Reversible Gating Controlled by Enzymes at Nanostructured Interface

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Supplementary Information

Experimental Section.

Chemicals and Materials.

The following chemicals were purchased from various companies and used as supplied: Urease from jack beans (EC 3.5.1.5, Sigma-Aldrich); L-glutamate dehydrogenase from bovine liver (EC 1.4.1.3, Sigma-Aldrich); poly(4-vinyl pyridine) (P4VP, M.W. 160,000 g·mol⁻¹, ρ = 1.101 g·cm⁻³, Sigma-Aldrich); adenosine-5'-diphosphate (ADP, Sigma-Aldrich); α-ketoglutaric acid (Sigma-Aldrich); β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH, Sigma-Aldrich); urea (Sigma-Aldrich); potassium hexacyanoferrate (II) trihydrate (Sigma-Aldrich); 4-nitrophenyl butyrate (Sigma-Aldrich); gold (III) chloride trihydrate (Sigma-Aldrich); (±)-α-lipoic acid (Sigma-Aldrich); N-hydroxysuccinimide (NHS, Sigma-Aldrich); N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma-Aldrich); ethyl butyrate (Fluka); bromomethyldimethylchlorosilane (Gelest).

Esterase from porcine liver (EC 3.1.1.1, Sigma-Aldrich) was purified according to the following procedure: 10 mg (270 Units) were dissolved in 500 µL of 100 mM PBS buffer (pH 7.4). After vortex stirring the sample was letting to sediment for 30 min at 4°C. Afterwards, 450 µL of the supernatant were taken into a 30-kDa Nanosep ultra-filtration tube for centrifugation at 12,000 rpm for 30 min at 4°C. The sediment was washed with 250 µL of Na₂SO₄, 0.1 M, at pH 6 and centrifuged again. This process was performed three times. After last centrifugation the sample was concentrated and ready to use.

Ultrapure water (18.2 MΩ cm) from NANOpure Diamond (Barnstead) source was used in all of the experiments.

Preparation of Gold Nanoparticles.

The gold nanoparticles (NPs) were prepared by the standard citrate method.[1] Briefly, 200 mL of 0.01% (w/v) HAuCl₄ were heated to boil, and then 7 mL of 1% (w/v) aqueous trisodium citrate solution were added under vigorous stirring. The color was changed to grayish-black and then to wine-red within a few minutes. The dispersion was allowed to cool down. Gold NPs obtained by this method showed an average size of ca. 19 nm in diameter, Figure SI-1(A), and the characteristic gold NPs surface plasmon resonance absorbance maximum at 520 nm.

Electrode modification.

The ITO-electrodes were chemically modified with P4VP-brushes using the “grafting to” method[2,3] according to the following procedure. The ITO-coated glass slides (20±5 Ω/sq surface resistivity, Sigma-Aldrich; geometrical area of 1.2 cm², roughness factor of ca. 1.6±0.1[4]) were cut into 25 mm × 8 mm
strips. They were cleaned with ethanol in an ultrasound bath for 15 min and dried under a stream of argon. The cleaning step was repeated using methylenechloride as a solvent. The initial cleaning steps were followed by immersing the strips into a cleaning solution (heated to 60°C in a water bath) composed of NH₄OH, H₂O₂, and H₂O in the ratio of 1:1:1 (v/v/v) for 1 hour. (Warning: This solution is highly reactive and extreme precautions must be taken upon its use.). Subsequently, the glass strips were rinsed several times with water and then dried under argon. The freshly cleaned ITO-strips were reacted with bromomethyltrimethylchlorosilane, 0.1% (v/v), in toluene for 20 minutes at 70°C. The silanized ITO was rinsed with several aliquots of toluene and dried under argon. Then 60 µL of the P4VP solution in nitromethane, 10 mg·mL⁻¹, were applied to the surface of each ITO glass strip, dried to form a polymer coating, and left to react in a vacuum oven at 140°C overnight. The final cleaning steps, to remove the unbound polymer, consisted of soaking for 10 minutes in ethanol, followed by additional 10 minutes in a dilute solution of H₂SO₄ (pH 3). Modified electrodes were stored under water. Si-wafers used in the ellipsometry measurements were cleaned and modified using the same procedure as for the ITO-coated glass. We did not observe any difference in the properties of the brushes prepared on the different substrates.

After formation of the P4VP-brush on the ITO-electrode (similarly on the Si-wafers), Au-NPs were deposited on polymer-modified electrode by simple physical adsorption. The surface of the adsorbed Au-NPs was further modified with a self-assembled monolayer of α-lipoic acid by the chemisorptions process from ethanol/water, 7:3 (v/v), solution containing 1 mg·mL⁻¹ of α-lipoic acid. Within 3 hours a self-assembled monolayer of carboxylated dithiol was formed. Then the surface was carefully cleaned by its washing with ethanol, followed by drying in air.

