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Properties of mixed alkanethiol–dendrimer layers and their applications in biosensing

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Abstract

We studied the properties of mixed alkanethiol–dendrimer layers on a gold support and their application in biosensing. We showed that properties of glucose sensor can be modified using a different ratio of 1-hexadecanethiol (HDT) and poly(amidoamine) dendrimer of first generation (G1). The cyclic voltammetry in the presence of the redox couple, $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, was used for estimating how effectively the layer blocks the redox probe's access to the electrode surface. A scanning electrochemical microscope (SECM) was used to image the resulting distribution of the organic compounds. We found that with increasing content of dendrimers, the integrity of the layers was improved.

Keywords: Hexadecanethiol; Dendrimers; Glucose oxidase; Cyclic voltammetry; Scanning electrochemical microscopy

1. Introduction

The self-assembled monolayers (SAMs) are widely used for modification of the solid surface. In this respect, the SAM of long-chain alkanethiols on a gold support are of special interest due to their strong chemisorption and high degree of thermal and chemical stability [1,2]. However, two-dimensional structure and limited number of terminal groups of alkanethiol SAM restrict their application in the fabrication of enzyme biosensors. Recently, a new class of macromolecules—dendrimers—have been synthesized [3,4]. It has been shown that highly branched structure of dendrimers can be used for enzyme immobilization. At the same time, the monomolecular layers formed by dendrimers revealed high mechanical stability and can be functionalised without lost of dendrimers from the surface. The large area of dendrimers allowing to increase the number of immobilized functional units, and thus to increase the sensor sensitivity. Examples are multilayer enzyme films composed of a glucose oxidase (GOX) and the fourth generation of dendrimers (G4) [5].

We have shown that even for lower generation of dendrimers (G0 and G1), good stability of the glucose sensor can be achieved, especially when dendrimers were adsorbed onto the gold support together with hexylmercaptan [6] or hexadecanethiol [7]. However, the question of how the physical and structural properties of these mixed layers depends on the alkanethiol–dendrimer ratio arises. In this work, we therefore studied the properties of the mixed alkanethiol–dendrimer layers and the influence of the alkanethiol–dendrimer ratio on the properties of the glucose oxidase, immobilized on a surface of these mixed layers. Cyclic voltammetry [8] and scanning electrochemical microscopy (SECM) [9–11] have been used to study the properties of SAM.

2. Experimental

2.1. Chemicals

GOX (EC 1.1.3.4, type VII, 144.8 units/g solid), isolated from *Aspergillus niger*, and glutaraldehyde, specially purified for electron microscopy, were purchased from Sigma, USA. 1-Hexadecanethiol (HDT), ethyl alcohol, chloroform and

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poly(amidoamine) dendrimer (PAMAM, generation 1—G1) were obtained from Aldrich, USA. Ferrocyanide, monobasic and dibasic potassium phosphate, potassium chloride, sulfuric acid and hydrogen peroxide were obtained from Merck, Germany. The buffer solution for the amperometric measurements was composed of a mixture of mono and dibasic potassium phosphate, concentration of the buffer was 0.1 M and pH 7.6. The electrolyte for SECM was composed of 4 mM $K_4Fe(CN)_6$/1 M KCl. All solutions were prepared with high-purity chemicals and by using deionized water (resistance >15 MΩ cm, ELIX 5, Millipore, El Paso, USA).

2.2. Preparation of the mixed hexadecanethiol/dendrimer layers and enzyme sensors

The gold electrode (Minerale, Poland) of 1.6 mm diameter was used for the preparation of the biosensor. The gold electrodes were cleaned as described elsewhere [12]. The SAMs were prepared by immersion of the gold electrodes in ethanolic solution containing different molar ratios of HDT and G1 (1:0.43; 1:0.82; 1:1.5; 1:3; 1:9) for 18 h. This procedure resulted in the formation of stable hexadecanethiol/dendrimer layers.

