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## Scanning tunnelling microscopy study of polypyrrole films and of glucose oxidase as used in a third-generation biosensor

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### Abstract

We have studied the surface morphology of different forms of the organic conductor polypyrrole, which serves as the environment for immobilization of the redox enzyme glucose oxidase. Scanning tunnelling microscopy (STM) images of polypyrrole films, obtained electrochemically on highly oriented pyrolytic graphite, STM images of polypyrrole microtubules and STM images of glucose oxidase molecules adsorbed on a gold facet are presented and discussed.

The conclusions from this study are: (i) the polypyrrole films, prepared chemically or electrochemically, exhibit disordered non-crystalline structure; (ii) the polypyrrole surface corrugations and the dimensions of the glucose oxidase molecule are of the same order of magnitude, allowing strong adsorption of the enzyme to the conducting polymer. On the basis of these data, a model for the direct interaction between glucose oxidase and polypyrrole in the biosensor is proposed.

### INTRODUCTION

Current efforts in the field of amperometric biosensors are directed towards the development of electrodes which communicate directly with a redox enzyme. The advantage of this design is that it allows the construction of reagentless biosensors, which are referred to as third-generation biosensors [1].

Interaction of redox enzymes with bare metal or carbon surfaces is often accompanied by large structural changes in the protein structure and concomitant loss of enzymatic activity. Therefore protein electrochemistry is usually performed

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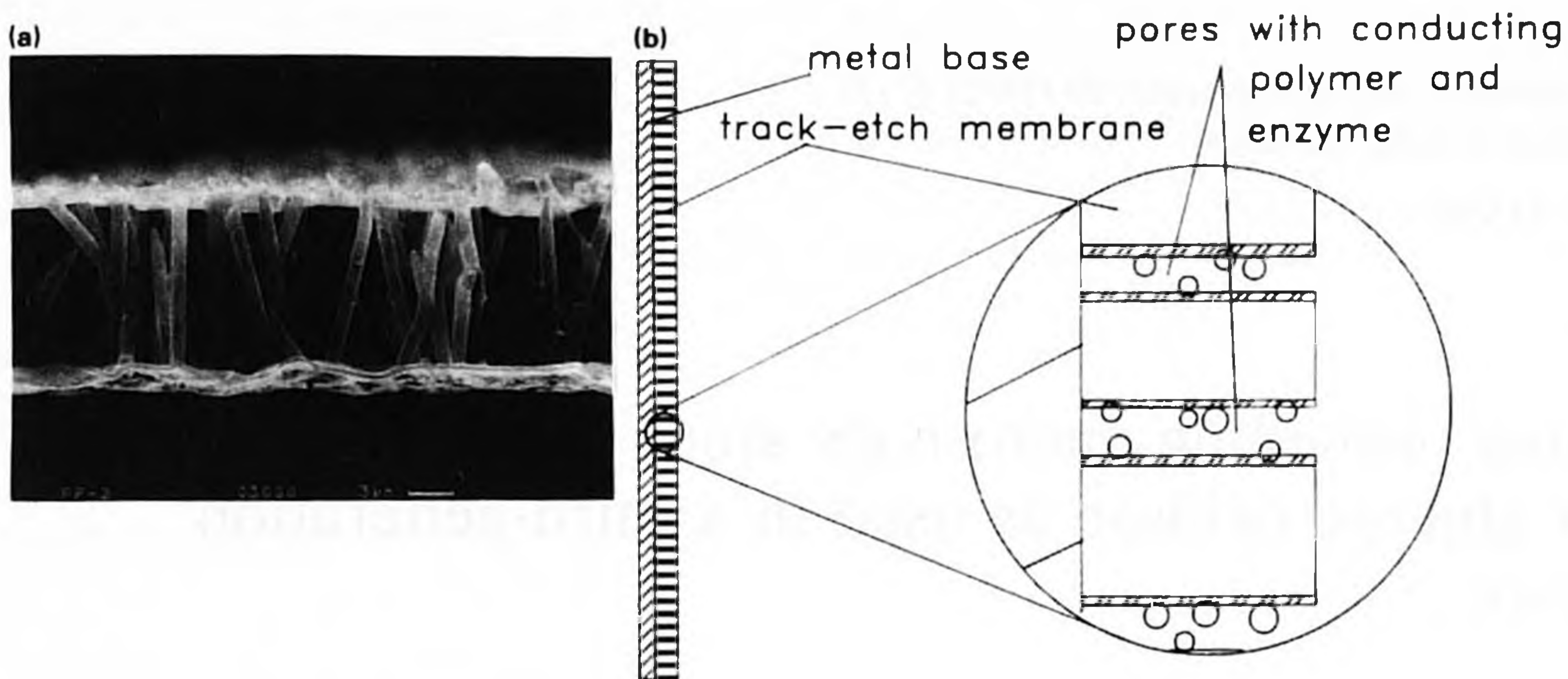


Fig. 1. (a) SEM image of polypyrrole microtubules. The polycarbonate template material was washed away with dichloromethane. The pore size of the original membrane was 1000 nm; the membrane thickness was 11  $\mu\text{m}$ . (b) Schematic drawing of the biosensor in which the microtubules are used.

with surface-modified electrodes [2]. Many research groups have applied organic conductors as surface modifiers [3–6], in particular polypyrrole [7–10]. The biosensors constructed with these electrodes do not work according to the principle of direct electron transfer between the redox enzyme and the electrode material. This electron transfer is mediated by a small and mobile redox species which is co-adsorbed with the enzyme.

