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Combined atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and quartz crystal microbalance (QCM) studies of glucose oxidase (GOx) immobilised onto self-assembled monolayer on the gold film

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Summary

In fabrication of biosensors, self-assembled monolayers (SAM) are an attractive method of immobilizing enzymes at electrode surface since it allows precise control over the amount and spatial distribution of the immobilized enzyme. The covalent attachment of glucose oxidase (GOx) to a carboxylic terminated SAM chemisorbed onto gold films was achieved via carbodiimide activation of the carboxylic acids to a reactive intermediate susceptible to nucleophilic attack by amines on free lysine chains of the enzyme. Atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and quartz crystal microbalance (QCM) measurements were used for characterization of GOx modified gold surfaces. Tapping mode AFM studies have revealed that GOx molecules form slightly disordered arrays of pentagonal or hexagonal clusters. Observed features of immobilised GOx are distributed as a submonolayer on the SAM surface which has allowed visualisation of native and unfolded enzyme structure. The presence of the SAM and enzyme on the gold surface was detected by XPS spectroscopy. Spectra show typical peaks for the C 1s, O 1s and N 1s regions. A kinetic study of the adsorption of GOx onto activated SAM using in-situ QCM allowed determination the amount of immobilised GOx on the layer and consequently the optimal immobilisation conditions. Performance parameters of the biosensor such as sensitivity to glucose concentration as a function of enzyme loading were evaluated amperometrically using the redox mediator *p*-benzoquinone.

Introduction

Biosensors are increasingly becoming practical and useful tools in medicine, food quality control, environmental monitoring and research. The coupling of biorecognition molecules such as enzymes, antibodies or whole cells with either electrochemical, optical or piezoelectric signal transducer is the basis of a biosensor.[1] Immobilisation of the biological molecule is a particularly demanding aspect of the fabrication biosensors as the immobilisation procedure must reproducibly maintain the biorecognition molecule close to the transducer surface while retaining its biological activity.[2] Many physical and chemical methods for immobilising enzymes on solid electrode surface, like adsorption and physical entrapment within membranes, have been widely used.[1] Recently covalent attachment on self-assembled monolayer (SAM) has been investigated as a new, promising strategy for fabrication of a variety of biosensors.[2] Self-assembled monolayers (SAM) can potentially provide a reproducible and robust method of fabricating immobilised enzyme layers with considerable control over the orientation and distribution of the enzyme[2]. The well-ordered monolayers formed by alkanthiols on metal surfaces can be used to immobilise of single layer of enzymes close to an electrode surface with a high degree of control over the molecular architecture of the recognition interface[3] Some techniques such as electrochemistry [4, 5], ellipsometry [6], scanning tunneling microscopy (STM)[7], atomic force microscopy (AFM) [8], FTIR and X-ray photoelectron spectroscopy (XPS) [9], surface plasmon resonance [10],

quartz crystal microbalance (QCM) [11] have been employed for examination of covalent immobilisation of enzyme onto the SAM.

Glucose oxidase (GOx) is a common enzyme used in electrochemical biosensors for determination of glucose in medical and industrial situations. Our pervious studies [3,4,11] have been concerned with optimilisation of a procedure to ensure reproducible enzyme loading and defined morphology which may result in fabrication of more reproducible devices.

This abstract presents study of glucose oxidase electrode as a biosensor fabricated by direct covalent attachment of GOx onto SAMs. A combination of techniques are used, such as AFM, XPS and QCM, for the characterization of the sensing interface in an attempt to correlate the surface structure with the amperometric response of the resultant enzyme electrode.

Experimental Section

The enzyme electrodes were fabricated by the self-assembly of 3-mercaptopropionic acid (MPA) onto gold from an ethanolic solution as described previously [2,5] (see Figure 1).

Figure 1 Schematic representation of the immobilisation of GOx to a SAM on a gold surface

Gold films were prepared by gold evaporation onto silanised glass slides by (3-mercaptopropyl) trimethoxysilane (MPS). [12]. The carboxylic acid terminated SAM was immersed in a solution of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) to activate the SAM for enzyme immobilisation. The activated electrodes were then exposed to an enzyme solution where free amine groups on the enzyme surface nucleophilically attacked the succinimidyl-terminated SAM thus giving covalent attachment of the enzyme. Applied concentrations of GOx enzyme were 0.2 μg/mL, 2μg/mL, 10μg/mL, 25μg/mL, 120μg/mL, 300μg/mL, 480μg/mL and 600μg/mL with incubation times of 5, 30, 60, 90 and 120 minutes. Electrodes were thoroughly rinsed with the buffer solution and water and then dried and stored in a desiccator.