GOX was then immobilized on a surface of the film according to the procedure described in detail elsewhere [6,7]. Briefly, a 15-μl drop of GOX dissolved in a phosphate buffer (concentration 1 mg/ml) was added to a surface of electrode covered by HDT/G1 layer. After the water was evaporated, the GOX molecules were cross-linked with glutaraldehyde. For this purpose, the electrode was immersed in a vacuum compartment (volume 10 cm^3) for 30 min (the pressure in a compartment corresponded to 30 mm of Hg column). The compartment contained small amount (1 ml) of glutaraldehyde dissolved in deionised water in a concentration of 5%. After this procedure, the enzyme sensors were stored in small PVC container in a refrigerator at 4 °C.

2.3. Experimental techniques

The amperometric and cyclic voltammetry experiments were performed by means of PGSTAT 10 (Eco Chemie, The Netherlands) controlled by GPES 4.7 software using a three-electrode system: A gold electrode served as a working electrode, a platinum electrode was used as a counter and saturated calomel electrode (SCE) as a reference electrode. Amperometric detection of enzymatic reaction was based on measuring the current of anodic degradation of hydrogen peroxide at a potential of 670 mV (positive terminal was on a gold working electrode) in an electrochemical cell 25 ml in volume.

The SECM allowed us to obtain an image of the surface immersed in the liquid phase by measuring the faradaic current produced by an electron-transfer reaction at a small tip. Usually, the sample studied is placed under a thick liquid layer containing a quasi-reversible redox couple, the so-called mediator. Therefore, the tip must be insulated, e.g., by glass. When the potential is applied between the tip and the working electrode, the diffusion current can be measured. At sufficiently large distance of the tip from the surface, the steady-state diffusion current, $i_{T\infty}$ is given by:

$$i_{T\infty} = 4nFDcr_T \quad (1)$$

where n is the number of transferred electrons ($n=1$ in our case), $F=9.65\times10^4$ C/mol is the Faraday constant, D is a diffusion coefficient, c is the bulk concentration of mediator, r_T is the exposed radius [8]. Current $i_{T\infty}$ serves as reference value for measurement taken with the platinum tip in close proximity to the sample surface. If the tip is within a distance $d<5r_T$ to an inert, insulating surface, current falls below $i_{T\infty}$ due to hindered diffusion of the mediator. This phenomenon is called negative feedback. Current larger than $i_{T\infty}$ can be observed above the sample area where the mediator is recycled by an electrochemical reaction. This mode is called positive feedback [9].

As a tip, we used platinum wire 25 μm in diameter and sheathed by glass. The sample was placed on a surface of gold electrode (diameter 250 μm) coated by glass, which served as a working electrode. A silver wire was used as a counter electrode. The experiments were performed in a cell 10 ml in volume. The electrolyte was composed of 4 mM $K_4Fe(CN)_6$ + 1 M KCl. The amplitude of potential between the gold support and the tip was 500 mV (negative terminal was on a gold working electrode).

3. Results and discussion

In the first series of experiments, we used cyclic voltammetry to study the integrity of the layers of different molar ratios of HDT/G1. For this purpose, the electrode covered by the layer was immersed in a solution containing a redox

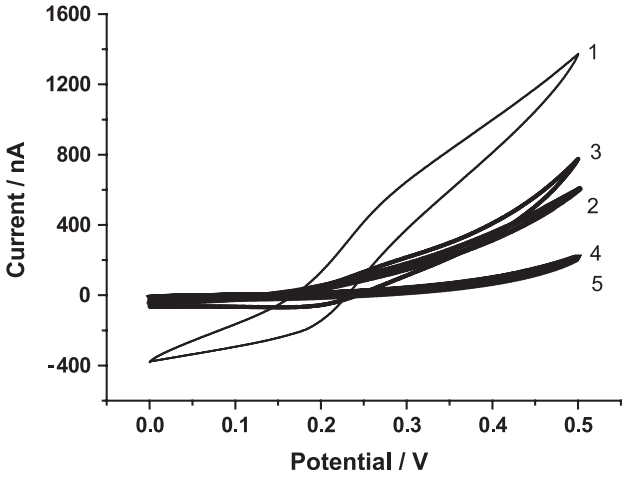

Fig. 1. Cyclic voltammograms of gold electrodes covered by mixed HDT/G1 monolayers of different molar ratios of HDT and G1 recorded in an aqueous solution of 4 mM $K_4Fe(CN)_6$ + 1 M KCl. Scan rate 0.03 V/s. (1) 1:0.43; (2) 1:0.82; (3) 1:1.5; (4) 1:3; (5) 1:9 mol/mol.