Recently we have reported that the redox enzyme glucose oxidase can very efficiently be immobilized on a new type of polypyrrole, namely in the form of microtubules which are synthesized using the pores of a track-etch membrane as templates. With these microtubules we have been able to construct a reagentless glucose sensor which displays direct electron transfer from the enzyme to the conducting polymer. A scanning electron microscopy (SEM) image of the polypyrrole microtubules applied in this biosensor is shown in Fig. 1. Further details of this amperometric biosensor are presented elsewhere [11,12]. Since the microtubular polypyrrole does not contain additional redox mediators (neither covalently anchored nor physically adsorbed), the intriguing question arises of why direct electron transfer takes place. To answer this question, we have undertaken a scanning tunnelling microscopy (STM) study of the two components of the biosensor, i.e. the conducting polymer and the redox enzyme.

The scanning tunnelling microscope is based on the tunnelling of an electric current from a metallic tip to the surface of a sample [13]. Therefore the electrical conductivity of the sample under investigation is of crucial importance. Up to now only a limited number of polymers have been studied by STM. They all are conducting polymers [14–16].

When the material to be studied is non-conducting, it is still possible to obtain STM images by depositing a thin layer of the material on a conductive substrate, such as highly oriented pyrolytic graphite (HOPG) or a noble metal surface. In this

way it has been possible to obtain STM images of e.g. lipids [17,18], DNA [19] and *n*-alkanes [20].

In this paper we present a study of the surface morphology of polypyrrole microtubules on a nanometre scale and for comparison also of polypyrrole electrochemically deposited on a HOPG substrate. In addition we present the first STM images of glucose oxidase molecules adsorbed on a gold facet. The results of this study are used to propose a model for the observed direct interaction between glucose oxidase and the conducting polymer.

## MATERIALS AND METHODS

Polypyrrole was electrochemically deposited on HOPG. To this end a freshly cleaved graphite surface was introduced into an aqueous solution containing 0.3 M pyrrole (Merck) and 0.15 M potassium chloride. The electrochemical treatment was a potential sweep from 0 to 1 V vs. an Ag/AgCl reference. The scan rate was  $0.1 \text{ V s}^{-1}$  (model CV-1B potentiostat, Bioanalytical Systems).

Polypyrrole microtubules were synthesized within the pores of Cyclopore track-etch filtration membranes (Cyclopore S.A., Belgium) according to a procedure described previously [21,22]. STM samples of the track-etch membranes, containing the polypyrrole microtubules, were prepared as follows. A small rectangular piece of the membrane material was placed on its side on the sample holder and glued down. After drying, the membrane was cut with a fresh razor blade as near to the holder surface as possible.

Glucose oxidase (GOd, E.C. 1.1.3.4) type II ( $25\,000 \text{ U g}^{-1}$ ) from *Aspergillus niger* (Sigma) was adsorbed on gold facets (atomically flat or only monoatomic steps on the surface) by introducing the gold in a potassium phosphate-buffered aqueous solution (pH 7.4) of  $5 \text{ mg GOd ml}^{-1}$  for 67 h. After adsorption, the gold facets were rinsed with buffer solution and either allowed to dry in the air or stored in buffer solution in the refrigerator.

The microscope used was a home-made STM constructed at the Research Institute for Materials at the University of Nijmegen. It was an ambient-air operating machine with a temperature-compensated two-piezotube construction and a differential screw tip approach system. The tip material was Pt-Ir and the microscope was in the constant-current mode. The set-up has been described in detail elsewhere [23,24].

## RESULTS AND DISCUSSION

### *Polypyrrole on HOPG*

Polypyrrole films were prepared electrochemically following the method described in the previous section. The different samples we studied were obtained by applying different numbers of electrochemical cycles. The samples obtained by cycling up to three times from 0 to 1 V (vs. Ag/AgCl) were used to study the initial