![Figure SI-1](image_url)

**Figure SI-1.** (A) TEM image of the Au-NPs. Inset: The bar-chart showing the size-distribution of the Au-NPs. (B) SEM image of the P4VP-functionalized ITO-surface loaded with the enzyme-modified Au-NPs.
**Enzyme immobilization on modified electrode.**

Esterase was covalently attached to the thiol-functionalized nanoparticles by activation of the carboxylic groups using EDC and NHS. The mixture containing 0.07 M NHS and 0.2 M EDC was placed on the surface for 1 h and then the solution was carefully removed from the surface. Then esterase, 10 mg·mL⁻¹, in 0.1 M Na₂SO₄ solution, pH 6, was placed on the surface of the electrode and incubated for 12 h at 2°C. The enzyme-functionalized surface was washed with 0.1 M Na₂SO₄ solution, pH 6, to remove the excess of the non-reacted enzyme. The second enzyme, urease, was immobilized on the top of the first enzyme using EDC and NHS. For this purpose, a mixture containing 0.07 M NHS and 0.2 M EDC was placed on the surface for 30 min and then the solution was carefully removed from the electrode. Then urease, 2 mg·mL⁻¹, in 0.1 M Na₂SO₄ solution, pH 6, was placed on the surface and incubated for 12 h at 2°C. The enzyme-functionalized surface was washed again with 0.1 M Na₂SO₄ solution, pH 6, to remove the excess of the non-reacted enzyme. Figure SI-1(B) shows a SEM image of the P4VP-functionalized ITO-surface loaded with the enzyme-modified Au-NPs. The activity of the immobilized enzymes was analyzed using standard assay procedures provided by Worthington BioChemical Corporation (urease), Sigma Aldrich (esterase).

**Enzyme activity.**

The activity of urease immobilized on the NPs/P4VP-ITO-electrode was measured by the decrease of the NADH absorbance at 340 nm (ε = 6,200 M⁻¹·cm⁻¹) for the double-enzyme configuration, Figure SI-2(A), and for urease being a single immobilized enzyme, Figure SI-2(C), according to the standard assay provided by Worthington BioChemical Corporation for this enzyme. Worthington has adopted an assay method where the hydrolysis of urea is measured by coupling the ammonia production biocatalyzed by urease to a glutamate dehydrogenase reaction where α-ketoglutarate and NADH are converted to glutamate and NAD⁺ respectively. The reacting solution (2.4 mL) consisted of 0.1 mL of 23 mM ADP, 0.1 mL of 7.2 mM NADH, 0.1 mL of 26 mM α-ketoglutarate, 0.1 mL of 1.8 M of urea and 0.1 mL of 500 units of glutamate dehydrogenase (the rest of the volume was 0.1 M potassium phosphate buffer, pH 7.6). The reaction was carried out in the presence of the two-enzyme/Au-NPs/P4VP-modified electrode in 0.1 M potassium phosphate buffer, pH 7.6 solution at 25°C.

The activity of esterase immobilized on the NPs/P4VP-ITO-electrode was measured by the reaction with 4-nitrophenyl butyrate. The activity was determined by measuring the absorbance increase at 420 nm, for the double-enzyme configuration, Figure SI-2(B), and for esterase being a single immobilized enzyme, Figure SI-2(D), upon hydrolysis of 4-nitrophenyl butyrate to 4-nitrophenol (ε = 5000 M⁻¹·cm⁻¹) biocatalyzed by esterase according to the standard assay provided by Sigma-Aldrich for this enzyme. The reaction solution (1 mL) consisted of 100 mM 4-nitrophenyl butyrate in 50 mM potassium phosphate buffer pH 7.5. The reaction was carried out at 25°C in the presence of the enzyme/Au-NPs/P4VP-modified electrode.

The results of the enzyme-assay are summarized in Table 1.

The immobilized enzymes operated on the modified surfaces at pH values significantly different from the optimum values. We analyzed the effect of the pH value on the enzyme activity in order to estimate the decrease of the activity caused by the non-optimum pH, Figure SI-3.
Figure SI-2. Enzyme-activity assay: (A) and (B) The activity of urease and esterase, respectively, in the double-enzyme configuration. (C) and (D) The activity of urease and esterase, respectively, in the single-enzyme configuration.

