JVSTB Journal of Vacuum Science & Technology B

Characterization of the crotalus durissus terrificus venom by atomic force microscopy

Vitor Baranauskas, Doroty Mesquita Dourado, Zhao Jingguo, and Maria Alice da Cruz-Höfling

Citation: Journal of Vacuum Science & Technology B **20**, 1317 (2002); doi: 10.1116/1.1486007

View online: http://dx.doi.org/10.1116/1.1486007

View Table of Contents: http://scitation.aip.org/content/avs/journal/jvstb/20/4?ver=pdfcov

Published by the AVS: Science & Technology of Materials, Interfaces, and Processing

Characterization of the *crotalus durissus terrificus* venom by atomic force microscopy

Vitor Baranauskas[a)]
Faculdade de Engenharia Elétrica e Computação, Departamento de Semicondutores Instrumentos e Fotônica, Universidade Estadual de Campinas (UNICAMP), 13 083-970 Campinas, SP, Brazil

Doroty Mesquita Dourado
Universidade para o Desenvolvimento do Estado e da Região do Pantanal (UNIDERP), Campo Grande, MS, Brazil

Zhao Jingguo
Faculdade de Engenharia Elétrica e Computação, Departamento de Semicondutores Instrumentos e Fotônica, Universidade Estadual de Campinas (UNICAMP), 13 083-970 Campinas, SP, Brazil

Maria Alice da Cruz-Höfling
Departamento de Histologia e Embriologia, Instituto de Biologia, (UNICAMP), Universidade Estadual de Campinas, 13 083-970 Campinas, SP, Brazil

(Received 7 November 2001; accepted 22 April 2002)

Atomic force microscopy was used to study the morphology of crude venom from the South American rattlesnake *crotalus durissus terrificus*. The results indicate that the crystallized crude venom has a high surface roughness with pores of diameters around 100 nm. Coiled filamentary structures of diameters of about 200 nm and lengths greater than 1000 nm, and large concave structures of diameter around 1000 nm, have also been observed dispersed over the main solid surface of the venom. Such structures may be related to aggregates of toxic enzymes or to bacteria or both. The coiled filamentary structures appear to be entangled on the pores of the venom surface in contrast to the large concave structures, which appear not to be bonded to the principal crystallized surface. © *2002 American Vacuum Society.* [DOI: 10.1116/1.1486007]

I. INTRODUCTION

Advances in the characterization of the complex composition of snake venom are important in the development of antidotes, which may have greater efficiency in the prevention of harmful traumatic effects when humans are poisoned. Most of the lethal accidents provoked by snake bites in Brazil are related to the *crotalus durissus terrificus* (CDT) rattlesnake, which is generally found in open fields, cleared forests, and dry lands. The primary activities of the crotalic venom in humans are systemic actions triggered by neurotoxic, myotoxic, and coagulative components of the venom. The neurotoxic symptoms are mainly produced by the crotoxin, which is a beta neurotoxin of pre-synaptic action that inhibits the release of acethylcoline at nerve terminals. The main result of this inhibition is neuromuscular immobility, which leads to the symptoms of neuro-motor paralysis and respiratory distress. The myotoxic action produces damage to the skeletal muscle fibers with the release of enzymes and myoglobin into the blood, and the coagulation process is affected by the direct conversion of the fibrinogen into fibrin. Fibrinogen consumption may lead to impaired blood coagulation, and death results by suffocation or acute renal insufficiency.[1]

The main toxins present in the CDT venom are crotamine (together with crotoxin responsible for muscle effects), gyrotoxin, convulxin, and crotoxin, a phospholipase $A_2(PLA_2)$, which is the major venom component responsible for the high toxicity of the venom. The CDT venom has been investigated, both by biochemical and pharmacological methods, and their biological effects examined with the separation and purification of their enzymes and toxins, as well as characterization of their action mechanism.[2,3] Such techniques are mainly based on phenomenological assays of the venom fractions and hence yield primarily a description of their overall or specific activities. However, for the development of a truly molecular model of the action of the venom on important mechanisms such as the hemorrhagic and fibrinolytic phenomena, several questions concerning to the lack of structural and morphological information of the toxins and enzymes must be answered.