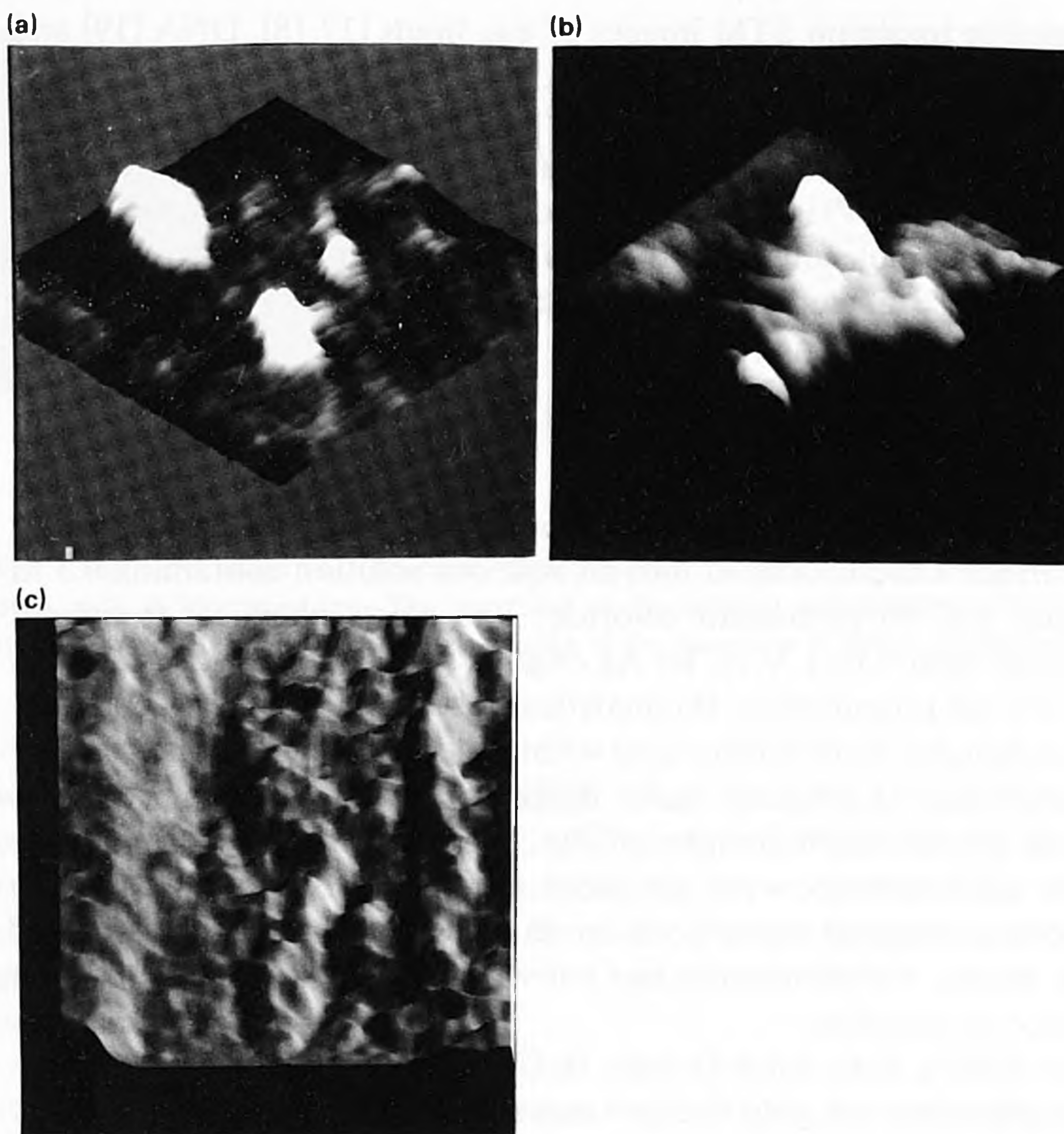


Fig. 2. STM images of polypyrrole films deposited on HOPG (tunnel current 100 pA, tip voltage  $-150$  mV): (a), (b)  $800 \times 800$  nm<sup>2</sup> image of a polypyrrole film at its initial stage of formation (the vertical scale range is 100 nm); (c)  $80 \times 80$  nm<sup>2</sup> image of the structures between the islands in (a).

steps of the polymerization reaction. The samples polymerized for longer times were used to study the final surface corrugation of the polypyrrole film as it is expected to be in the amperometric biosensor mentioned in the Introduction. The thickness of these electrochemically polymerized films was estimated to be 100 nm. This estimation is based on the fact that a charge of  $24 \text{ mC cm}^{-2}$  leads to a film of 100 nm [25].

Figure 2 shows the images of polypyrrole deposited on an HOPG surface after three electrochemical cycles. Some individual islands are visible in Fig. 2(a) and a much denser structure in Fig. 2(b). These images were taken from the same sample but at different places, which were at different distances from the platinum counterelectrode during the polymerization. Our explanation of these features is the following. The polymerization reactions occur at numerous and isolated sites all over the electrode surface. If there are only a few nucleation sites, the polymer grows slowly into dome structures. These domes eventually impinge on each other.

When the number of growth sites increases, the overall structure becomes smoother and more parallel to the original electrode surface [15,26].

The dimensions of the islands in Fig. 2(a) are up to 250 nm in the lateral direction and up to 50 nm in height. The structures which impinge upon each other (Fig. 2(b)) are up to several hundreds of nanometres long and up to 100 nm in height. The structures between the islands in Fig. 2(a) are chain like, with a width of 10 nm at the base and up to 1 nm in height. These chains are presented at higher magnification in Fig. 2(c). They seem to exhibit randomly distributed zigzag structures in accordance with the molecular organization of the films reported by Mitchell and Geri [26]. We failed to obtain atomic or individual molecular orbital resolution of our samples. In all our measurements the tunnel current was noisy and trials to scan with higher current (the tip closer to the sample surface) led to oscillations or to dragging effects.

Figure 3(a) presents a  $50 \times 50 \text{ nm}^2$  STM image of a polypyrrole film with a thickness corresponding to the films used in the amperometric biosensor. The image is a representative picture. It shows rows of polypyrrole chains elongated in one preferential direction, but with different widths and heights along these rows. A cross-section of the image height is given in Fig. 3(b). The typical values of the width and height are approximately 5–6 nm and up to 2.5 nm respectively. Figure 3(c) shows the image obtained at the highest magnification with a reproducible structure for different scanning directions and speeds. This picture suggests that two rows of the same chain type are present, 3 nm wide and 1.5 nm high, which impinge on one another.

### *Glucose oxidase*

Gold facets with adsorbed glucose oxidase molecules were imaged by SEM and STM. The enzyme molecules were adsorbed in the way described earlier. On the SEM images the adsorbed enzyme layer was clearly visible.