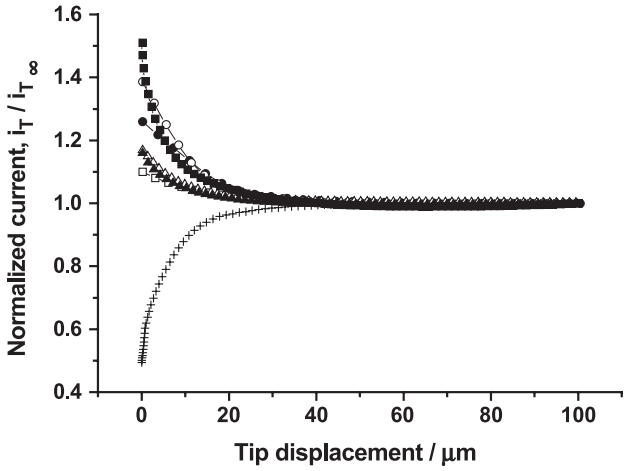

Fig. 2. Dependence of the tip current i_T normalized to the diffusion current $i_{T\infty}$ on relative tip displacement over a conductive gold surface without coverage (■) and that covered by hexadecanethiol SAM (+) or a mixed HDT/G1 layer of a different HDT/G1 molar ratio: (●) 1:0.43; (○) 1:0.82; (□) 1:1.5; (Δ) 1:3; (▲) 1:9 mol/mol in a solution containing 4 mM $K_4Fe(CN)_6$ + 1 M KCl.

couple, $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$. The shape of cyclic voltammogram depends on the integrity of the layers, i.e., how effectively they block the redox probe's access to the electrode surface. Fig. 1 shows typical cyclic voltammograms for electrodes covered by mixed HDT/G1 layers of different HDT/G1 molar ratios. It is seen that by increasing the content of dendrimers, there is a tendency to decrease the current flowing across the mixed layer and to smoothen the redox waves. Absence of the redox waves close to the formal potential of the probe indicates that the layer is completely impermeable for redox probe species. On the other hand, the presence of the redox wave (Fig. 1, curve 1, HDT/G1 ratio is 1:0.43) shows that the layer is loosely ordered or contains numerous structural defects and thus can be easily penetrated by external molecules.

The results of SECM experiments are shown in Fig. 2, where the plot of the normalized current between the gold surface and the tip vs. the relative tip displacement over a gold support for a different coverage of the gold electrode is presented. We can see that for the layer composed of HDT, the current decreases when the tip approaches the surface of the working electrode. This decrease is caused by diffuse limitation of the current [13]. However, for uncovered gold surface as well as that covered by a mixture of HDT/G1 layers, an increase in the current was observed. This effect was obviously most intensive for uncovered gold electrode. The diffusion current at the distance far from the surface of working electrode was approximately 1.4 nA in this case. Using Eq. (1) and considering that the diffusion coefficient for 4 mM $K_4Fe(CN)_6$ is approximately 6.3×10^{-6} cm^2 s^{-1} [9], we obtained for effective radius of the tip the value 14.4 μm, i.e., the diameter is 28.8 μm, which is close to the geometric diameter of the tip (i.e., 25 μm). The considerable increase of the current similar to that characterized for uncovered gold surface took place also for mixed layers of HDT/G1 ratios equal to 0.43 and 0.82 mol/mol. For higher content of dendrimers, the current increase was less expressed. The SECM method allowed us to obtain two-dimensional image of the regions of different conductivity. This is demonstrated in Fig. 3. As an example, we selected the layers composed of a mixture of higher conductivity, i.e., HDT/G1 = 1:0.82 mol/mol as well as the layer characterized by lower conductivity, i.e., HDT/G1 = 1:1.5. We can see that in the case of lower content of dendrimers, the considerable area of higher current on the image indicates a lot of defects in the layer (Fig. 3a). However, the image presented in Fig. 3b is characterized by considerably lower currents, which indicate no visible defects or pinholes.

