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Development of bio-nanowire networks using phage-enabled assembly for biological sensor application

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ABSTRACT

This paper proposes a new approach to detect an electrochemical reaction using a working area consisting of bio-nanowires from genetically modified filamentous phages and nanoparticles. Use of the nanomaterials on the working electrode is a vital consideration in biological sensor development, because the biosensor sensitivity heavily depends on the material used. Here we use that fd-tet p8MMM filamentous phages displaying the MMM peptide on the major coat protein pVIII (designated p8MMM phages) were immobilized on the active area of an electrochemical sensor through chemical binding. The bio-nanowires composed of p8MMM phages and silver nanoparticles facilitated sensitive, rapid detection of particular molecules. We performed the experiment for observing electrochemical glucose detection to estimate the possibility of using one or other characterized-biological sensor. The current response of the bio-nanowire sensor reached sufficiently high signals at various glucose concentrations (10^{-7} to 10^{-4} M). The cyclic voltammetry peak current I_p and peak potential E_p were 689 μA/cm^2 and 280 mV, respectively. The filamentous nanophage-based electrode displayed a high sensitivity and good stability under various pH and temperature in enzyme determination. As a result, it may have wide application in analytical systems, label-free detection and biological sensors.

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1. Introduction

Bio-nanobinary convergence technologies are of considerable interest due to their potential application in biodegradable product, portable detector, and biomedical systems. Biosensors using nanowires to acquire biochemical signals play a key role in portable or implantable electrochemical sensor applications [1]. The system technology of biological sensor in particular is evoked toward spontaneous favorable responses that have molecular recognition and can use the nanoassembly structure for detecting an electrochemical reaction. Especially, biological molecular assembly is an excellent model system using nanoengineered approach for electrochemical sensors.

Filamentous phages such as M13 and fd phage have a thread-like shape of ~900 nm in length and <10 nm in diameter. These phages can infect many Gram-negative bacteria. The two major components of phages are the genetic material and the protein coat. Small foreign peptide and protein gene sequences such as antibody fragments and enzymes can be fused to coat protein VIII or III of filamentous phages and can be displayed on the phage surface [2–4]. Recently, the study reported a biologically active molecular network consisting of filamentous phage directly assembled with gold (Au) nanoparticles [5]. A phage-coated electrode was also developed to measure electrochemical impedance [6,7]. Thus, the phage entity can be modified to allow certain functionality and to form hetero-complexes with organic nanomaterials to develop accurately positioned nanobio templates for sensor application.

The highly selective and sensitive measurement of the electrochemical potentiometric or amperometric response is vital for clinically diagnostic glucose detection. The ultimate goal of biological sensor development is to produce a versatile, reproducible, and highly selective sensor [8–10]. It is particularly important to determine the optimal conditions for each electrode that allow the immobilized biological sensing components to monitor electrochemical signal variations [11]. However, several conditions, such as monolayer dispersal, self-assembly, and sampling control, are needed to apply nanostructured materials to biosystems [12,13].

In this work, we synthesized and assembled a nanocomposite bio-nanowire with silver nanoparticle-binding bacteriophages, and showed that the networks are biocompatible and preserve the internalization attributes mediated by a displayed peptide. Also, we investigated the possibility of use for biological sensor by taking advantage of silver (Ag) that has the highest electrical conductivity of any metal.