At the present stage in the development of STM imaging of biological molecules it is not possible to draw definite conclusions concerning the dimensions of biomolecules [27]. The interaction of the needle tip with biomolecules is still unknown. Therefore our STM results with regard to glucose oxidase should be interpreted with some caution. Figures 4(a) and 4(b) show the STM line and intensity images of glucose oxidase molecules on gold facets. The imaged area is  $38 \times 38 \text{ nm}^2$  and the vertical range is about 3 nm. The picture displays a conglomerate of enzyme molecules or a stack of several of these molecules. The individual molecules are oval shaped, with a dimension of 14–18 nm along the main axis and 5–8 nm along the perpendicular direction. These structures were reproducibly present in both scanning directions. The height of the images was about 1–2 nm. The molecules had the appearance of a ring with a 2 nm wall thickness and a small hole in the middle.

The lateral dimensions of the observed molecules are in agreement with the dimensions obtained from ellipsometry data on glucose oxidase adsorbed on a gold

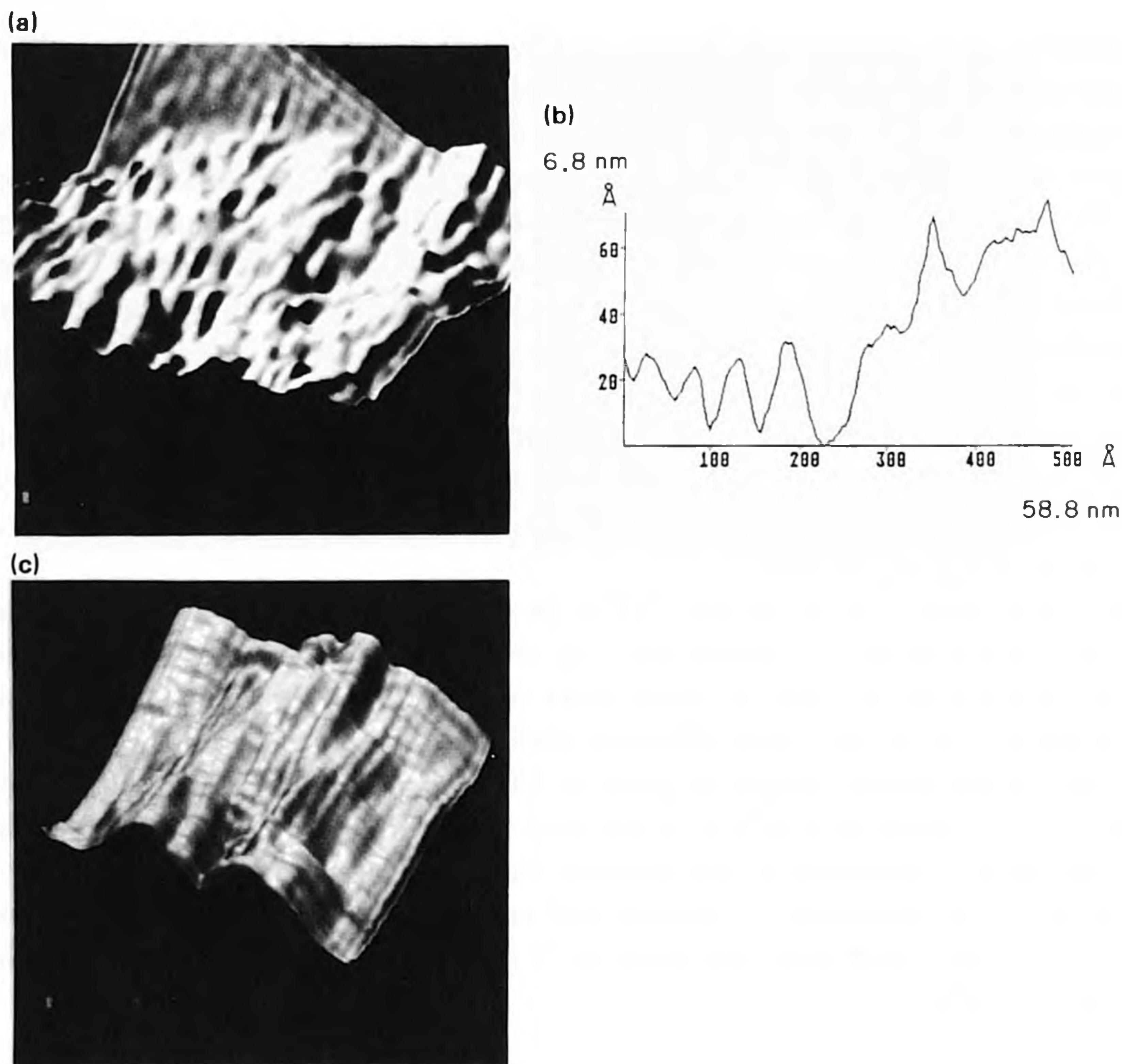


Fig. 3. STM images of a polypyrrole film surface with a thickness according to polypyrrole as used in the amperometric biosensor (tunnel current 50 pA, tip voltage  $-150$  mV): (a)  $50 \times 50$  nm<sup>2</sup> (the vertical full scale is about 20 nm); (b) cross-section of the lower right part of the image in (a); (c)  $10 \times 10$  nm<sup>2</sup> image (the vertical scale is about 4 nm).