It is evident from above that conductivity of the layer in general tends to decrease with increasing content of dendrimers. However, from known properties of the den-

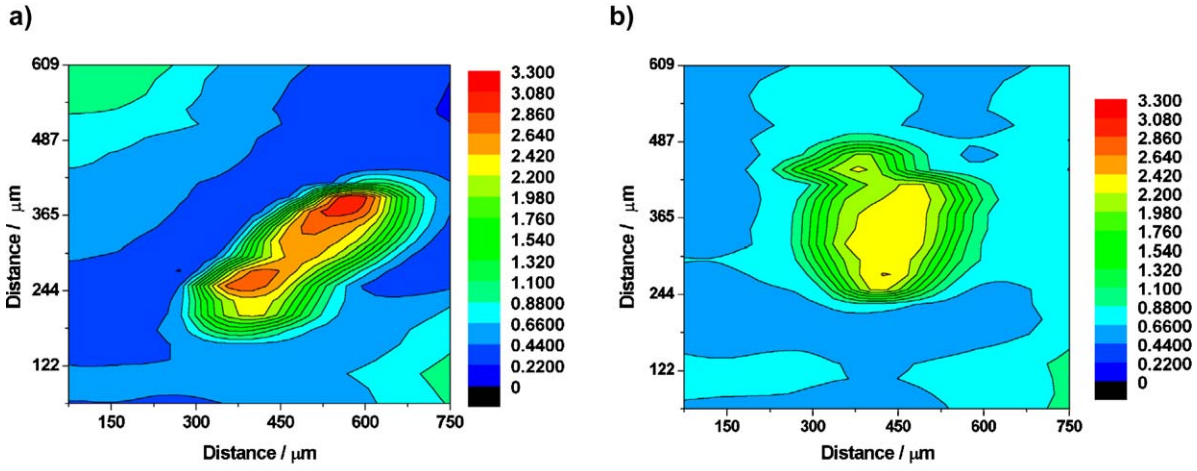

Fig. 3. SECM image of the layers formed from the HDT/G1 mixture in a molar ratio of (a) 1:0.82 (I_{max} = 3.02 nA) and (b) 1:1.5 (I_{max} = 2.43 nA). The regions of different color (colour available in the on-line version) are characterized by different amplitudes of the current between the tip and the working electrode—see scale of the current (nA) at the left side of the figures.

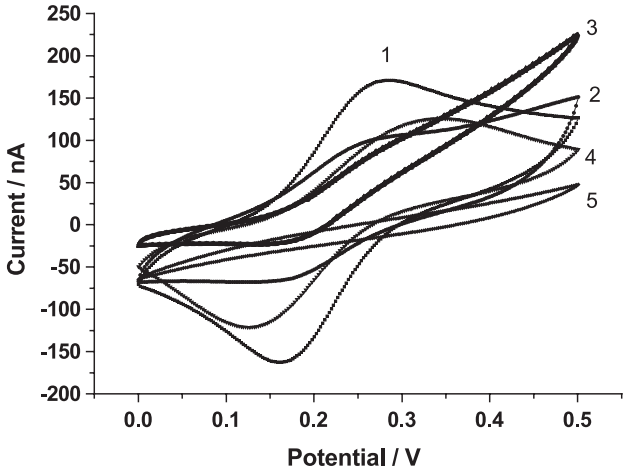

Fig. 4. Cyclic voltammograms of gold electrodes covered by mixed HDT/G1 monolayers of different HDT/G1 molar ratios with immobilized GOX recorded in an aqueous solution of 4 mM $K_4Fe(CN)_6$ + 1 M KCl. Scan rate 0.03 V/s. (1) 1:0.43; (2)1:0.82; (3) 1:1.5; (4) 1:3; (5) 1:9 mol/mol.

