the peak located around room temperature is decreased by a factor of ten, compared with bone. Second, the residues dielectric responses below 0 °C are different from the bone response. As shown on Fig. 3, two peaks are observed below 0 °C; these peaks, labelled β and γ, are located around −150 and −80 °C, respectively. Note that the lower temperature peak obtained in bone at around −130 °C has disappeared. Consequently, at the early stages of demineralization the complex TSC spectrum of the mineral phase vanishes and the dielectric response must be associated with molecular movements of 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 also were resolved using fractional polarizations. 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 temperatures deduced from analysis of these relaxation processes are, respectively, 100, 90 and 100 °C. This fine structure confirms that in the early 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 is observed in residues R2 to R10: all relaxation processes isolated between liquid nitrogen temperature and 0 °C are characterized by three compensation phenomena with T_c temperatures around 100 °C.

3.2.3. Demineralization with EDTA + GuHCl

For the last stages of extraction, the complex TSC spectra of the residues (RG1 → RG4) are analogous to those of the previous residues (cf. Figs 3 and 5). However, as shown on Fig. 6, analysis of relaxation times

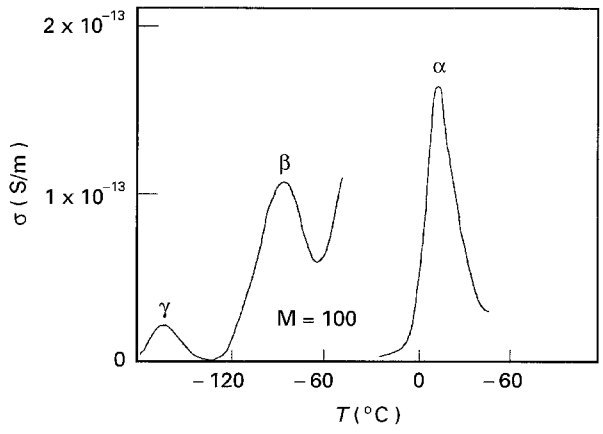

Figure 5 Complex spectrum of RG1 residue.

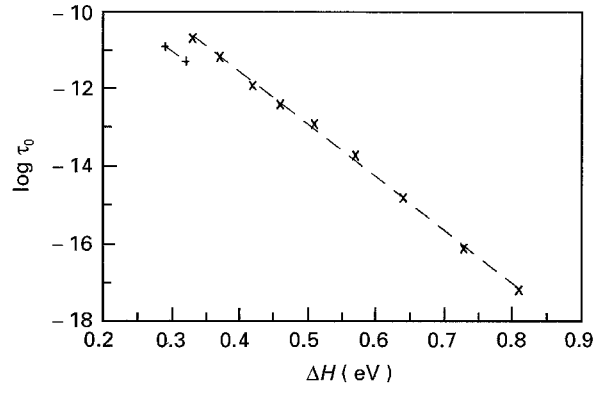

Figure 6 Compensation diagram of RG1 residue: + $\gamma(T_c = 100\,°C;\ \tau_c = 1 \times 10^{-7}\,s)$; × β $(T_c = 100\,°C;\ \tau_c = 1 \times 10^{-6}\,s)$.

shows that the RG1 residue presents only two compensation phenomena. As it is well known that guanidinium chloride isolates collagen from other non-collagenous proteins, the RG1 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, etc.).

These latter proteins probably ensure the interface between organic collagen and mineral apatite. As for the other β and γ compensation phenomena, previous investigations [16] 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.

4. Discussion

4.1. Dynamic mechanical properties of the filler

The TSC spectra of bone and bone residues at the first stage of demineralization show the existence of only one compensation phenomenon. The correlation of all relaxation times isolated below 0 °C, suggests the existence of local movement precursors of the solid–solid transition above 100 °C. It is important to recall that the temperature and parameters are characteristic of, respectively, the barrier jump energy and the activation entropy of the system, so the comparison with stoichiometric hydroxyapatite (HA) compensation parameters indicates that, in calcified tissues, defects are located inside channels. The presence of intracrystalline defects underlines the existence of a mineral–organic interface.

The mechanical static modulus of apatite and bone being of the same order of magnitude ($7 < E(\text{GPa}) < 30$ for bone and $40 < E(\text{GPa}) < 110$ for HA) the role of the filler seems principally to increase the mechanical properties of the connective tissues. But our experiments show that to phenomenologically reproduce the same behaviour the filler must be linked to the matrix.

4.2. Dynamic mechanical properties of the matrix

In calcified tissues, collagen forms the matrix of the composite. The relaxation map analysis of collagen indicates that γ and β localized movements of, respectively, "apolar" and "polar" sequences have relaxation times which converge at around 100 °C. This result shows that collagen is characterized by a glass transition around 100 °C and consequently, that collagen is in the glassy state at physiological temperature.

So, when collagen is replaced by another polymeric material, it is important to ensure that the polymer is thermostable. Polymer families such as polyethylene, polyamide, polymethylmethacrylate, polyetheretherketone, generally used in biomaterials satisfy this condition.

4.3. Matrix/filler interphase

In calcified tissues the collagen/apatite interphase is not direct. Multiple proteins ensure the linkage between organic and mineral phases. Results of this work confirm that some proteins, such as osteocalcin, are preferentially bound to the apatite, while other proteins, such as matrix-Gla proteins, are principally linked to the collagen. These proteins ensure the cohesion of the composite but numerous other protein components, such as proteoglycans, have also been detected [1–6]. In fact, in earlier work [17] we demonstrated that cartilage proteoglycans are characterized by a glass transition at around −50 °C. This result shows that proteoglycans play the role of elastomeric material. Moreover, as shown on Fig. 7, extrapolation of the relaxation times towards physiological temperatures indicates that straight lines cut the 100 Hz frequency. As this frequency corresponds to the average shock frequency, these experiments confirm that proteoglycans serve as shock absorbers in connective tissues. As in a synthetic composite, the elastomer ensures ductility of the tissues.

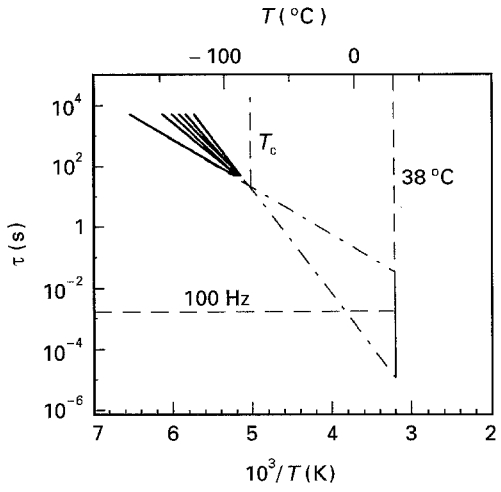

Figure 7 Relaxation map of proteoglycans.

5. Conclusions

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.

From a physicochemical point of view, composites for bone replacement must be biphasic material, the filler reinforces the thermoplastic matrix and the cohesion and ductility are ensured by elastomers. The filler role seems to increase the static modulus while the elastomer could play the role of adapting dynamic strain to the stress.

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