Particular attention has been given to the enzymes phospholipases $A_2(PLA_2)$, which are subdivisions of crotoxin, to obtain a complete sequence of their amino acids for the future identification of a possible simple neutralization process of the toxic properties of crotoxin.[4–7] In addition to the biochemical identification, preliminary fluorescence[5] and crystallographic studies[6,7] have been made with the purification and crystallization of these enzymes. They are characterized by a structure with an N-terminated 12-residue α helix, a relatively long-loop segment containing the calcium binding site, a pair of large nearly parallel α helices, a short antiparallel double-stranded β sheet, and a C-terminal loop.[8,9] However, morphological studies of the venom compounds themselves using scanning electron microscopy (SEM) or transmission electron microscopy are scarce, because of dif-

[a)]Author to whom correspondence should be addressed; electronic mail: vitor@dsif.fee.unicamp.br

FIG. 1. *Cascavel*-South American rattlesnake (*crotassus durissus terrificus*) collected from Pantanal region, Mato Grosso do Sul, Brazil, for use in the present work.

ficulties in the analysis of these components in the vacuum environment required for electron microscopy.

In this work, a preliminary analysis of the morphology of crude CDT venom using atomic force microscopy (AFM) is discussed. AFM provides a direct measurement of the forces between the surface of the venom crystals and a sharp probe tip, which is passed over the sample to produce a three-dimensional (3D) computer image of the sample topology. AFM is a relatively new imaging technique that has the advantage of manipulating samples outside a vacuum environment,[10] and also without the need for the dehydration and metal coating procedures usually required for SEM analysis.[11,12]

II. EXPERIMENTAL DETAILS

Figure 1 shows a picture of a wild adult animal collected from the Pantanal region, Mato Grosso do Sul, Brazil, which was one of the specimens used in our experiments. To stimulate the production of the natural toxic effects of the CDT venom, the animals remained with water *ad libitum* and without food for 30 days before the venom was extracted. The crude venom was collected by a specialist, who pressed the animal's fangs directly on a clean glass beaker. Specimens for AFM analysis were prepared by transferring of small amounts of the solidified venom (dried at room temperature) onto the surface of soda-lime glass slides, following safety guidelines for handling toxic powders.

AFM measurements were made using a Nanoscope II (Digital Instruments) operating at ambient pressure and employing cantilevers with a nominal force constant of $k = 0.022\ \mathrm{Nm}^{-1}$.

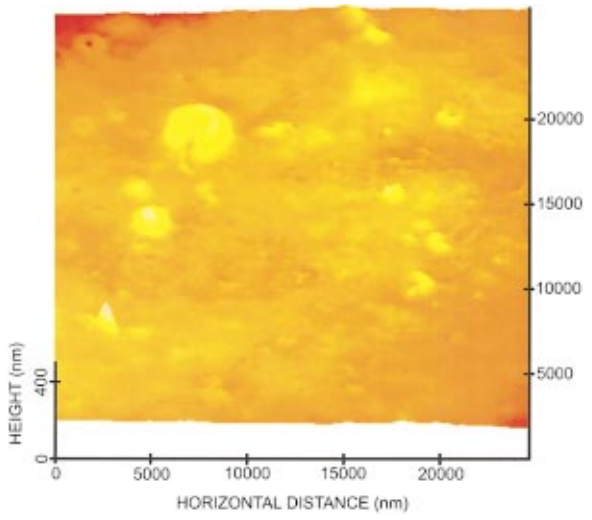

FIG. 2. Typical low-magnification AFM image of the 3D surface of the crude CDT venom.