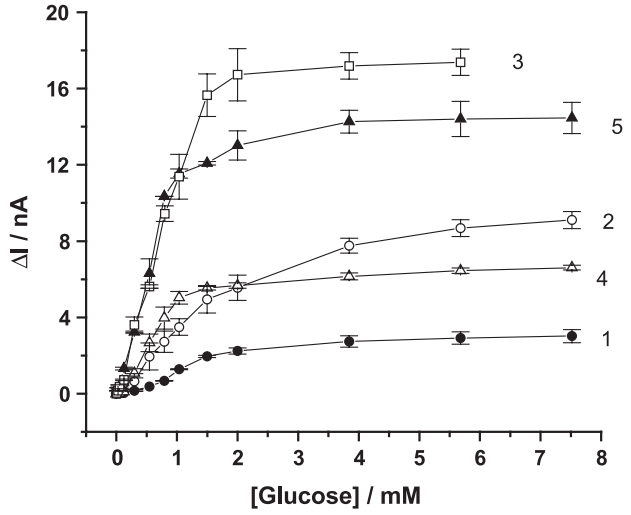

Fig. 5. Changes of the current ΔI ($\Delta I = I - I_0$, where I_0 is the initial current prior addition of glucose) vs. concentration of the glucose for GOX immobilized on a mixed layer of different HDT/G1 molar ratios: (1) 1:0.43; (2) 1:0.82; (3) 1:1.5; (4) 1:3; (5) 1:9 mol/mol.

drimers, it is evident that the pure dendrimer films should be more conductive than that composed of alkanethiols. In fact, it is evident from Fig. 2, i.e., the diffusion current flowing through HDT layer is smaller than that flowing through mixed HDT/G1 film with relative small content of dendrimers. Thus, based on the above assumption, we would expect an opposite effect, i.e., the conductivity of the films on a gold support should increase with increasing G1/HDT ratio. The obtained opposite effect is difficult to explain. We can, however, speculate that at lower content of G1 in the G1/HDT mixture, the dendrimer molecules play a role of certain impurities that restrict the formation of compact HDT layers. In contrast with HDT, which interacts with gold by chemisorption, dendrimers adsorb to the gold surface physically. They probably could also adsorb to that part of the gold surface that are not available for alkanethiol molecules, i.e., containing some impurities and therefore resulting in so-called pinholes. If the above assumption is true, then increased concentration of dendrimer molecules in a mixed G1/HDT layer should result in coverage of the gold surface that contained pinholes. Thus, the coverage of pinholes should result in the decrease in the conductivity of the film.

Having the information about the properties of the mixed HDT/dendrimers layers, it is interesting to compare the properties of the glucose sensor formed by means of immobilization of GOX on mixed layers of a different HDT/G1 ratio. Fig. 4 shows the cyclic voltammograms of the gold electrode covered by mixed layers composed of a different ratio of HDT and G1 with immobilized GOX. After the immobilization of glucose oxidase on the amino-terminated groups of dendrimers, the resulting faradaic current decreased for each tested biosensor (compare with Fig. 1). It is evident that the layers composed of higher content of dendrimers (HDT/G1 = 1:1.5–1:9) are characterized by lower conductivity in comparison with layers of higher HDT content (HDT/G1 = 1:043–1:0.82) (Table 1). These results are in agreement with that presented above for the HDT/dendrimer layers without GOX. The overall decrease of the current following immobilization of GOX also evidence that attachment of GOX molecules to the film did not cause desorption of the G1 and HDT molecules from the gold surface.