A Dimension 3100 AFM (Digital Instrument, Santa Barbara, CA) using Si_3N_4 cantilevers was used for imagining using tapping mode where tip lightly "taps" on the sample surface during scanning, contacting the surface at the bottom of its swing, see Figure 2.

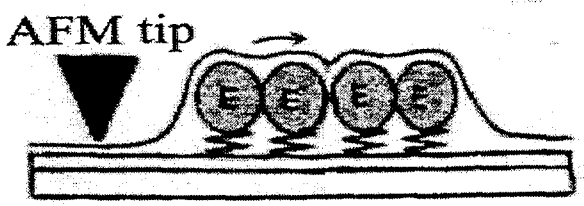


Figure 2 Idealized illustration of contact AFM tip and GOx enzyme covalently attached onto the SAM.

AFM images of bare gold, MPA modified gold and MPA-modified gold with immobilised with different concentration of GOx were obtained in air with optimally adjusted parameters of force curve, tip frequency, scan size and scanning speed. High-resolution images were difficult to achieve due to high interaction of tip as well as the highly flexible and soft surface of the GOx interface.

The enzyme electrode employed in the XPS studies were formed as described earlier. The analysis took place 1-3 days after the samples were prepared using XPS spectrometer with X–ray source of monochromated Al Kα line (1486.6eV).

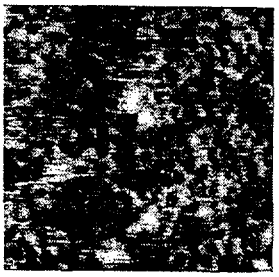
The kinetics of the adsorption of GOx onto EDC/NHS-activated self-assembled monolayer of MPA were monitored in situ using a home made QCM. The enzyme loading was estimated by using a "dip-and-dry" method that involved the measurement of the resonant frequency of a quartz crystal before and after enzyme immobilisation. Additionally, the amount of GOx immobilised was determined by stripping the active center from the enzyme, flavine adenine dinucleotide (FAD) out of the enzyme and fluorometrically measuring the concentration of FAD in solution [4].

The response of the enzyme electrode to glucose concentration was monitored amperometricaly at

+ 500 mV vs Ag/AgCl reference electrode in phosphate buffer with p-benzoquinone employed as a redox mediator.

Results and Discussion

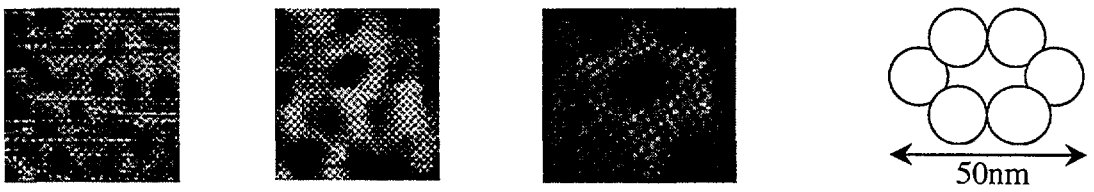
AFM characterisation



Surfaces of different samples of fabricated enzyme electrode made using different concentrations of GOx were studied by taping mode AFM in air. A typical image is presented in Figure 3 which shows the presence of array of pentagonal to hexagonal clusters with size of about 50nm in diameter.

Figure 3. A typical image of GOx enzyme immobilised on SAM concentration of 300µg/ml and deposition time of 60 minutes, scanning area 1um²

Profile analysis of enzyme surface shows an average height of 2-3nm which is much less then native GOx molecule. This may indicate that structures are in unfolded form but could also be an artifact of the AFM tip to provide a true profile of the surface relief. This is further confirmed by the apparent larger molecular size of individual GOx molecule in the AFM images. From the magnified images of linked group of clusters and isolated clusters shown in Figure 4a, 4b and 4c, a proposed model of immobilised GOx is shown in Figure 4d. Five to six GOx molecular units are linked in one ring and linked with other clusters to give an array structure of enzyme monolayer.



a) b) c) d)

Figure 4 . Magnified AFM images of GOx clusters with linked a) ten clusters b) two clusters c) one isolated hexametric cluster d) and proposed model.