electrode [28]. The vertical dimensions of our imaged molecules, however, need some further comments. Ellipsometry suggests that the glucose oxidase molecule is an ellipsoid with a minor axis up to 5 nm long. The vertical value we measured is smaller, but we expected this to be so for two reasons. First, it is known from other investigations [28] that the interaction between glucose oxidase and gold is very strong. This will lead to an irreversible adsorption of the enzyme on the gold surface. During this process the conformation of enzyme molecules slowly changes, exposing more and more amino acid groups capable of forming bonds to the gold substrate. This results in defolding of the tertiary structure and in deactivation of the protein. Secondly, the tunnelling current is not only dependent on the distance between the tip and the sample but also on the local work function. Therefore, when the tip is scanning badly conducting material, the feedback loop makes the tip go into the examined surface to keep the tunnel current constant. This is one of

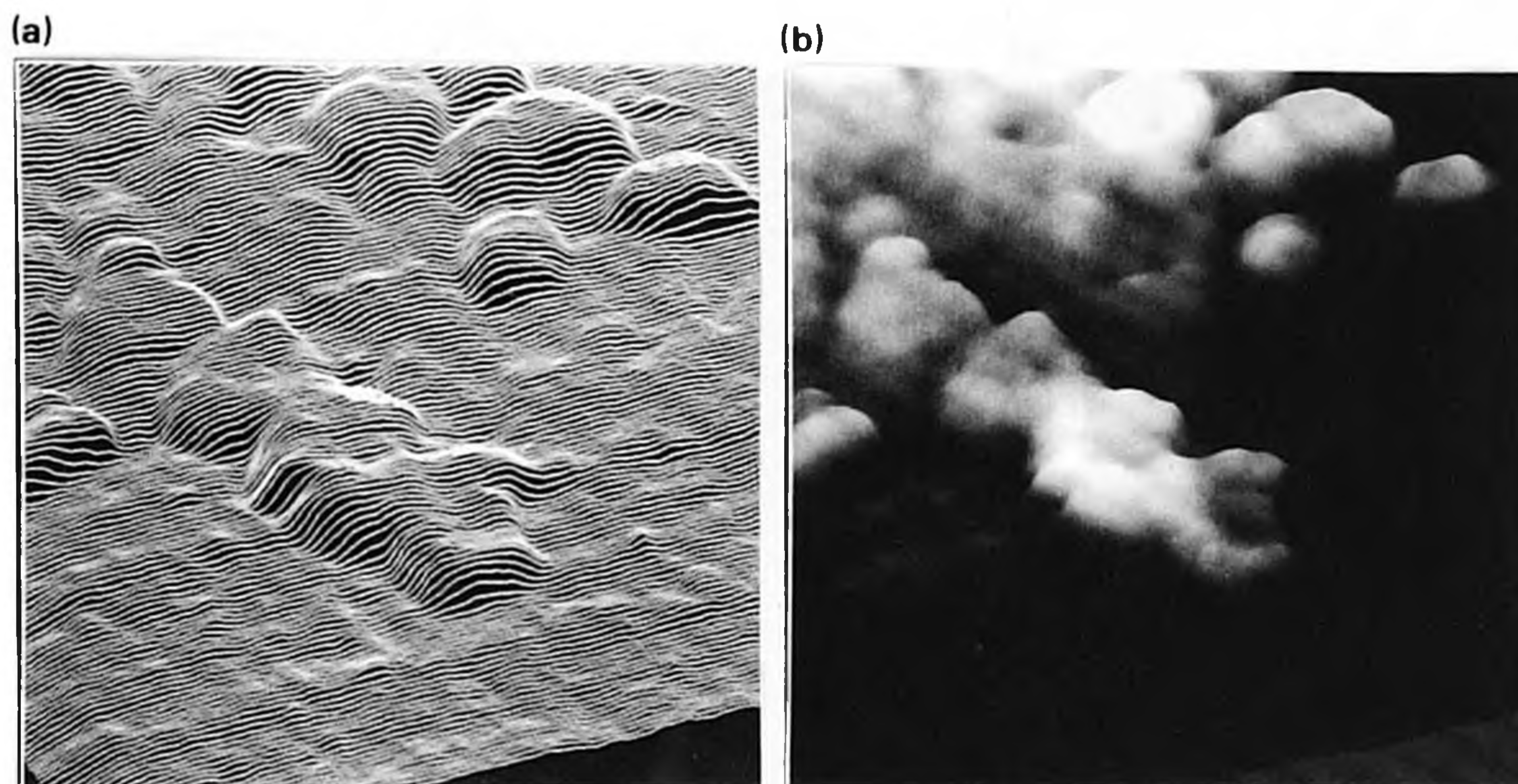


Fig. 4. (a) Line and (b) intensity STM images of glucose oxidase molecules adsorbed on a gold facet (area  $38 \times 38 \text{ nm}^2$  with vertical scale range about 3 nm, tunnel current 50 pA, tip voltage  $-150 \text{ mV}$ ).

the fundamental reasons why the dimensions of STM images from biological samples should be interpreted with caution (see above) [27].