In order to compare the properties of glucose sensors based on GOX immobilized on a mixed G1/HDT layer, we

Table 1
Comparison of the properties of the glucose biosensors based on GOX immobilized on a SAM composed of a mixed hexadecanethiol/dendrimer (G1) of a different molar ratio

HDT/G1 ratio, mol/mol	K_m, mM	Enzyme turnover, s^{-1}	Sensitivity, nA cm^{-2} mM^{-1}	Specific conductance, 10^{-5} Ù$^{-1}$ cm^{-2}	Number of experiments
1:0.43	1.51 ± 0.27	0,27 ± 0.03	52.2 ± 14.4	3.5 ± 0.5	8
1:0.82	1.64 ± 0.08	0,81 ± 0.04	164.9 ± 23.6	3.6 ± 0.3	6
1:1.5	1.01 ± 0.27	1.71 ± 0.34	531.9 ± 50.9	1.4 ± 0.3	8
1:3	0.52 ± 0.14	0,58 ± 0.01	243.3 ± 16.0	2.2 ± 0.5	5
1:9	0.75 ± 0.22	1.33 ± 0.15	558.2 ± 112.1	1.7 ± 0.3	6

Specific conductance has been determined from cyclic voltammograms in an electrolyte 4 mM $K_4Fe(CN)_6$ + 1 M KCl for voltage range 0–0.1 V. The results were obtained on five to eight independently prepared electrodes. The results represent means ± S.D.

measured the current response of the sensor as a function of glucose concentration. The corresponding plot of the current vs. glucose concentration for the sensors studied is presented in Fig. 5. We can see that the plot for each mixed layer has typical shape characteristic for enzyme activity at the electrode surface. Using the results presented in Fig. 5, we calculated the basic kinetics parameters of the enzyme reaction for all systems studied (the method of the calculation has been described in detail elsewhere [7]). The results of calculation are presented in Table 1. There was a tendency of decrease in the Michaelis–Menten constant, K_m, with increased content of dendrimers. The values of K_m were, however, lower than that for free GOX in a solution (33 mM, see Ref. [14]), which may be connected with a slower rate of glucose oxidation at the amphiphilic surface as it is revealed from considerably lower value of enzyme turnover in comparison with that for free GOX in a solution (approximately 340 s^{-1} [14]). This result proves the reduction in the degree of the conformational freedom of GOX molecules at a sensor surface. The GOX at neutral pH is negatively charged with 11 charges. Thus, the electrostatic interaction with the substrate could result in dissociation of the GOX dimers, and thus should change the kinetic parameters of enzymatic reaction [15]. As it is seen in Table 1, by increasing the content of the dendrimer, there was also a certain tendency for enzyme turnover and sensitivity of the GOX sensor to increase. Both effects could be connected with the fact that increased content of dendrimer allows to immobilize more GOX molecules at the sensor surface. Relatively low enzyme turnover as well as the sensitivity of the sensor at higher content of amphiphilic compound—HDT—could evidence certain unfolding of the enzyme at the more hydrophobic surface [16]. It is interesting that obtained sensitivity in this case was similar with that reported by Sun et al. [16] for the GOX sensor not stabilized by glutaraldehyde and deposited on a platinum support by Langmuir–Blodgett technique.

4. Conclusion

The obtained results evidence that with increasing content of dendrimers in a mixed HDT/dendrimer layers, there is improved coverage of the electrode surface. On the other hand, the sensitivity of the sensor increases with increasing dendrimer content. This effect can be explained by increased number of GOX molecules at the sensor surface. For practicality purposes, it is important to prepare enzyme biosensor that is composed of the supported layer, which will provide not only optimal immobilization of enzyme molecules, but also preserve gold support from possible interferences of the species from the solution. According to the obtained results, it is possible to select the composition of HDT/dendrimers that will fit the above mentioned requirements, i.e., the most compact layer with no substantial restriction of enzyme activity and sensitivity. In our case, this feature holds for the layers with a HDT/G1 ratio of 1:1.5 or for higher dendrimer content. However, for other generation of dendrimers as well as for other alkanethiols, the optimal layer composition could be different.

Acknowledgements

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