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Article

Spectroscopic and thermal characterization of alternative model biomembranes from shed skins of *Bothrops jararaca* and *Spilotis pullatus*

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Recently, there has been an interest in the use of shed snake skin as alternative model biomembrane for human stratum corneum. This research work presented as objective the qualitative characterization of alternative model biomembranes from *Bothrops jararaca* and *Spilotis pullatus* by FT-Raman, PAS-FTIR and DSC. The employed biophysical techniques permitted the characterization of the biomembranes from shed snake skin of *B. jararaca* and *S. pullatus* by the identification of vibrational frequencies and endothermic transitions that are similar to those of the human stratum corneum.

Uniterms: Biomembrane/alternative models. *Bothrops jararaca*/skin/qualitative characterization. *Spilotis pullatus*/skin/qualitative characterization. Human *stratum corneum*/similars.

Existe atualmente interesse no uso da muda de pele de cobra como modelos alternativos de biomembranas da pele humana. O presente trabalho apresentou como objetivo a caracterização qualitativa de modelos alternativos de biomembranas provenientes de mudas de pele de cobra da *Bothrops jararaca* e *Spilotis pullatus* por espectroscopia Raman (FT-Raman), espectroscopia fotoacústica no infravermelho (PAS-FTIR) e calorimetria exploratória diferencial (DSC). As técnicas biofísicas FT-Raman, PAS-FTIR e DSC permitiram caracterizar qualitativamente os modelos alternativos de biomembranas provenientes das mudas de pele de cobra da *B. jararaca* e *S. pullatus* e identificar freqüências vibracionais e transições endotérmicas similares ao estrato córneo humano.

Unitermos: Biomembranas/modelos alternativos. *Bothrops jararaca*/pele/caracterização qualitativa. *Spilotis pullatus*/pele/caracterização qualitativa. Estrato córneo humano/similares.

INTRODUCTION

The intact human skin is considered as a barrier against the penetration and permeation of agents such as particles, chemical substances, radiations and microorganisms (Williams, Barry, Edwards, 1994). It is of utmost importance for the survival of human beings, despite being an obstacle for the action of active substances applied over the skin (Kalia *et al*., 2004).

Cutaneous penetration and permeation of active substances may be firstly limited by the epidermis, especially the *stratum corneum* (SC), due to its barrier function and to the fact that it is the first layer of contact with the outer side. The epidermis is divided into two portions: *(1)* the internal portion, formed by cells that are constantly proliferating; *(2)* the external portion, formed by the same cells that originate the SC after its keratinization and death (Moser *et al*., 2001).

Skin penetration studies using *in vitro* evaluation present advantages such as: economy, fast obtainment of results, control of the experimental conditions and the possibility to evaluate a larger number of replicates, among others. The ideal situation would be to use human skin as a model, but the lack of this material, the need to submit the

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experiment to an Ethics Committee, the storage difficulties and high cost, and the viability of this membrane model limit its usage (Baby *et al.*, 2009; Schmook, Meingassner, Billich, 2001; Rigg, Barry, 1990).

As alternative biomembranes, experimentation animals' skin, synthetic membranes and three-dimensional cultures are used to experimentally simulate epidermis. There is interest in using shed snake skin in alternative models of human skin biomembranes, and researchers have been evaluating its applicability in penetration / permeation studies, obtaining favorable responses. Shed snake skin is composed by pure SC without viable epidermis and follicles (Rigg, Barry, 1990). It offers a barrier similar to human SC and can be obtained abundantly without the death of the animal. Shed snake skin can be easily stored and does not tend to be contaminated nor microbiologically degraded, as it does not present living tissues (Itoh *et al*., 1990; Baby *et al*., 2007; Baby *et al.*, 2008a).