Figure SI-3. Enzyme-activity assay at different pH values: (A) For urease, 16.8 mU, in a solution of 0.1 M phosphate buffer at pH 7.6 (curve 1) and the same amount of the enzyme in a solution of 0.1 M of sodium sulfate at pH 4.5 (curve 2). (B) For esterase, 3.6 mU, in a solution of 0.05 M phosphate buffer at pH 7.5 (curve 1) and the same amount of the enzyme in a solution of 0.1 M of sodium sulfate at pH 4.5 (curve 2).
Table 1. The enzyme-activity derived from the assay performed with the modified electrodes.a

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Activity of immobilized urease (nmole min(^{-1}))(^{b})</th>
<th>Activity of immobilized esterase (nmole min(^{-1}))(^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-enzyme configuration</td>
<td>16.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Single-enzyme configuration</td>
<td>12.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

\(^{a}\) The assay of urease was performed according to the standard procedure.\(^{[5]}\) The assay of esterase was performed according to the standard procedure.\(^{[6]}\)

\(^{b}\) Activity of urease corresponds to the biocatalyzed decrease of NADH.

\(^{c}\) Activity of esterase corresponds to the biocatalyzed generation of 4-nitrophenol.

The following conclusions were derived from the comparison of the enzyme activities:

a) The activity of the enzymes in the double-enzyme configuration is not much different from the activity of the same enzyme being alone on the modified interface. Therefore, the immobilization procedure does not decrease significantly the enzyme activity. (It was confirmed by comparison between enzymatic activity for immobilized enzymes and activity of the same concentration of the non-immobilized enzyme).

b) The enzyme activity at pH values used in the experiments with the immobilized enzymes is at least 3-fold lower than for the same enzyme at the optimum pH value.

The long time (ca. 30 minutes) required for the ON-OFF switching process of the electrode upon the biocatalytic transformations originates from the following reasons listed in the order of decreasing importance:

(i) The amount of the immobilized enzyme on the interface is small. Production of butyric acid by the esterase immobilized on the electrode is 3.6 nmole min\(^{-1}\). The bulk pH changes (10 mL cell) from pH 4.5 to 6.0 would require 875 minutes. For the immobilized urease (production of urea at the rate of 16.8 nmole min\(^{-1}\)) this process would take 200 minutes. Therefore, the pH changes in a thin-film proceeding for 30 minutes are at the expected time-scale.

(ii) The enzymes operate at pH values which are far from the optimum values.

(iii) The pH changes biocatalytically generated in a thin-film at the interface propagate diffusonally to the bulk solution – this process reduces the effect at the surface and requires longer time to accumulate ∆pH needed for the polymer switch.

(iv) The restructuring of the pH-switchable polymer support is a time-consuming process.

The molecular dimensions of the enzymes were estimated using commercial software HyperChem (Release 2) for Windows. The protein structures were taken from the Protein Data Bank (Brookhaven National Laboratory, http://www.rcsb.org/pdb). The error in the calculated dimensions of the proteins is estimated to be ±5 Å.

**Instruments and methods.**

All optical measurements were performed using UV-2401PC/2501PC UV-VIS spectrophotometer (Shimadzu, Tokyo, Japan). The electrochemical measurements were carried out with an ECO Chemie Autolab PASTAT 10 electrochemical analyzer using the GPES 4.9 (General Purpose Electrochemical System) software package for cyclic voltammetry. The pH measurements were performed with Mettler Toledo® SevenEasy pH-meter. The layer thickness and the amount of the grafted material were evaluated at the wavelength of 633 nm and at the angle of incidence of 70° for the Si-wafers (modified similarly to the ITO-electrode and used for the ellipsometry measurements) using an Optrel Multiscop (Berlin, Germany) null-ellipsometer equipped with an XY-positioning table for mapping the sample surface (lateral resolution is defined by the beam spot of about 2 mm). From the ellipsometry measurements we
calculated the grafting amount of the polymer, \( A = H \rho \), and the grafting density, \( \sigma = \frac{AN_A}{M_w} \), where \( H \) is the ellipsometric thickness of the dry brush sample, \( \rho \) is the density of the polymer (\( \rho = 1.101 \text{ g} \cdot \text{cm}^{-3} \) for P4VP), \( N_A \) is the Avogadro’s number. The error in calculation for the ellipsometrically measured thickness is no larger than ±5% for the 5 nm thick films since the difference in the refractive indexes of all organic ingredients is small.

**Electrochemical measurements.**

All electrochemical measurements were performed at an ambient temperature (23±2°C) in a standard three-electrode cell (ECO Chemie). The working electrode was a two-enzymes/Au-NPs/P4VP-modified ITO-electrode with a geometrical area of 1.2 cm². A Metrohm Ag/AgCl/KCl, 3M, electrode served as a reference electrode and a Metrohm Pt wire was used as a counter electrode. The background aqueous electrolyte solution was composed of 0.1 M Na₂SO₄. Cyclic voltammograms were recorded in the presence of 0.2 mM potassium ferrocyanide, \( K_4[Fe(CN)_6] \), in the potential range from 0 V to 0.4 V after equilibration for 5 s at the starting potential. The potential scan rate was 100 mV·s⁻¹. Peak currents for each measurement were obtained from a second scan.

**References:**