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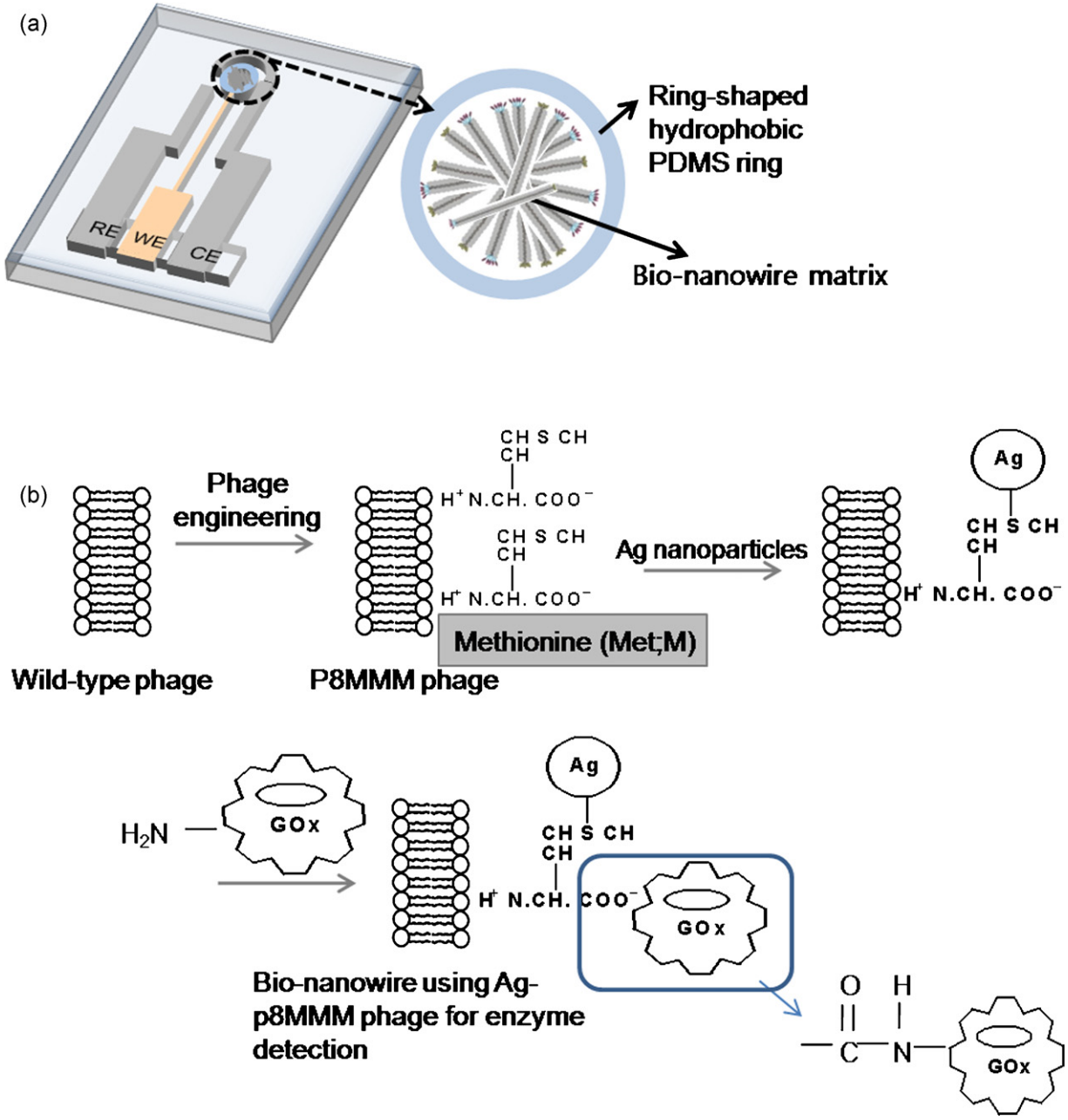

Fig. 1. Ag-p8MMM phage conjugates and schematic tri-electrode biosensor using Ag-p8MMM phage conjugates. Tri-electrode system with hydrophobic ring (a) and chemical reaction about phage engineering (b) are shown.

2. Experimental

2.1. Apparatus

Transmission electron microscopy (TEM) was performed with a Tecnai 20 (FEI Co., The Netherlands; 200 kV acceleration voltage) to observe the physical state of the electrode with the bio-nanowire during operation. Images were recorded with a CCD camera (Gatan MSC 794) at a resolution of at least 2048 × 2048 pixels. Cyclic voltammetry (CV) data was acquired using a computer-controlled potentiostat/galvanostat (Model M273, EG&G, USA). Fig. 1 shows a schematic diagram of the p8MMM phage, Ag-p8MMM, used for bio-nanowire composition, and the tri-electrode biosensor system used to detect the glucose concentration.