Shed snake skin have similarities with human SC, such as: *(1)* tissue thickness (human SC = 13-15 μm; shed snake skin = 10–20 μm); *(2)* protein structure (keratin type α and β); and *(3)* lipid composition (human SC = 2.0–6.5%; shed snake skin = approximately 6.0%, involving the presence of cholesterol, free fatty acids, glycol ceramides and phospholipids, among others) (Rigg, Barry, 1990).

Various biophysical methods have been employed to study the morphology and dynamics of the SC, aiming to understand the correlation between its structure and function. Among these methods: X-ray diffraction, electron paramagnetic resonance, nuclear magnetic resonance, DSC (Differential Scanning Calorimetry), FT-Raman, and PAS-FTIR (Lafleur, 2001; Baby *et al.*, 2006a; Baby *et al.*, 2008b).

This research work aimed at characterizing qualitatively alternative models of biomembranes from *Bothrops jararaca* and *Spilotis pullatus* shed snake skin through FT-Raman, PAS-FTIR and DSC. It is also worth to mention that the usage of shed snake skin as alternative model of biomembrane to study cutaneous permeation contemplates the aspect of Experimental Ethics in Animals and Human Beings, besides being ecologically correct.

MATERIAL AND METHODS

Sample preparation

Ventral portions of *Bothrops jararaca* and *Spilotis pullatus* shed snake skin, gently donated by Butantan Institute, São Paulo, were cut and washed in abundance with distilled water. The samples were immersed in distilled water for eight hours in order to hydrate. After samples' hydration, the excess of water was removed with smooth compression using quantitative filter paper (Baby *et al*., 2006b). The samples were kept between two microscopy laminas and maintained in a desiccator until the time of analysis with FT-Raman, PAS-FTIR and DSC.

FT-Raman

Raman Bruker® RFS 100/S Spectrometers with OPUS® software were used. The samples were put in the analytical compartment and 256 co-additions corresponding to the spectra between 3500 and 200 cm^{-1} were obtained. The conditions were the following: *(1)* laser potency: 250 mW; *(2)* resolution: 4 cm^{-1}; *(3)* slit: 7; and *(4)* gain: 4 (Baby *et al.*, 2007; Baby *et al*., 2006b). Three replicates were used.

PAS-FTIR

The samples were adequately cut so as to fulfill the area of the photoacoustic cell in a model MTEC® 200 spectrometer. In order to avoid the movement of the cell, the samples were covered by a metal device. Then, a flow of helium was used for 2 min to remove water and carbon dioxide molecules. After sealing the cells, vacuum was made in the spectrometer. The 64 co-additions were controlled using the Bomem® PCDA program, in the spectral range of 4000–400 cm^{-1}. A rubber composite was used as standard. The experimental conditions were: *(1)* resolution: 4 cm^{-1}; *(2)* slit: 10; *(3)* gain: 4; and *(4)* speed of the mobile mirror: 0.05 cm/s (Baby *et al*., 2007; Baby *et al*., 2006a). The measurements were done using three replicates.

DSC

Samples of approximately 2.0 mg were sealed in a semi-hermetic crucible and were submitted to the following conditions (Baby et al., 2006b) in the DSC 10 equipment: *(1)* initial temperature: 25.0 °C; *(2)* cooling ramp: 20.0 °C/min to 0 °C *(3)* isotherm: 5 min *(4)* heating ramp: 10.0 °C/min to 160 °C *(5)* cooling ramp: 20.0 °C/min to 0 °C *(6)* isotherm: 5 min *(7)* heating ramp: 10.0 °C/min to 160 °C; and *(8)* final temperature: 25.0 °C (Baby *et al*., 2006b).

The transition temperatures were determined considering the minimum values of the endothermic peaks observed in the heating curves. The DSC curves were built with the values of the heat flow as a function of the temperature. The measurements involved three replicates.