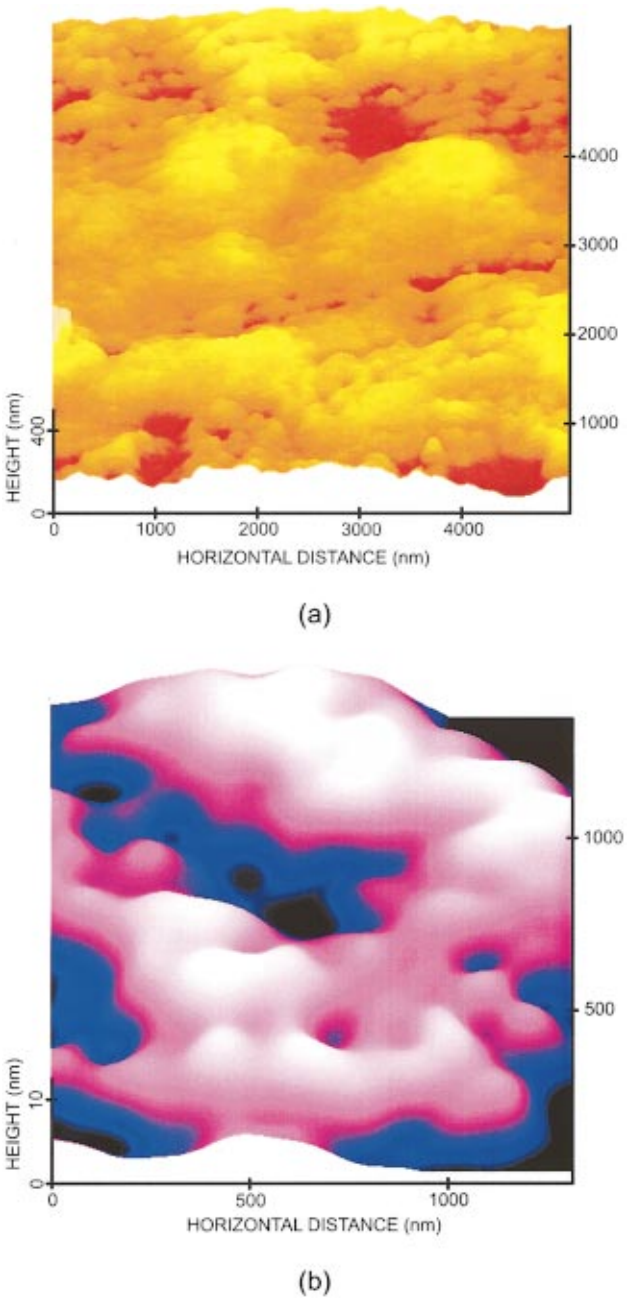

FIG. 3. AFM images of the main solid surface of the crude CDT venom. Image (b) corresponds to a greater magnification of image (a).

