# REHYDRATION MECHANISMS IN COLLAGEN AS SEEN BY THERMALLY STIMULATED CURRENT

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**Abstract**

The objective of this work is to suggest a quantitative model of natural skin aging. This model is expected to carry out tests for pharmaceutical or cosmetic products to achieve skin firmness purposes. We have defined an experimental protocol to highlight mechanism of rehydration and molecular mobility of collagen. Thermally stimulated currents in skin collagen have been investigated in the temperature range of -180°C to 60°C. We highlighted principally two types of molecular movements. α relaxation mode at approximately 20°C is associated with intermolecular mobility of tropocollagen molecules. Intermolecular mobility of polar sequences characterized by β relaxation mode is highly sensitive to hydration. This mode shows in the hydrated state two components: β1 and β2. During dehydration, there is firstly a significant decrease in the intensity of α and β1 modes and the disappearance of the β2 sub-mode. Finally, γ mode registered at -160°C is associated with non-polar amino acids.

**Keywords:** Thermally stimulated current, collagen, TSDC method

## Introduction

The connective tissue is the subject of much research due to the complexity of its structure but also to the importance of its functionality. Improved knowledge in this area is based firstly on the progress of biochemistry and secondly on the introduction of new techniques that have been adapted to the characterization of these materials. We are interested in the problem of skin aging. In this approach, the collagen, which is the main component of the skin and responsible for its structural integrity and mechanical strength, has been the subject of our investigations.

Thermally stimulated depolarization currents (TSDC) measurements are used to characterize our material. Since Van Turnhout's pioneering works (J. Van Turnhout 1973), TSDC has been frequently employed to investigate the molecular motions in polymeric materials and biomaterials (M.T. Viciosa and al. 2010; M. Arnoult and al. 2007; V.M. Gun'ko and al. 2009; Nery, S and al. 2001). The high interest in TSDC technique is due to the low equivalent frequency of about $10^{-3}$ Hz (A. Gourari and al. 1985)and its capability to resolve complex dielectric transitions.

**Experiment**

**Experimental part**

Before extraction, samples of skin are shaved and cut into small pieces. Neutro-soluble collagen is extracted in a buffer of 1M NaCl/0.05M tris/PH 7.2 for 24 hours at 4 °C. After centrifugation, the residue is suspended in a solution of 0.5 M acetic acid and digested with pepsin for 24 hours at 4°C. The insoluble material is separated by centrifugation (10.000 g x 1 h) and supernatant is fractionated by adding 0.7 M NaCl. Thus we will have in the final residue, practically only type I collagen (localized in the skin, the bones and the tendons) and type III collagen (located in the fetal skin and vessel walls. Hydroxyproline rate is high with the presence of cysteine). The percentage of collagen obtained is 80%. This percentage remains practically constant from 3 weeks of the animal life.

The sample of collagen to be studied is compressed in the form of 8mm diameter pellet and about 0.5 mm thick. Two states were studied: The "hydrated" sample represents the lyophilized collagen pumped only in primary vacuum and for a time less than 15 minutes. The "dehydrated" sample is placed in the cell in an atmosphere of helium and then heated at 120 °C for 10 min.

TSDC measurements were carried out with an apparatus developed in our laboratory from the concept outlined by Bucci and Fieschi (C. Bucci and al. 1966.) and described elsewhere(N. Benrekaa and al. 2006; N. Benrekaa and al. 2004). The sample with electrodes shorted is located in the TSDC measurement chamber, which was maintained at a constant, and specified pressure of $10^{-3}$ mbar with an inert $N_2$ atmosphere to avoid parasitic effects such as oxidation or possible degradation processes following repeated heating-cooling sequences. The current was measured with an electrometer (Keithley 617) and recorded by using X–Y. The electrometer was also coupled to a PC for data collection. A platinum temperature sensor Pt100, mounted in the sample holder and adjacent to the film, allowed the temperature measurement with a precision of 0.05°C. The heating rate of 8°C/min was used and controlled by a temperature regulator (BT300/302 CLTS). The complex TSDC spectrums

were obtained by submitting the samples to a constant electrical field Ep=$10^6$ V/m during 2 min at a polarization temperature of Tp=25°C. The sample is then cooled, with a ramp of 20 °C/min, to liquid nitrogen temperature in the presence of the electrical field. The field was then suppressed at around -180 °C and the electrodes were short-circuited for 5 min, finally the sample was heated at 8 °C/min to 80°C. All measurements were repeated to verify the reproducibility and the accuracy of the results.