RESULTS AND DISCUSSION

FT-Raman spectroscopy is useful to study biological materials, as water presents minimum interference, when compared, for instance, to infrared spectroscopy (Anigbogu *et al*., 1995; Casper *et al*., 2001). In the SC study, the vibrational frequencies of particular interest are: C-H (3100–2700 cm^{-1}), C=O (around 1650 cm^{-1} - amide I) originated from the α-keratin, C-N (1274 cm^{-1}) and N-H angular deformation of protein (amide III) (Anigbogu *et al*., 1995; Baby *et al.*, 2008b; Diem, 1993).

Raman spectra of the SC samples of *Bothrops jararaca* and *Spilotis pullatus* shed snake skin (Figures 1 and 2, respectively) presented spectral profiles similar to the ones described in the literature (Williams, Barry, Edwards, 1994). Characteristic vibrational spectral regions of the SC were observed, with intense signal bands between 3100-2700 cm^{-1}, referring to distensions originated from the lipid carbonic chains (symmetric and non-symmetric CH_3, symmetric and non-symmetric CH_2 and CH).

There was a band in the region between 1650-1672 cm^{-1}, related to the distention of the C=O of the α-keratin's amide I and, possibly, β-keratin. Besides, there was a band in the region between 1450-1460 cm^{-1}, probably originated from the angular deformation from C-H to CH, CH_2 and CH_3.

When comparing the Raman spectra from the SC of both species, there are similarities. However, the intensity of the signal appears to be greater for *Spilotis pullatus*, especially as far as the band between 3100-2700 cm^{-1} is concerned, what indicates differences in the biomembrane surface's morphology among these species.

Raman spectroscopy registers signals due to the spreading light that strikes on the sample's surface. Therefore, differences in signals' intensities are related to alterations in the sample's surface morphology caused by modifications in the molecular density per area unit of the

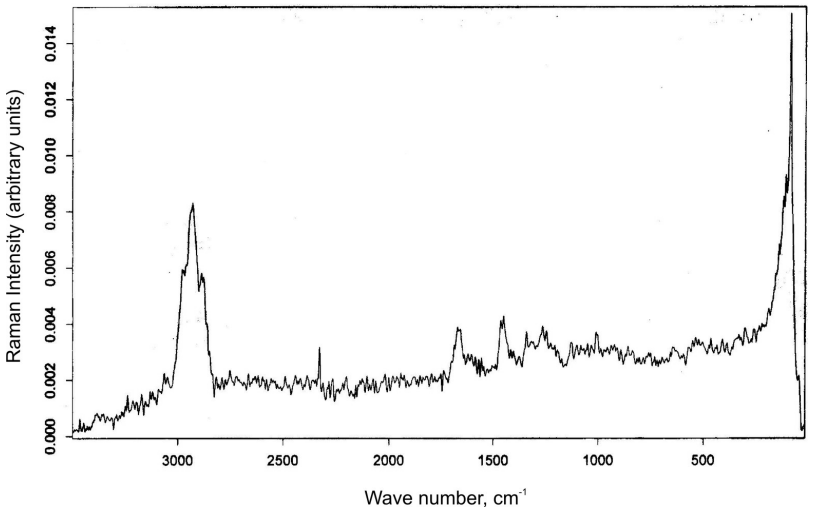

FIGURE 1 - FT-Raman spectral profile of the *Bothrops jararaca* biomembrane model.

