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Self-assembling monolayer formation of glucose oxidase covalently attached on 11-aminoundecanethiol monolayers on gold

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Glucose oxidase (GOx) has been attached covalently to form uniform enzyme monolayers on self-assembled monolayers (SAMs) from 11-aminoundecanethiol (AUDT) by taking advantage of chemical oxidation of GOx carbohydrate residues followed by coupling the resulting 'aldehydic' enzyme with the terminal amino group in the SAM as characterized by AFM imaging, IR, QCM, and electrochemical measurements.

The specific attachment of biomolecules such as proteins or DNA onto solid supports is of substantial interest for research in biotechnology. Recent activities in this area have focused on patterned, or spatially arranged, assemblies of biomolecules[1] since such a molecular level design is key for the development of high-performance biosensors and devices to mimic biological cell membranes. Among a wide range of biomolecules, glucose oxidase (GOx) is the most widely used enzyme in biosensor applications due to its robustness and stability. In the present paper, we will report self-assembly of GOx covalently attached on SAMs from **AUDT**.

In an electrochemical biosensor application, it is essential to provide intimate contact between the enzyme molecules and the sensing surface while maintaining, even improving, enzyme stability. In this regard, several physical and chemical schemes[2] can be used involving reconstitution of an apoenzyme[3] while SAMs have presented an attractive environment for gentle, stabilizing immobilization of biomolecules.[4] Now, we have achieved a stable attachment of GOx by oxidizing its carbohydrate residues with periodic acid[5] followed by coupling the 'aldehydic' enzyme with the terminal amino group in the SAM (Fig. 1). To date, much of the work has dealt with the methodology of chemical modification of amino residues in GOx. In viewing the specific structure of GOx, however, the mannose type carbohydrate resides which represent ~16% in weight and are not implicated in catalytic action should find a role in enzyme immobilization.

Oxidation of the GOx-bound sugar residues was performed with sodium metaperiodate according to the established procedure.[6] An enzymic assay which was made spectrophotometrically at 460 nm by the coupled peroxidase/*o*-dianisidine system[7] showed that this type of chemical modification lowered the specific enzymic activity, but only slightly; two independent enzyme preparations gave activity loss of 5.1% (255 unit mg^{-1} for native and 242 unit mg^{-1} for aldehydic GOx) and 11% (210 and 186 unit mg^{-1}, respectively).[8]

Evaporated gold films with monotonically high steps were resistively evaporated onto mica and used for atomic force microscopic (AFM) measurement.[9] The gold substrate was first treated with **AUDT**[10] solution (1 mM in ethanol) and the SAM was allowed to form for 24 h at room temperature. The substrate was rinsed thoroughly with ethanol followed by water, then further treated with the aldehydic GOx solution (12–13 μM in 40 mM phosphate buffer, pH 6). The reaction was kept for 5 h at 5 °C. Finally, the substrate was gently but thoroughly rinsed with water, dried under vacuum, and used for experiments immediately.

Fig. 2 shows a representative AFM image obtained for the gold substrate with the GOx/**AUDT**/Au interfacial structure. Although some aggregated domains are observed in part, the substrate surface is covered almost completely by finely distributed, compact spheroidal particles. When we used an aldehydic GOx solution with lower concentration, surfaces were observed having many defects in the adlayer; in a qualitative sense, the fraction of the defected structure was deemed to increase with decreasing concentration of the enzyme. For control, neither the combined use of 1) native GOx and **AUDT**, nor 2) the aldehydic GOx and undecanethiol gave noticeable immobilization of the particle. Thus, we can conclude that each compact spheroid appearing in the AFM image is a single GOx molecule.

Hecht *et al*. reported the corresponding dimensions for GOx in its dimeric form to be 60 Å × 52 Å × 77 Å by X-ray crystallographic analysis.[11] Now, if we assume lateral deformation of the GOx molecule introduced by the finite dimension of the tip (10 nm curvature radius),[12] we obtain an apparent length of 27 nm for a spheroidal particle of 7.7 nm in length. Cross

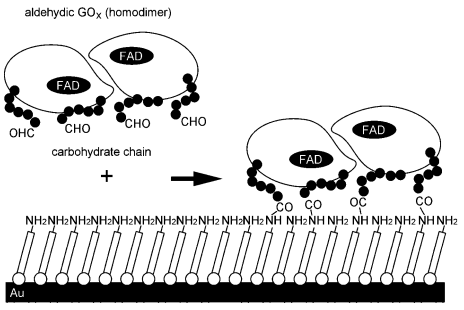

Fig. 1 Formation of a GOx/**AUDT** conjugated structure by the coupling reaction between the activated carbohydrate residues in GOx and the terminal amino groups in the SAM.

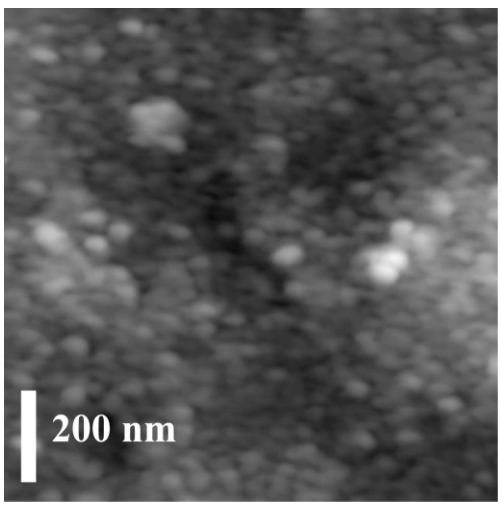

Fig. 2 AFM image of the gold substrate with the GOx/**AUDT**/Au(111) interfacial structure.