**Basic considerations for peak analysis**

In general, the buildup of polarization at a constant temperature T can be described by the equation (C. Lacabanne and al. 1994)

$$P(t) = P_0\left[1-\exp\left(-t/\tau\right)\right] \tag{1}$$

where τ and P0 are respectively, the relaxation time of dipoles and the maximum amount of polarization possible at temperature T with,

$$P_0 = \left(N\mu^2 E_p / 3k_B T\right) \tag{2}$$

where N is the concentration of dipoles, Ep is the local electric field and μ is the dipole moment which is independent of temperature, kB is Boltzman's constant .

We may represent the temperature variation of the relaxation time τ by the Arrehinus type equation (C. Lacabanne and al. 1994)

$$\tau\left(T\right) = \tau_0 \exp\left(w/k_B T\right) \tag{3}$$

where $\tau_0^{-1}$ is the characteristic frequency factor for a vacancy jump from a lattice site to another for the orientation of the dipoles and is independent of temperature, w is the activation energy.

The current density released during a thermally stimulated measurement can be expressed (C. Lacabanne and al. 1994)

$$J'(T) = -\frac{N\mu^2 E_p}{\tau_0 3k_B T}\exp-\left(-\frac{w}{k_B T}+\frac{1}{\beta\tau_0}\int_{T_0}^{T}\exp\left(-\frac{w}{k_B T}\right)dT'\right) \tag{4}$$

where β =dT/dt is the heating rate.

By introducing
$$J'\left(T\right) = P_0 J(T)/P(T) \tag{5}$$

We obtain
$$J'\left(T\right) = \text{constant} - \left(w/k_B T\right) \tag{6}$$

The plot of ln(J′) as function of 1/kBT is a straight line, whose slope gives the value of w and ln(τ0).

For the determination of the characteristic parameters of the process, i.e., the activation energy and the pre-exponential factor, the initial rise method is mostly used.

The integral in Eq.(4) can be approximated as an asymptotic expansion (C. Lacabanne and al. 1994)

$$\ln J(T) = \ln A - \frac{w}{k_B T} - \frac{B}{\beta}\int_{T_0}^{T} \exp\left(\frac{-w}{k_B T'}\right) dT' \tag{7}$$

where $A = P_0 / \tau_0$; $B = 1/\tau_0$ (8)

by introducing

$$I = \int_{T_0}^{T} \exp\left(\frac{-w}{k_B T}\right) dT \cong \left\{ T E_2\left(\frac{w}{k_B T}\right) - T_0 E_0\left(\frac{w}{k_B T_0}\right)\right\} \tag{9}$$

where En(z) is the integral exponential function (M. Abramowitz and al.1970) given by

$$E_n(z) = \int_1^{\infty} \frac{e^{-zt}}{t^n} dt \quad , \qquad \mathrm{Re}(z)>0 \tag{10}$$

we obtain

$$\ln j(T) \cong \ln A - f - \frac{BT}{\beta f}\left(1 - \frac{2}{f} + \frac{6}{f^2} - ...\right)\exp(-f) \tag{11}$$

with f=w/kBT (for T<Tg) or f=w/kB(T-T∞) (for T>Tg)

The use of Eq.(11) presents an advantage of using more experimental points particularly in the region where the current reaches its maximum. From the values obtained for A, w and B from the fitting, the characteristic parameters of the process may be determined.

The relaxation time associated with an elementary peak may be deduced from

$$\tau(T) = \frac{P(T)}{J(T)} \tag{12}$$

**Results**

Figure 1 represents the complex TSC spectrum of collagen and its modification according to hydration.

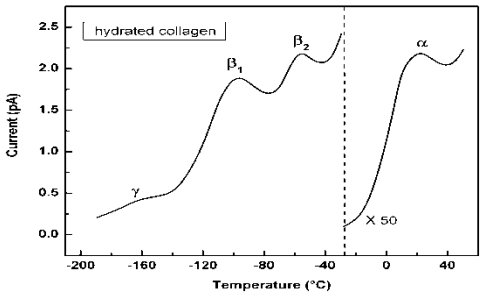


**Figure 1.** TSC spectrum from −180 to 50 °C in inert N2 gas for hydrated collagen.

The complex spectrum was obtained in the hydrated state (10%) after polarization at room temperature. It highlights the existence of three peaks of energy losses which express dielectric relaxation modes.

