Polypyrrole microtubules and their use in the construction of a third generation biosensor

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Abstract
Conducting polypyrrole microtubules have been prepared by template synthesis inside track-etch membranes. The interiors of these microtubules can adsorb the redox enzyme, glucose oxidase. The enzyme-coated tubules have been employed in the construction of a third generation amperometric biosensor for the determination of glucose. With this biosensor, glucose concentrations in the range 0.1–250 mM can be measured easily. The polypyrrole microtubules have been characterized by different microscopic techniques, including scanning tunneling microscopy. Based on the microscopy data, a model is presented for the interaction between the conducting polymer and the glucose oxidase molecules.

Introduction
Nowadays, much effort is being directed toward the development of so-called third generation biosensors [1–7]. These sensors detect an analyte amperometrically without the need of a co-substrate or a low molecular weight mediator as in first [8, 9] and second [5, 10, 11] generation biosensors. In a third generation biosensor the redox enzyme (e.g., glucose oxidase) communicates freely with the electrode. In general, a redox enzyme does not interact directly with an electrode material as its active center is surrounded by an insulating protein shell [12]. To overcome this, modified electroconductive polymers have been studied as mediators in recent years. In such polymers the enzyme can easily be entrapped during the electrochemical polymerization process [13, 14].
However, the results have been disappointing in so far as the enzyme loses its activity.

In a previous communication we reported that glucose oxidase can be adsorbed irreversibly in conducting microtubules of polypyrrole, while retaining its activity [15]. Polypyrrole microtubules are prepared by diaphragmatic oxidative polymerization of pyrrole inside the pores of a track-etch filtration membrane [16, 17]. During the polymerization reaction the membrane acts as a template for microtubule formation. We have described an amperometric glucose sensor in which these microtubules are successfully used as mediators between enzyme and electrode [18]. Apparently, the microscopic space inside the pores of the track-etch membranes, and the morphology of the interior of the polypyrrole microtubules, are important for realizing the direct electron transfer from the enzyme to the conducting polymer. In this paper we report on the surface morphology of the polypyrrole microtubules as studied by scanning electron microscopy (SEM) and scanning tunneling microscopy (STM). It will be shown that the dimensions of the polypyrrole surface corrugation matches the dimensions of the glucose oxidase molecules [19]. This opens the possibility of close contact between enzyme and polymer and provides a pathway for direct electrochemical interaction.

**Experimental**

Details regarding the construction of the biosensor have been described elsewhere [18]. Polymerization of pyrrole inside the filtration membranes was carried out as described by Cai and Martin [16].

All electrochemical measurements were performed with an Autolab potentiostat controlled by an Olivetti M24 Personal Computer and General Purpose Electrochemical System (GPES)-software (Eco Chemie, The Netherlands). Current output was recorded on a Yew 3556 pen recorder. Electron micrographs were made on a CAMSCAN scanning electron microscope (Cambridge Instruments). Convocal laser scan microscopy and scanning tunneling microscopy images were made on home-made instruments.

To perform amperometric analysis, the enzyme membrane was placed as working electrode in a three-electrode flow cell (Sparc Holland). An Ag/AgCl electrode was used as reference. The base of the flow cell acted as auxiliary electrode (glassy carbon). Phosphate-buffered saline solution was driven through the cell at a rate of 1.75 ml min$^{-1}$ (Watson Marlow 101U peristaltic pump). The potential of the membrane was set at 0.350 V versus the Ag/AgCl reference. During flow injection analysis the buffer solution was replaced for 20 s by a glucose solution and the resulting current transient was monitored.
Results

Polypyrrole tubules synthesis

Polyester and polycarbonate track-etch filtration membranes (10 \( \mu \text{m} \) thickness) were treated with pyrrole in a manner described in our previous paper [18]. Membranes with pore sizes of 600, 800 and 1000 nm were chosen for the template synthesis of the polypyrrole microtubules. The inner diameter of the microtubules was controlled by varying the time of the polymerization reaction. Scanning electron microscopy (SEM) showed how the pores of the membranes became gradually filled with conducting polymer with increasing polymerization time (Fig. 1). On increasing the polymerization time the porosity of the membranes decreased in addition to the electrical resistance over the membrane. After 4 min, non-porous membranes were obtained with an electrical resistance of 1.9 \( \Omega \).

Refluxing a polycarbonate membrane in dichloromethane for several hours caused the membrane material to dissolve, and the polypyrrole microtubules could be isolated by filtration of the solvent. Figure 2(a) shows a photograph of isolated polypyrrole microtubules as imaged by SEM. Figure 2(b) shows a further enlargement of some isolated tubules. It is clearly visible that the microtubules are hollow. Furthermore, from the length of the tubules it can be concluded that the complete pores of the track-etch membrane have been coated with polypyrrole.