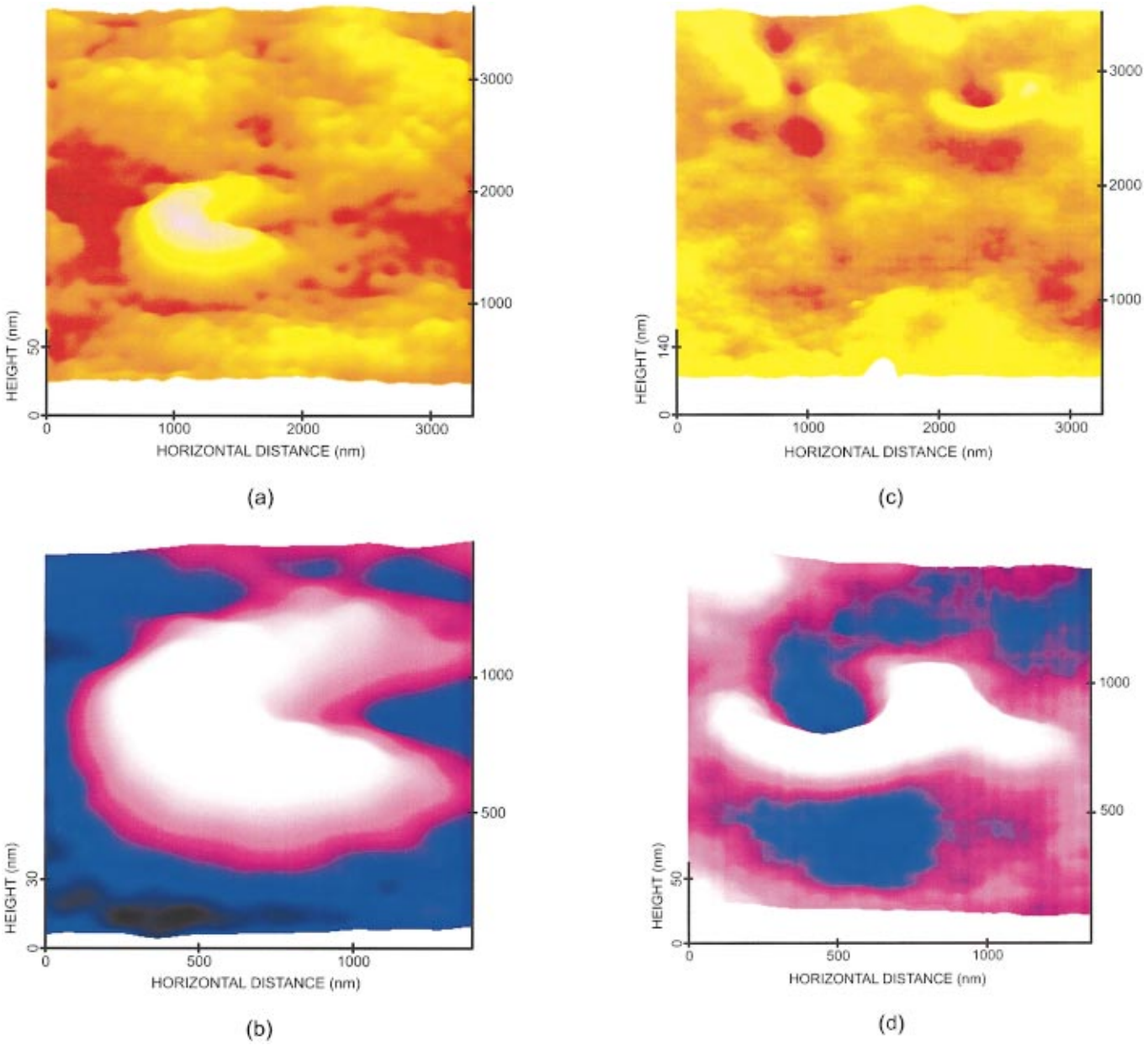

FIG. 4. Three-dimensional AFM images of the concave (a, b) and coiled (c, d) structures observed over the main solid surface of the crude CDT venom.

III. RESULTS AND DISCUSSION

Figure 2 shows a typical three-dimensional AFM overview image of the surface of the crude CDT venom. One can see a nonhomogeneous distribution of round structures over a rough and porous surface. Most of the large structures appear as short coiled filaments or as concave shells.

Further amplification of the surface of the CDT venom [see Figs. 3(a) and 3(b)] revealed round pores and aggregates of diameters around 100 nm. Such features may be related to complex biochemical structures. However, in these rough and soft surfaces it was not possible to increase the AFM resolution beyond the scale shown in Fig. 3(b) without the introduction of artifacts from the tip geometry, or from deformations due to the force used on the cantilever.

Some of the typical large structures present on the solid venom surface are depicted in Figs. 4(a)–4(d). Concave structures of diameter around 1000 nm appear frequently on the CDT venom surface [see, for instance, Figs. 2 and 4(a)]. They are not well fixed on the venom crystal surface, blurring the AFM imaging as the tip scans its surface, especially when a high magnification is used [see Fig. 4(b)]. Coiled filamentary structures are also common on the samples but they seem to be entangled in the porous surface [see Fig. 4(c)]. The diameters of these coiled filaments are about 200 nm and their lengths are greater than 1000 nm, as shown in the typical 3D image presented in Fig. 4(d). Such structures may be related to aggregates of toxic enzymes and/or to bacteria which in the accidents are co-responsible for the local effects of the injured tissue.