The intensity of α mode, observed at 30 °C, is about one fifteen times higher than that of modes β and γ. The β mode in hydrated state shows two components $\beta_1$ and $\beta_2$ observed respectively at -60 °C and -100 °C. At last, a lowest γ mode is observed at -160 ° C.

The spectrum (fig.2) was obtained in the same experimental conditions, on the same sample after dehydration by heating at 110 °C and pumped under high vacuum for 12 hours.

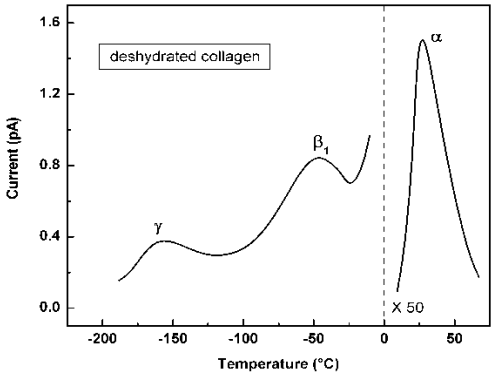


**Figure 2.** TSC spectrum from −180 to 50 °C in inert N2 gas for deshydrated collagen.

Thermal windowing method (J.A. Diego and al. 1999) was applied to resolve experimentally the complex TSC spectra around α mode of collagen into elementary spectra (figure 3). Each elementary spectrum can be considered as a Debye peak, and thus is associated with a single relaxation time. The study of the fine structure of the α relaxation mode allowed its attribution to the relative movements of the tropocollagen molecules to each other.

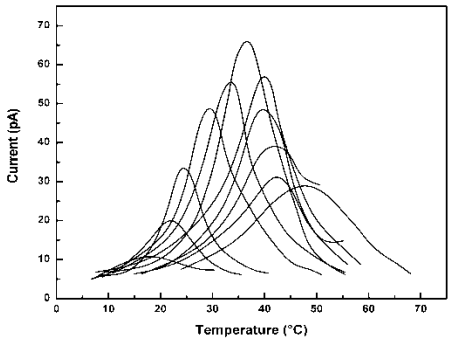


**Figure 3.** TSC peak profile of dehydrated collagen obtained by thermal windowing technique (polarization window ΔT = 5 °C, Tp varies from 15 to 55 °C, polarization field 106 V/m, heating rate 8 °C/min) in the α range.

β mode as shown in Figure 2 is strongly affected by moisture, had originated in movements of collagen hydrophilic amino acids constituting the collagen polar sequences.

$\beta_1$ sub-mode relaxation, observed at -50 °C in the dehydrated collagen is associated with cooperative movements of ionic side chains located between the triple helixes. These chains movements are released by the breaking of hydrogen bonds that were established with the intermolecular water molecules.

As for the sub-mode $\beta_2$ observed at -100 °C in the hydrated collagen only, it is due to intramolecular rearrangements of chains segments linked by hydrogen bonds to intramolecular water molecules.

To follow the evolution of the TSC complex spectrum of collagen with age, several thermograms are recorded on the same sample to verify the reproducibility of results. Thus , during recordings, we were able to record high collagen sensitivity of immature rats with low levels of hydration. Indeed, we can observe from the second run, a shift of the peak $\beta_1$ to low temperatures. Figure 4 shows the evolution during two successive passages of the TSC spectrum of immature rats (5 weeks) dehydrated collagen.

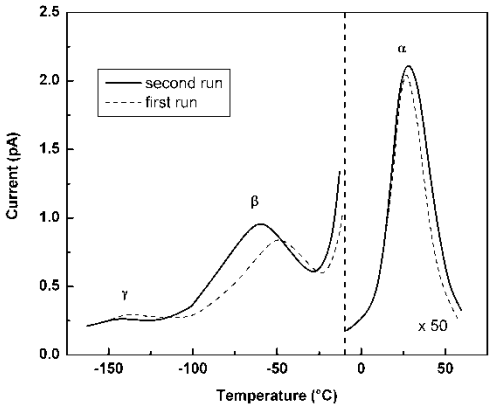

**Figure 4.** Superposition of two complex spectra of 5 weeks old rat collagen.

We first note a good reproducibility of the complex spectrum with three modes α, β and γ and a slight increase in the intensities of $\beta_1$ and α. modes. We also observe a significant shift of the $\beta_1$ mode maximum to low temperatures, whereas the temperatures of α and γ modes are unchanged.