This observation is in agreement with other studies of native GOx molecular structure[7,13] where the enzyme is described as dimeric protein containing one tightly bound flavin dinucleotide (FAD) per monomer and has ellipsoid shape with average diameter of 10nm per molecule. Similar images with same features are obtained for all samples with different concentration of GOx except for concentration of 0.2µg/ml which demonstrates that immobilisation from this concentration did not make uniform enzyme monolayer onto SAM. It is possible that small areas of assembled enzyme still exist on the surface but these were not

observed in the present experiment. Control experiments where the surface was repeatedly washed and then imaged with the AFM showed the clusters were robustly adhered to the surface. Furthermore, the AFM images obtained of bare gold, gold films with adsorbed MPA and activated MPA by coupling agent EDC/NHS did not show any of these surface features and possessed significantly different tip-sample interaction , thus providing strong evidence that these clusters are due to immobilized GOx.

The AFM images obtained in this way clearly distinguish arrays of linked GOx enzyme molecules which constructs a single monolayer on the SAM. These results correlate well with data from QCM and amperometric characterisation of electrode which shows good enzyme sensitivity to glucose as measured by a mediator redox system.

XPS, QCM and functional characterization

The steps in fabrications of enzyme electrode from assembly of MPA, activation with EDC/NHS and finally immobilisation of enzyme were assessed using XPS. The appearance of a N 1s signal (centered at 400.3eV) after activation step, a new component in the C 1s region (centered at 286.6eV) assigned to carbon atoms linked to sulfide group of NHS and an O 1s peak (centered at 531.6eV) assigned to oxygen atoms in the sulfonate group indicates that NHS was present on the surface. The presence of GOx immobilized on the surface of the SAM could be confirmed by changes of peaks in C 1s region attributed to the presence amino acid side chains of polypeptide backbone and in cabrbohydrate residues of its polysaccharide shell. The N 1s peak at 400.1eV was assigned to nitrogen atoms present in the amide bonds in the polypeptide chain, amino acid chain and N-acetylglucosamine residues.

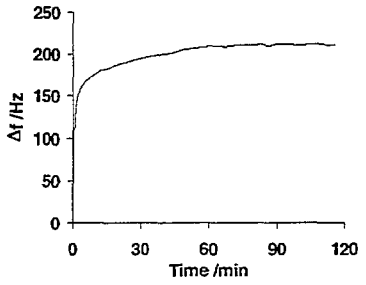


Figure 5. Response frequency change as function of time for adsorption of GOx

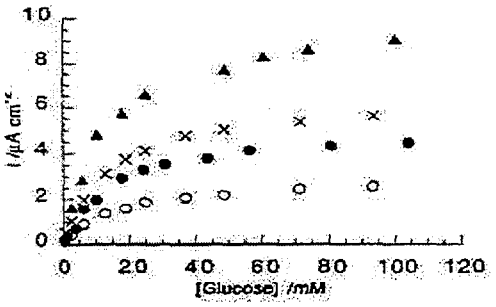


Figure 6. Influence of enzyme loading on the response to glucose concentration

The QCM study of kinetics of the adsorption of GOx onto DDC/NHS activated self-assembled monolayer of MPA monitored in situ is shown in Figure 5. These adsorption experiments indicate that extreme care must be taken when selecting the condition for enzyme immobilisation for reproducible sensor fabrication. It is clear that an initial 30 minute immersion period would have been insufficient to result in complete monolayer coverage. The optimal enzyme immobilisation procedure was chosen to be 90 minutes incubation in 480μg protein/ml of pH 5.5 buffer. These conditions would ensure complete monolayer coverage without a significant extension of the incubation period. Using this enzyme incubation time between electrode variability was reduced to less than 10%, reproducibility superior to that achieved with polymer immobilization [13]. Finally, the electrode prepared using optimised conditions exhibited a significantly improved reproducibility compared to those previously fabricated as illustrated in Figure 6 which shows the influence of enzyme loading (o) 0.08pmol/cm ^{2}, (•) 0.13pmol/cm^2, (x) 0.019pmol/cm^2 and (_) 0.29pmol/cm^2 versus the response of the enzyme electrode to glucose monitored amperometricaly with p-benzoquinone as a redox mediator.

Conclusions

AFM images of glucose oxidase covalently attached to self-assembled monolayers on gold were obtained. These give useful qualitative information about immobilized layer of the enzyme. Molecules of enzyme are assembled in single layer which shows pentametric or hexametric cluster structures of about 50nm in diameter which suggest a native and unfolded enzyme structure. In correlation with other techniques such as QCM, XPS and amperometric examination, it was shown that reproducibility and sensitivity of biosensor depends significantly on the procedure used in immobilisation of enzyme to the electrode. The covalent immobilisation of enzyme with alkanthiols is very promising immobilisation technology in fabrication of biosensors.

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