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section analysis showed that the average length of the spheroid was 34 ± 2.7 nm (n = 20) while the height was estimated to be *ca*. 2 nm. The determined spheroidal length seems to be consistent to the theoretical one. To date, only a limited number of studies have been reported on AFM imaging of GOx.[13] Quite recently, Calvo *et al.* reported imaging of GOx molecules which were electrostatically adsorbed on cystamine-modified gold surface.[1] It is noteworthy that we have achieved for the first time single molecular imaging and at the same time, self-assembly of GOx in a covalently immobilized state.

Formation of the GOx/**AUDT** conjugated structure on gold was further evidenced by reflection–absorption Fourier-transform infrared spectroscopy (FT-IRRAS) and microgravimetric analysis using a quartz-crystal microbalance (QCM). First, the IR-RA spectrum of the substrates treated with **AUDT** showed a spectrum fundamentally similar to the transmittance spectrum of **AUDT** in its isotropic sample; it showed C–H stretches in asymmetric and symmetric mode at 2924 cm^{-1} and 2850 cm^{-1}, respectively. Upon treatment with the aldehydic GOx, three new characteristic IR bands appeared at 1661 cm^{-1}, 1587 cm^{-1} and 1262 cm^{-1}; they can be ascribed to amide bands characteristic of protein molecules.

Next, definite mass increments are associated with SAM formation as well as GOx immobilization as determined by QCM measurements. We observed that a frequency decrease of 67 ± 7.5 Hz (n = 3) of the Au-quartz crystal was attained by treatment with **AUDT** solution. This is equivalent to 1.8 ± 0.20 nmol cm^{-2} of surface coverage when Sauerbrey's equation is adopted. In the meanwhile, a theoretical coverage of 0.76 nmol cm^{-2} is suggested because it is the value corresponding to the $\sqrt{3} \times \sqrt{3}$ overlayer structure for alkanethiols adsorbed on (111) surfaces.[14] Iodine desorption electrochemistry[15] gave a roughness factor of 1.6 for the Au electrode deposited on the quartz crystal. Thus, we have obtained the effective surface coverage of 1.1 ± 0.13 nmol cm^{-2} which was somewhat larger but not markedly than the theoretical one.

Subsequent treatment of the **AUDT**/Au substrate with the aldehydic GOx solution further decreased the oscillating frequency of the tip. Three different runs showed a frequency decrease of 155 ± 27 Hz which in turn gave a surface concentration of 5.5 ± 0.62 pmol cm^{-2} of GOx. If one takes into consideration of the effect of roughness, one obtains an actual surface concentration of 3.5 ± 0.39 pmol cm^{-2}. The occupied area for a single GOx molecule (46 nm^2)[11] gives a theoretical surface concentration of 3.6 pmol cm^{-2} with which the experimental datum agrees correctly. This result means that the present immobilization chemistry enables uniform, mono-enzymic film formation of GOx molecules on the SAM surface.

Finally, we investigated electrocatalytic oxidation of D-glucose at the GOx-electrode. Ferrocenemethanol used as redox mediator gave well-defined redox waves on the GOx-electrode as shown in Fig. 3. Upon addition of glucose, the anodic current observed with the GOx-electrode increased significantly. The steady-state current, I_s, at a given potential increased with increasing concentration of glucose in the sample solution (Fig. 3 inset). From the slope and the intercept of the double-reciprocal plot of the data, the maximum current, $I_{s,max}$, and the apparent Michaelis constant, K_{app}, were determined to be 12.3 μA and 199 mM, respectively. Dividing $I_{s,max}$ by surface concentration of GOx (2.9 pmol cm^{-2}), number of electrons involved, effective electrode area (0.59 cm^2), and the faraday, we obtained the apparent turnover number of 37 s^{-1} which means slower reaction kinetics than that of physically adsorbed GOx (3.6×10^2 s^{-1} for solution state and 2.3×10^2 s^{-1} for adsorbed state)[16] but still of particular significance.

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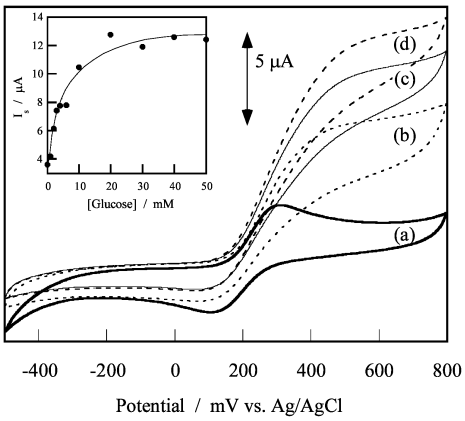

Fig. 3 Cyclic voltammograms for 0.1 mM ferrocenemethanol on a GOx-electrode in the presence of various concentrations of glucose. Glucose concentrations (mM) are (a) 0, (b) 3, (c) 10, (d) 50. Inset: plots for the magnitude of the steady-state current (I_s) *vs.* the corresponding glucose concentration. Electrolyte solution, aqueous 40 mM KH_2PO_4–K_2HPO_4 (pH 5.6); scan rate, 100 mV s^{-1}; temperature, 25 °C.

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9 A 200 nm thick gold film with (111) texture was resistively evaporated onto freshly cleaved mica at a rate of 0.1 nm s^{-1} in a home-built evaporator equipped with a turbo pump. The pressure was maintained below 1×10^{-6} Torr and the substrate temperature was kept at 300 °C. The gold film formed were further annealed in a muffle furnace at 550 °C for 3 h. AFM imaging was performed under air atmosphere operated in AC mode using a JSPM-4210 scanning probe microscopy (JEOL) equiped with a NCS12 cantilevers (spring constant 4.5 N m^{-1}, resonant frequency 150 kHz).

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