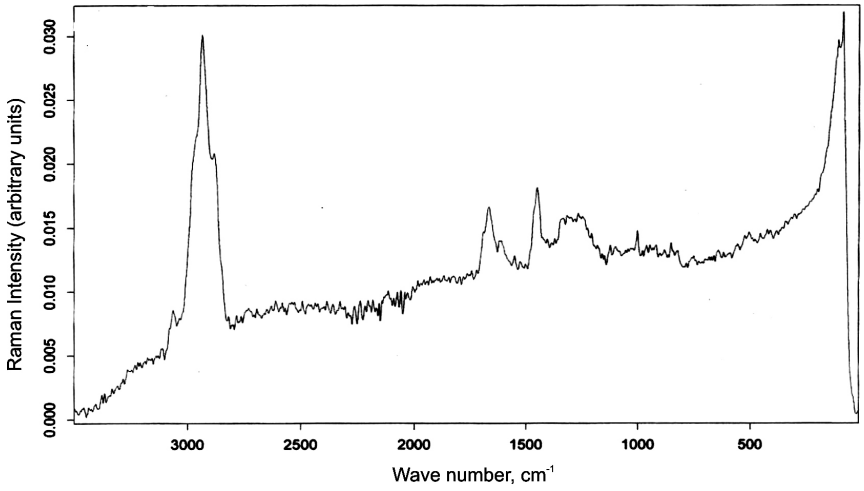

FIGURE 2 - FT-Raman spectral profile of the *Spilotis pullatus* biomembrane model.

surface region involved in the spreading of light, and by alterations in its topography (Ingle, Crouch, 1988).

In Figures 3 and 4 (PAS-FTIR spectra), we can observe the typical profiles of the hydrated biological material, with bands in 1650 cm^{-1} (C=O distension of amide I) and in 1550 cm^{-1} (C-N distension and N-H deformation of amide II). In the band between 3600-3300 cm^{-1}, we can observe the presence of water in the species' SC (Golden *et al.*, 1986; Lin *et al*., 1992).

The usage of DSC enables the study of structure and organization of the SC though the observation of the characteristic temperatures of the endothermic events related to the lipid fraction, e.g. phase transitions, and to the protein fraction of biomembranes that involve dehydration and keratin denaturation phenomena (Lin, Duan, Lin, 1996).

The isolated human SC has four characteristic endothermic transitions. The phase transition of the lipid bilayer from crystalline to gel state is attributed to the temperature of 40 °C. The phase transition of the lipid bilayer from lamellar gel to liquid state is attributed to the temperature of 75-85 °C. The transition that occurs at 105 °C represents the dehydration and denaturation of the protein fraction of the SC and the presence of a certain amount of water in the sample is required for its detection by the technique (Baby *et al*., 2006b; Golden *et al*., 1986, Ashton *et al*., 1992; Leopold, Lipppold, 1995).

In the DSC curve from *Bothrops jararaca* shed skin (Figure 5), not all the lipids endothermic transition peaks were observed. However, a transition of small magnitude was observed at, approximately, 58 °C. The endothermic peak referring to the transition involving dehydration and keratin denaturation occurred clearly at 130 °C.

Spilotis pullatus shed skin biomembrane presented a DSC curve similar to *B. jararaca*. The endothermic transi-

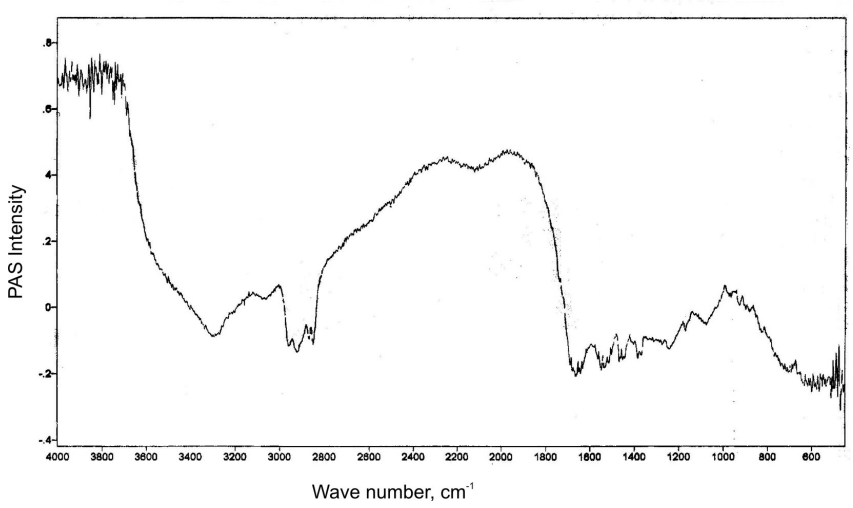

FIGURE 3 - PAS-FTIR spectral profile of the *Bothrops jararaca* biomembrane model.