These spectra show that the intermolecular movements of the $\beta_1$ mode require, in the second thermogram less energy to reorient: Water plasticizes movements, that is to say, it removes the chains and facilitates their relaxation. In addition, we found that this difference in temperature between the $\beta_1$ mode maxima of the two passages, decreases gradually as age increases.

The shift of temperature in $\beta_1$ mode caused by the mechanism of hydration decreases between 3 and 8 weeks. After 8 weeks, this shift continues to decline and vanish at the age of 3 months.

We have shown in Figure 5 the superposition of two consecutive spectra of mature rat collagen of 3 months old recorded during the first two thermograms on the dehydrated state.

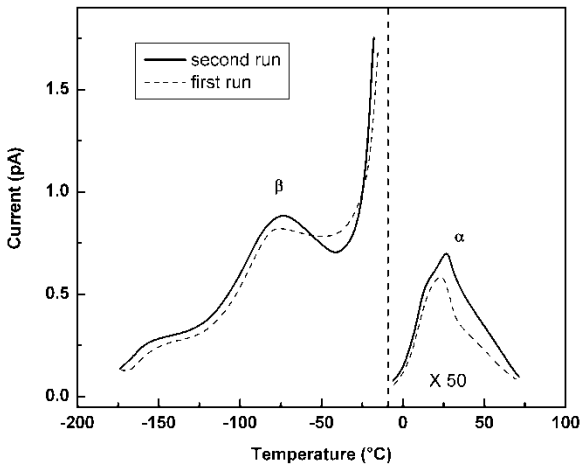


**Figure 5.** Superposition of two complex spectra of 12 weeks old rat collagen.

We note that from three months old, the effect attributed to collagen rehydration stops. In fact, we no longer note the intramolecular modes throughout adulthood. Figure 6 shows two TSC complex spectra for dehydrated collagen extracted from the rat's skin aged 18 months during two consecutive thermograms.

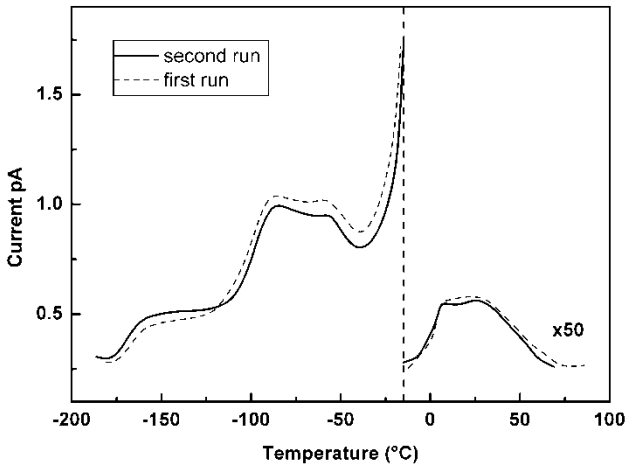


**Figure 6.** Superposition of two complex spectra of 18 weeks old rat collagen.

This figure shows that the observed modes reappear at the same temperatures with almost the same amplitudes, in particular, $\beta_1$ mode collagen which evolved for immature rat collagen is very stable here: the sample does not rehydrate between two successive passages. This same instability was observed for 21 and 24 months.

It is logical to think that cross links, once formed stop the water molecules to penetrate inside the tropocollagen molecule. The diameter of the microfibril no longer increases and therefore the plasticizing of intra and intermolecular movements can not occur.

**Discussion**

During the course of aging, the growing difference between the two passages noted $\delta_\beta$, reflects the evolution of rehydration power of the collagen with age (Fig. 7)