Inspection of the crude CDT venom by the AFM tech-

nique revealed very complex morphologies. Considering the variety of the biochemical and biological components of the venom, however, further refinements are necessary to improve the AFM analysis. One of these is the fractionation of the CDT venom into its basic compounds prior to the AFM imaging, to allow comparison of the morphological characteristics of the components with those of the crude venom. Special attention may be given to selected enzymes or other toxic agents of physiological relevance. The concave shells and the coiled structures revealed in the present images must also be elucidated since their natural morphology (which may be destroyed during the purification of the venom) may be important to our understanding of the complex action of the CDT venom in humans.

The main limitation of the AFM technique is the lack of chemical identification of the surface analyzed. However, the use of specific molecules adsorbed on the AFM scanning tip surface may provide, in the future, unique experimental possibilities for *in situ* chemical identification of the venom components. Any characterization technique has the potential to produce complementary information, and AFM may provide useful quantitative and qualitative information on the venom surface structure at the molecular scale, in parallel with studies with the classical techniques employed today.

IV. CONCLUSIONS

In summary, our preliminary experiments indicate that AFM has an important application to obtain the 3D morphologies of the complex mixture of toxins present in the crude CDT venom. AFM imaging of purified toxins, which is currently in progress, together with other techniques such as amino-acid sequencing, should help to provide information on the influence of morphology on the activity of the components of the venom.

ACKNOWLEDGMENTS

The authors thank Professor Ieda Maria Novaes Ilha for providing the picture shown in Fig. 1, and Hélder Antonio de Souza for his technical assistance. Both are from UNAERP - Campo Grande, Mato Grosso do Sul, Brazil. The authors also would like to thank Dr. S. F. Durrant for useful discussions and the Brazilian funding agencies CNPq and FAPESP for financial support.

Presented at the IUVSTA 15th International Vacuum Congress, the AUS 48th International Symposium and the 11th International Conference on Solid Surfaces, San Francisco, CA, 28 October–2 November 2001.

[1]M. M. Azevedo Marques, P. Cupo, T. M. Coimbra, S. E. Hering, M. A. Rossi, and C. J. Laure, Toxicon **23**, 631 (1985).
[2]E. E. Sanchez, L. A. Soliz, M. Susana Ramirez, and J. C. Perez, Toxicon **39**, 523 (2001).
[3]A. M. Soares, A. C. Mancin, A. L. Cecchini, E. C. Arantes, S. C. Franca, J. M. Gutierrez, and J. R. Giglio, Int. J. Biochem. Cell Biol. **33**, 877 (2001).
[4]V. Choumet, P. Lafaye, C. Demangel, C. Bon, and J. C. Mazie, Biol. Chem. **380**, 561 (1999).
[5]S. A. Sanchez, Y. Chen, J. D. Muller, E. Gratton, and T. L. Hazlett, Biochemistry **40**, 6903 (2001).
[6]S. Sharma, T. Jabeen, J. K. Singh, R. Bredhorst, C. W. Vogel, C. Betzel, and T. P. Singh, Acta Crystallogr., Sect. D: Biol. Crystallogr. **57**, 596 (2001).
[7]M. C. Nonato, R. C. Garrat, Y. P. Mascarenhas, W. D. P. Jesus, M. T. Assakura, S. M. T. Serrano, and G. Oliva, Biol. Crystallogr., D **57**, 599 (2001).
[8]R. K. Arni and R. J. Ward, Toxicon **34**, 827 (1996).
[9]D. H. F. Souza, H. S. Selistre-deAraujo, and R. C. Garratt, Toxicon **38**, 1307 (2000).
[10]G. Binning, C. F. Coate, and G. Gerber, Phys. Rev. Lett. **56**, 930 (1986).
[11]V. Baranauskas, B. C. Vidal, and N. A. Parizotto, Appl. Biochem. Biotechnol. **69**, 91 (1998).
[12]V. Baranauskas, I. Garavello-Freitas, Z. Jingguo, and M. A. Cruz-Hofling, J. Vac. Sci. Technol. A **19**, 1042 (2001).