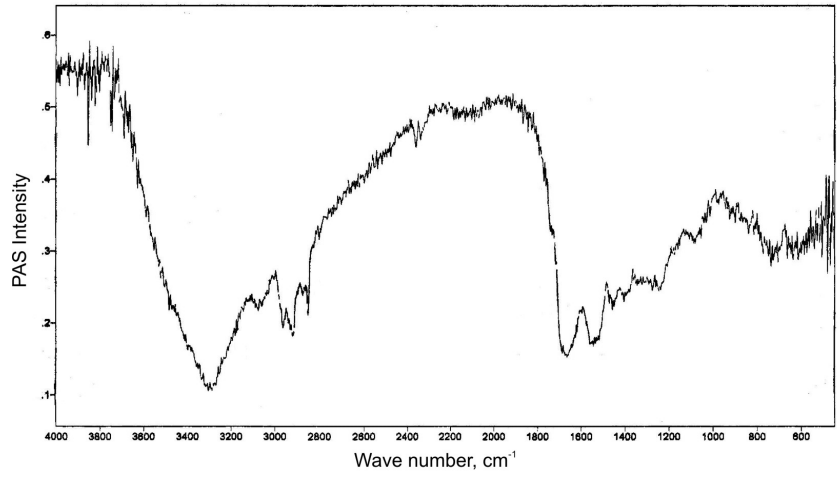

FIGURE 4 - PAS-FTIR spectral profile of the *Spilotis pullatus* biomembrane model.

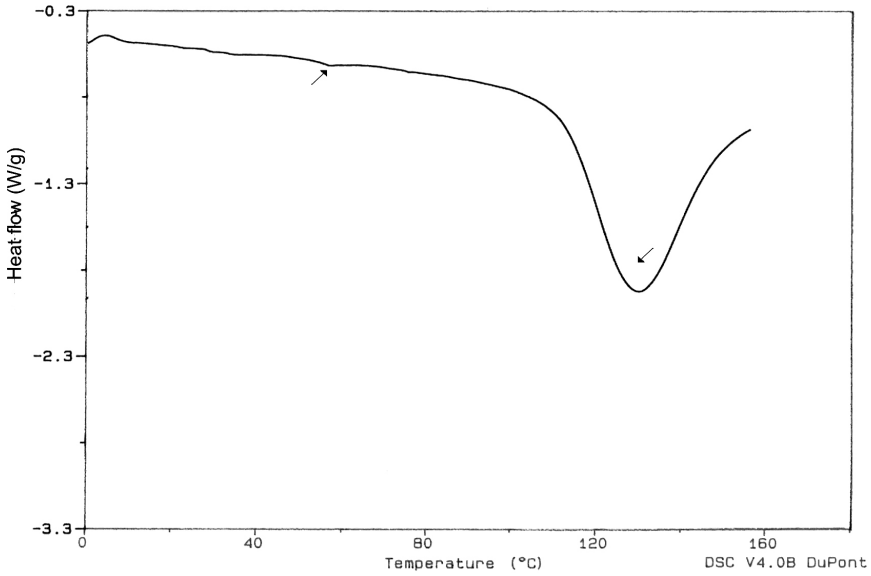

FIGURE 5 - DSC curve of the *Bothrops jararaca* biomembrane model.

tions related to lipids were of reduced intensity, observed at 38 and at 58 °C. The transition related to dehydration and denaturation of the protein fraction occurred at 129 °C.

CONCLUSIONS

The biophysical techniques FT-Raman, PAS-FTIR and DSC allowed the qualitative characterization of biomembranes alternative models from *Bothrops jararaca* and *Spilotis pullatus* shed snake skin, and the identification of the vibrational frequencies and endothermic transitions similar to those of the human SC.

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