2.2. Reagents and phage synthesis

We used the fd-tet phage vector (gifted by Dr. Philipp Holliger) and *E. coli* strains DH10β and K91BluKan (K91BK)(gifted by Dr. George P. Smith). To clone and prepare phage DNA, *E. coli* DH10β was grown in 2× TY medium. The fd-tet p8MMM phage vector, which has three methionine codons (MMM) at the beginning of the pVIII gene, was constructed using assembly PCR, restriction enzyme cutting, and ligation. The constructed vector was confirmed by sequencing (Eurofins MWG Operon).

For phage amplification, *E. coli* K91BK transformed with fd-tet p8MMM phage vector was grown in LB medium containing tetracycline (20 μg/ml) and kanamycine (100 μg/ml). Phages in the supernatant were concentrated by two cycles of PEG (16.7%)/NaCl (3.3 M) precipitation at 4 °C and redissolved in PBS (pH 7.4).

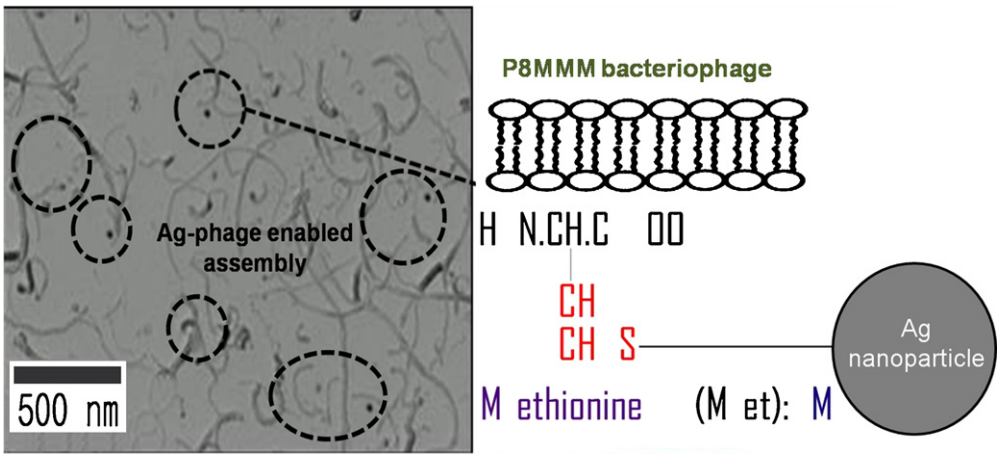

Fig. 2. TEM image of the sensor surface with Ag-p8MMM (TEM) phages and the chemical reaction between Ag nanoparticles and p8MMM phages are shown.

The titre of p8MMM phages infected into K91BK, measured as tetracycline-resistant (20 μg/ml) and kanamycine-resistant (100 μg/ml) colony forming units (cfu), was 3.5×10^{10} cfu/ml.

To design a bio-nanowire electrode using a combined Ag-p8MMM phage, p8MMM phages (3.5×10^{10} cfu/ml) were incubated with 10 μg/ml Ag nanoparticles (>99% 6–7 nm hydrophobic powder, PlasmaChem GmbH, Berlin) in tris-buffered saline (TBS) for 18 h at room temperature in an orbital shaker (2:1 ratio). After diluting with TBS, Ag-p8MMM phage conjugates were collected by ultracentrifugation (for 20 min, at 4 °C, 0.1 g or 20,000 rpm) for further analysis. The persistence of the bio-nanowires made with the Ag-p8MMM phages was checked by various microscope systems.