Figure 5 shows an image of glucose oxidase molecules obtained at a different bias voltage of the scanning tip. The lateral dimensions remain the same as before, but now there is no evidence of a ring structure. One explanation is that, by changing the bias voltage (from  $-150$  to  $-50 \text{ mV}$ ), different molecular orbitals are

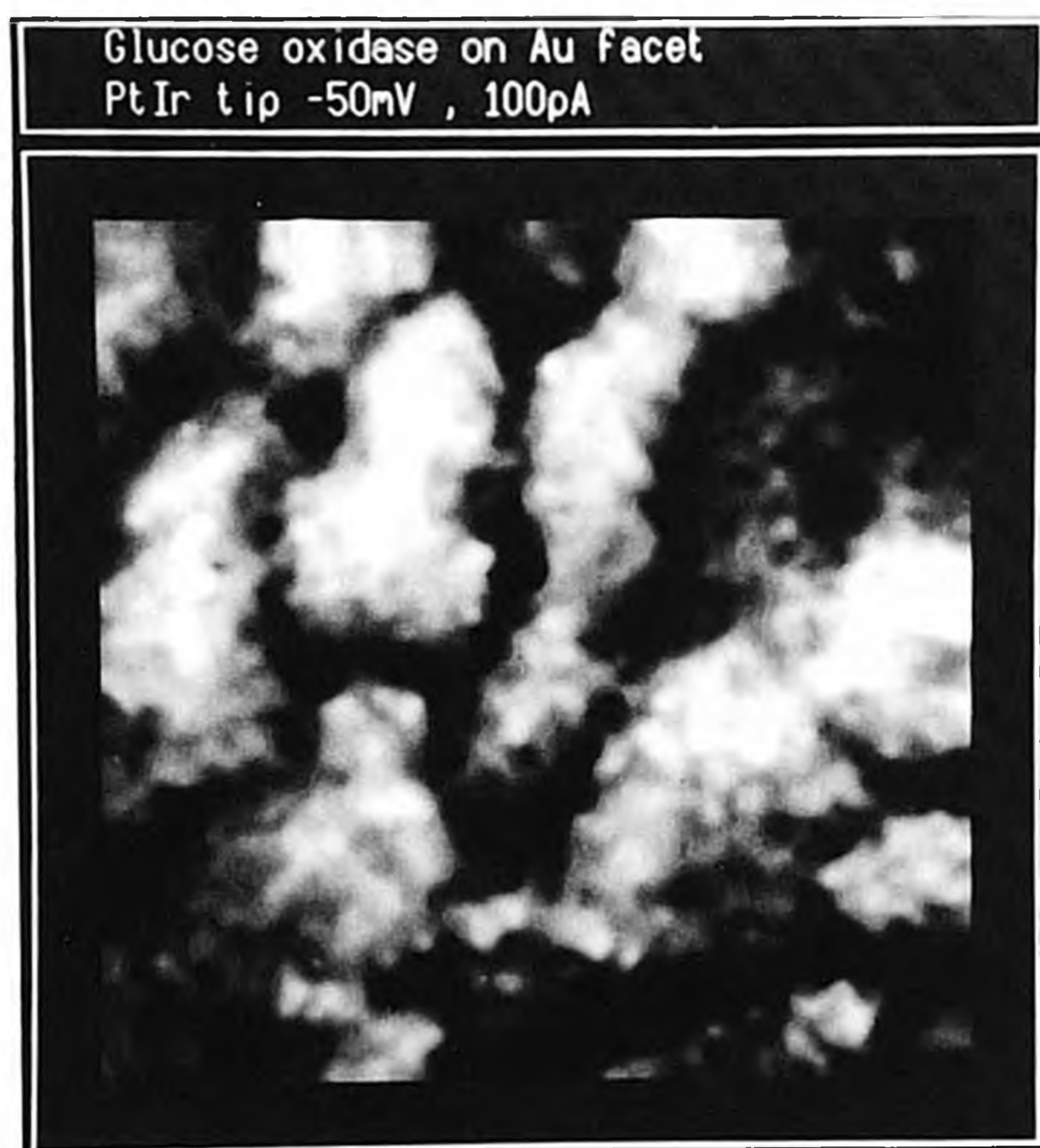


Fig. 5. STM top-view image ( $36 \times 36 \text{ nm}^2$ ) of glucose oxidase adsorbed on a gold facet (tunnel current 100 pA, tip voltage  $-50 \text{ mV}$ ). Imaged under ambient air, high humidity (greater than 70%).