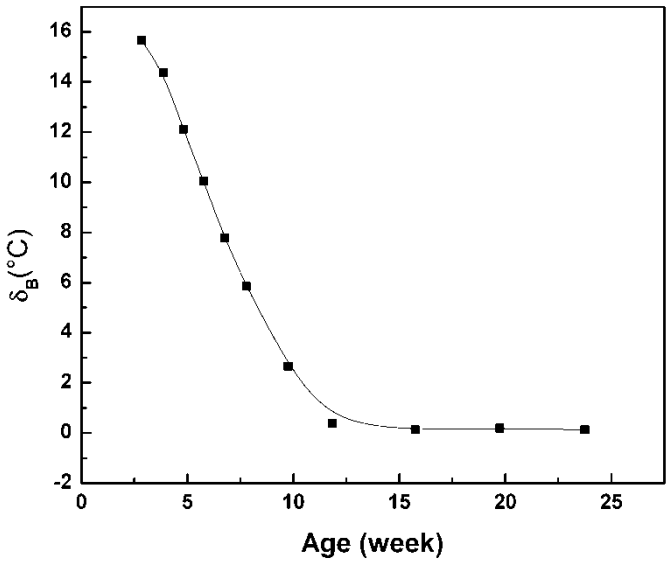


**Figure 7.** Evolution diagram of the power of rehydration during collagen maturation.

This evolution is almost sigmoidal: rapid decline up to 7 weeks and then gradual decrease up to 3 months. From 3 months, the first and second passages are identical, collagen can no more rehydrate.

This loss of rehydration power is linked to the formation and stabilization of cross links. Until puberty (7 weeks in the rat), the growth of the animal implies a significant synthesis of collagen. In this newly formed collagen, tropocollagen molecules are well aligned and shifted by approximately one quarter of their length, almost independent of each other. But soon, cross links will be established between polar residues of amino acids belonging to neighboring chains. It is therefore logical to assume that it is the formation of these cross links, which causes the decrease of hydrophilic sites accessible to water. In addition, these cross-links will, over time, become progressively irreducible and collagen increasingly insoluble.

The decrease in $\delta_\beta$ after puberty would be the consequence of the stabilization of collagen molecules. The formation of increasingly stable cross links block the interchain distance which will hinder the penetration of water molecules within the microfibril and collagen will therefore no longer be hydrated.

Figure 8 shows the variation of the activation enthalpy of the elementary peaks of collagen as a function of temperature. The criterion of Starkweather (W. Howard and al. 1981) can be used to analyze the nature of the molecular mobility.

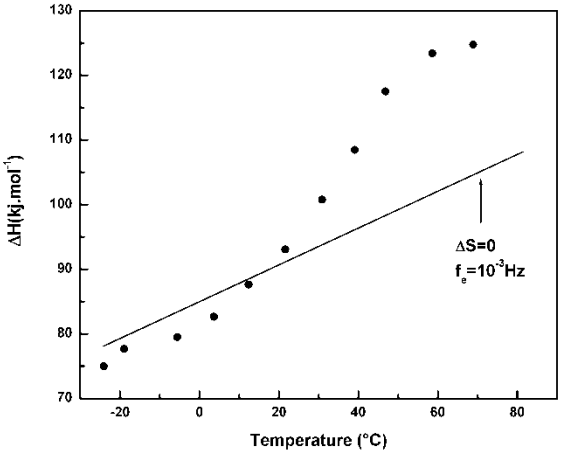


**Figure 8.** Activation enthalpy of the elementary relaxation times of collagen as a function of the temperature of the peak; Starkweather's function corresponding to an equivalent frequency and 5x10-2 Hz is superimposed on the Figure as a continuous line.

To discriminate between cooperative process, Starkweather's function (G. Marlena and al. 2011) (continuous line) has been superimposed on the figure; it corresponds to activation enthalpies derived from the activated state theory at null activation entropy. It can be observed from figure 8 that the lowest temperature processes of the main relaxation of the proteins obey the null entropy prediction these relaxation processes can be considered as simple. It reflects the movement of small molecular segments without modification of the environment. The activation enthalpies of the low temperature processes are close to the $\Delta S=0$ line, suggesting that the corresponding molecular mobility is localized and does not involve cooperativity. Only a slight increase of $\Delta H$ is observed above 25°C, indicating the rising of cooperativity. The maximum value of $\Delta H=78$ kJ mol-1 is found at Tm=30°C. So, it is noteworthy that the elementary mobilities of collagen below Tg have the similar features; they are indicative of the increasing size of the probed entity when temperature is increased until the maximum of the complex relaxation mode.

**Conclusion**

We studied an aspect of the evolution of molecular mobility, which characterizes collagen rehydration mechanisms and the density of cross links. The dynamics of inter and intramolecular chains was analyzed by thermally stimulated current technique. The study of collagen-water interactions showed that the power of rehydration of collagen gradually decreases during growth to completely disappear in early adulthood. The recorded sigmoidal evolution is associated with the formation and stabilization of collagen cross links. Finally,it will be of interest to study the strong-fragile transition that can occur for a protein. The collagen system should be a good model for such an investigation.

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