Immobilization of glucose oxidase

Glucose oxidase or fluorescein-labeled glucose oxidase was physically immobilized inside the polypyrrole microtubules by adsorption. To this end the polypyrrole-containing track-etch membranes were soaked in a 10 mM phosphate buffer solution (pH 7.4) containing the enzyme. After enzyme treatment, the membrane was rinsed in buffer solution to remove any enzyme material adsorbed on the surface of the membrane, and dried. This leaves only irreversibly-adsorbed enzyme. Confocal laser scan microscopy (CLSM) in fluorescence mode was used to image the immobilized fluorescein-labeled glucose oxidase [20]. Figure 3 shows a typical CLSM image of an enzyme-treated membrane. It can be seen that adsorption of enzyme has preferentially taken place on the walls of the polypyrrole microtubules. Membranes which had not been modified with polypyrrole did not show this behaviour. The CLSM image even suggests that the enzyme has penetrated the microtubular walls. This may lead to strong interaction between the enzyme and polypyrrole (vide infra).

Construction of biosensor

We constructed a biosensor electrode from the polypyrrole-modified membrane by coating one of its sides with platinum before enzyme immobilization (Fig. 4) [15]. The membrane was placed as the indicator elec-
trode in a flow-injection set-up. Amperometric measurements were performed by introducing glucose samples of various concentrations in the carrier stream and recording the resulting current transient. All experiments were carried out under an argon atmosphere at a potential of 0.35 V (versus Ag/AgCl). Catalase was present as a hydrogen peroxide scavenger in all solutions. Under these conditions oxygen mediation and accidental hydrogen peroxide detection can be excluded. Figure 5 shows the amperometric response of a typical membrane sensor to glucose concentrations in the range 0.1–250 mM. We found that glucose concentration in this range can be measured easily with good and reproducible results.

Fig. 1. Scanning electron micrographs of track-etch membranes after various polymerization times: (a) untreated membrane, original pore size 800 nm; (b) polymerization time 2 min.; (c) polymerization time 5 min.
STM study

Figure 6(a) shows a scanning-tunneling micrograph of the surface inside the polypyrrole microtubules. A depth profile on nanometer scale is presented in Fig. 6(b). This profile reveals that the polypyrrole surface is strongly corrugated. Typical values of the width and height of the corrugations are approximately 5–6 nm and up to 2.5 nm, respectively. The individual glucose oxidase molecules, as imaged by STM, have an oval shape with a dimension of 14–18 nm along the main axis and 5–8 nm
Fig. 3. Confocal laser scan image in the fluorescence mode of a membrane, containing polypyrrole microtubules of 1 μm dia., treated with fluorescein-labeled glucose oxidase. Only the microtubular walls are fluorescent (see arrows).

Fig. 4. Schematic representation of the biosensor.

along the perpendicular direction (Fig. 6(c)). These dimensions are in agreement with the literature values [21, 22]. The most interesting finding is that the dimensions of the polypyrrole corrugations match the molecular dimensions of glucose oxidase. A complete scanning-tunneling-microscopy study on the surface morphology of polypyrrole in microtubular form, and of individual glucose oxidase molecules, will be presented elsewhere [19].
Fig. 5. Plot of the measured anodic current response as a function of glucose concentration. A track-etch membrane with 800 nm pores was treated with pyrrole and iron chloride oxidizing agent for 1 min and incubated with glucose oxidase. The measurements were carried out at 0.350 V vs. Ag/AgCl under argon atmosphere. A phosphate-buffered saline solution with 25 U ml⁻¹ catalase was used.

Fig. 6. (a) STM image of the interior surface of a polypyrrole microtubule, area ~ 80 x 80 nm², vertical scale 30 nm; (b) cross section of the image in (a); (c) top view of glucose oxidase molecules adsorbed on an atomically-flat gold surface, area 36 x 36 nm².
Discussion

Immobilization of glucose oxidase by adsorption inside the polypyrrole microtubules appears to be irreversible. Enzymatic activity is retained. Apparently, the concave structure of the tubules is essential in this respect. It probably allows the polypyrrole to interact strongly with glucose oxidase from different directions. Interaction will be further enhanced by the nature of the two species involved. Polypyrrole in its conducting state is a polycation [23], whereas glucose oxidase at neutral pH is a negatively-charged species [21]. The negatively-charged groups on the enzyme molecules can replace the anions originally present in the polypyrrole structure. This results in electrostatic attraction, which may be very strong.

The shape and dimensions of the glucose oxidase molecules are such that these molecules can easily be adsorbed in the ditches on the surface inside the microtubules (Fig. 6(a)–(c)). This ensures that the electroactive sites on the conducting polymer surface are in the contacting range of the redox centers of glucose oxidase, resulting in direct electronic interaction.

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References