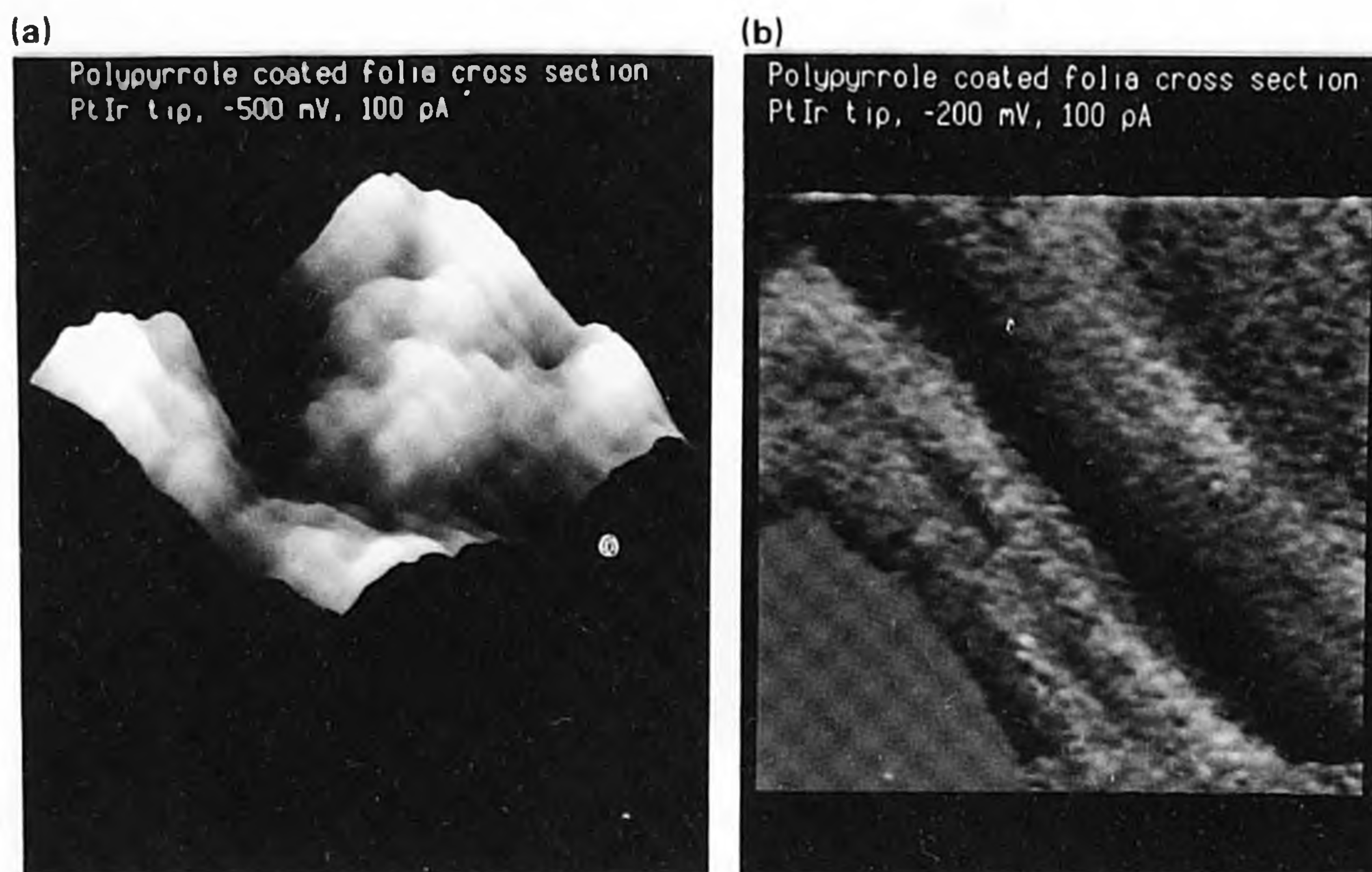


Fig. 6. STM images of a cross-section of a polypyrrole microtubule (tunnel current 100 pA, tip voltage  $-500$  mV): (a)  $80 \times 80$  nm<sup>2</sup> area with about 40 nm vertical scale range; (b)  $40 \times 40$  nm<sup>2</sup> filter image (gradient in  $x$  direction).

observed. Another important factor may be the ambient air humidity. This plays an important role when biological bulk specimens (generally non-conductive) are imaged. In line with reports by Yuan et al. [29], we obtained the most reproducible results when the air humidity was high (greater than 70%). Imaging at high humidity led to structures with more pronounced depth profiles (not shown). Glucose oxidase on gold was also imaged while placing the tip in a droplet of water on the gold surface. In this way we obtained the same results as for imaging in high humidity (greater than 70%) on a dry gold surface. The samples changed significantly when we placed the STM inside a glass bulb which was flushed with dry nitrogen. Imaging under these conditions led to “dragging effects” (see above). Therefore no STM images could be obtained under an atmosphere of low humidity.

### *Polypyrrole microtubules*

Figure 6(a) shows a typical image of the cross-section of the polypyrrole microtubules inside a track-etch membrane. This membrane is the kind of polypyrrole modified membrane that is used in the biosensor. The area is  $80 \times 80$  nm<sup>2</sup> and the vertical range is about 30 nm. The surface is strongly corrugated with ditches up to several nanometres deep and with corrugated walls, as also observed for polypyrrole on HOPG (see above). Fig. 6(b) shows a filtered image (gradient in  $x$  direction) of another location inside the polypyrrole tubules, which clearly visualizes the long ditches and steps on the surface. This image is a strong indication that inside the polypyrrole tubules the surface is sufficiently corrugated to adsorb

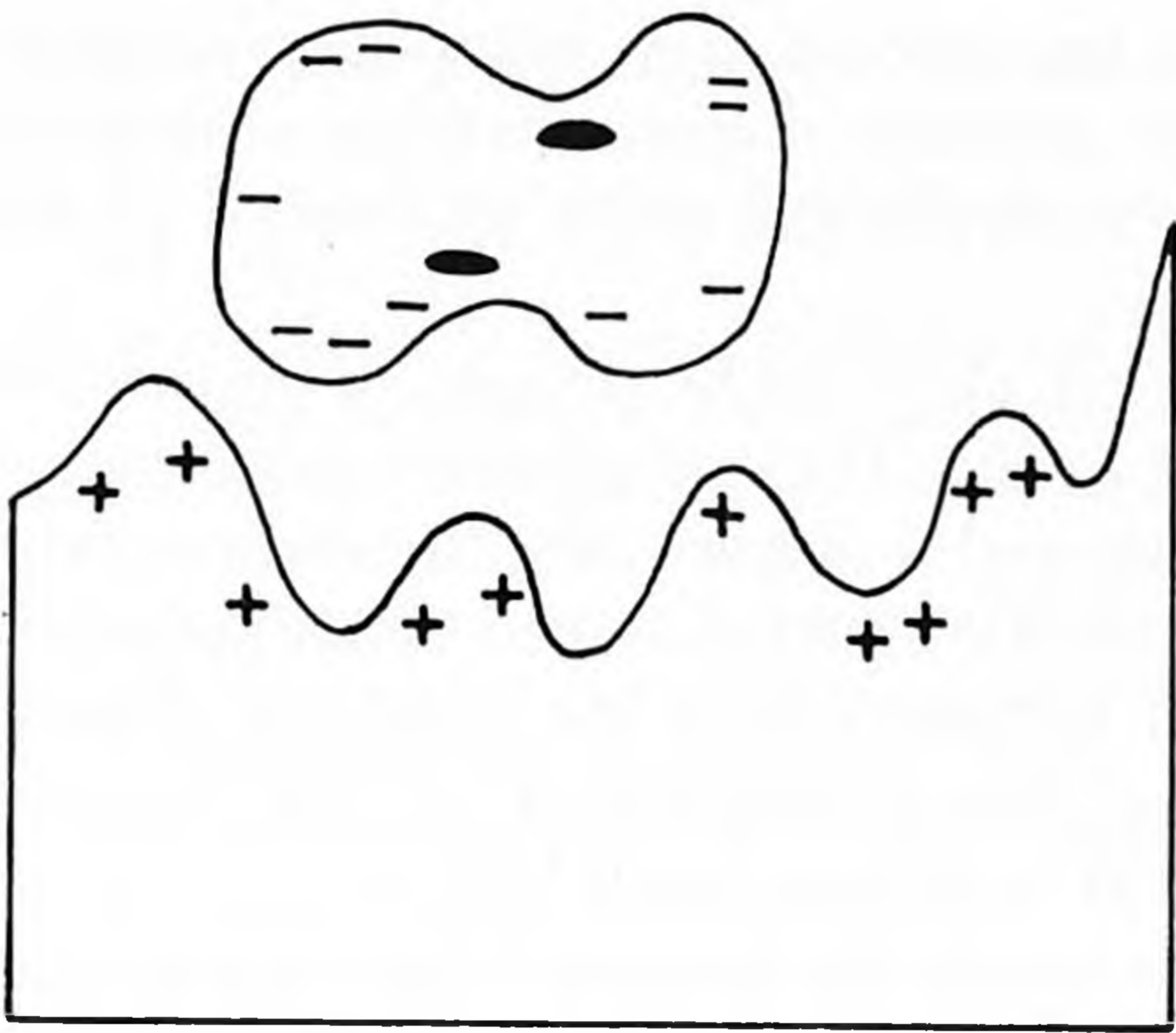


Fig. 7. Model for the interaction of glucose oxidase with the interior of the polypyrrole microtubules in the biosensor.

glucose molecules in such a way that the conducting polymer can get very close to the active centre of the enzyme. The most interesting feature is that the dimensions of the polypyrrole corrugations are in accordance with the molecular dimensions of glucose oxidase. If we assume that the native enzyme molecule is shaped like an ellipsoid or an "8", with the active centres in the grooves, the polypyrrole matrix can be imagined to interact with the surface of the enzyme as well as with the active centres.

The surface interaction between glucose oxidase and polypyrrole is most likely electrostatic in nature, since polypyrrole in its conducting state is a polycation [30] and glucose oxidase at neutral pH is a polyanion [28]. Experimental evidence for this electronic interaction has been presented in a previous paper [12]. Apparently, the concave structure of the tubules is very important. It allows the polypyrrole to interact with glucose oxidase from different directions. This multisite interaction may keep the enzyme biologically active while at the same time increasing the chance that direct electron transfer occurs [2,31]. A model for the strong interaction between glucose oxidase and polypyrrole is presented in Fig. 7. This figure shows a depth profile of an STM image of the polypyrrole microtubules and a drawing of a glucose oxidase molecule on the same scale. The dimensions of the enzyme molecules and the polypyrrole surface corrugation inside the microtubules match sufficiently to make electron transfer feasible.

## CONCLUSIONS

(1) We have obtained STM images of polypyrrole films synthesized electrochemically under various polymerization conditions on an HOPG electrode. The electrochemical deposition appears to occur at numerous and isolated sites all over the HOPG electrode surface. Initially, only a few polymerization sites are present,

which slowly grow into dome structures that impinge on each other. The polypyrrole films that are eventually obtained exhibit disordered non-crystalline structures with many ditches up to several nanometres in depth and width and with 1–2 nm corrugations on the walls.

(2) We have been able to obtain the first clear STM images of individual glucose oxidase molecules adsorbed on gold facets. These molecules show lateral dimensions of 14–18 nm along the longer axis and 5–8 nm along the shorter axis. These horizontal dimensions are in accordance with data obtained by ellipsometry [28]. The vertical dimensions are somewhat different from the expected dimensions. This can be attributed both to the strong adsorption of the enzyme molecules on the gold surface and to the STM technique itself [27].

(3) STM images of the polypyrrole surface inside the microtubules suggest that the conducting polymer may have a strong multisite interaction with the glucose oxidase molecules. This interaction accounts for the observed direct electronic communication between polypyrrole and the redox enzyme and may explain why no denaturation of the enzyme occurs.

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