2.3. Preparation of the Ag-p8MMM phage-modified tri-electrode

We developed an integrated electrochemical tri-electrode biosensor consisting of a reference electrode (Ag/AgCl thin film), counter electrode (Pt thin film), and nanoscale Ag-p8MMM phage composite working electrode. The glass of a Pyrex 7740 wafer was prepared as a substrate. Organic contaminants, including grease, dust particles, silica gel, etc., were removed by dipping the glass in a concentrated sodium hydroxide solution (60 °C) for 30 min. The glass wafer was then sonicated in acetone, ethanol, and de-ionized (DI) water for 10 min each, and subsequently dried with a stream of high purity N_2 gas. A thin Pt film (~2500 Å thick) was deposited by RF Sputter (Anatech Co.) on a Ti layer (~200 Å thick).

The counter electrode (CE) was patterned using UV-photolithographic steps. The glass wafer was coated with photoresist (PR) to define the pattern of Ag thin film deposition. The electrode shape was configured by removing the PR with acetone. An AgCl layer was fabricated on the film surface by dipping the chip into a 0.1 M $FeCl_3$ solution for ~1 min, followed by rinsing with DI water [13].

To immobilize Ag-p8MMM phages on the working electrode, the chip surfaces were modified with chemical treatments while simultaneously performing passivation of the other surfaces, as shown in Fig. 1b. The chip was then immersed in a solution of 3-aminopropyltriethoxysilane for 1 h at 50 °C to form covalent bonds between the Ag-p8MMM phage suspension and working area. The chip was then thoroughly rinsed with DI water.

2.4. Activated enzyme electrode for glucose detection

To investigate use of the Ag-p8MMM phage biosensor for electrochemical detection, we measured the enzyme detection of different glucose concentrations. Glucose oxidase (EC 1.1.3.4., type VII-S, Sigma) molecules were oxidized as described in the literature [14,15]. The modified working electrode was immersed in a glucose oxidase (GOx) solution mixed with $[Ru(NH_3)_6]^{3+}$ and a 0.1 wt% carboxy-methylcellulose (CMC) solution to detect the glucose concentration. *In situ* staining was performed in 5 mL PBS (pH 7.4) at 25 °C for ~10 min to obtain an enzyme-activating electrode at various glucose concentrations.

3. Results and discussion

3.1. Electrochemical properties of Ag-p8MMM phage-modified working electrodes

Many nanowire types exist, including metallic (e.g., Ni, Pt, Au), semiconducting (e.g., Si, InP, GaN), and insulating (e.g., SiO_2, TiO_2). Here we created bio-nanowires using a phage engineering technique. Three methionine (MMM) peptides are displayed on the surface of the major coat protein pVIII particles of the fd phage. Metal nanoparticles, especially Au and Ag, are easily deposited onto displayed methionine. Fig. 2 shows the TEM images of phage complex-attaching Ag nanoparticles. Most of the Ag nanoparticles closely bonded to p8MMM phages.

The use of nanoscale-engineered phage materials for electrodes might be an effective method to enhance device performance, since it markedly increases the effective area (A_{eff}) of the working electrode, thereby altering biosensor sensitivity. Working electrodes made from bacteriophages are characterized by a strong adhesion and are thus amenable to easy chemical signal acquisition. Therefore, we next compared the physico-chemical and electrical properties of the working electrode with or without Ag-p8MMM phages.

Fig. 3 shows an image of the biosensor with the proposed electrode strips for electrochemical detection and the tri-electrode system consists of a reference electrode (RE; a), counter electrode (CE; b) with hydrophobic ring and working electrode (WE; c). A well-dispersed Ag-p8MMM phage solution was spread and dried on the modified surface of the working area. The working area was firmly specified using ring-shaped hydrophobic polydimethylsiloxane. In this electrode, the electrochemical A_{eff} was determined from the CV results of redox reactions on the electrode surface, and was compared with the A_{eff} about the Au nanoparticle electrode surface. The CV experiment was performed in 200 ml of 0.06 M phosphate buffer and 0.1 M KCl (pH 7.4) mixed with a prepared solution of 5 mM $K_3[Fe(CN)_6]$ at room temperature. The Randles–Sevcik equation was used to relate the peak

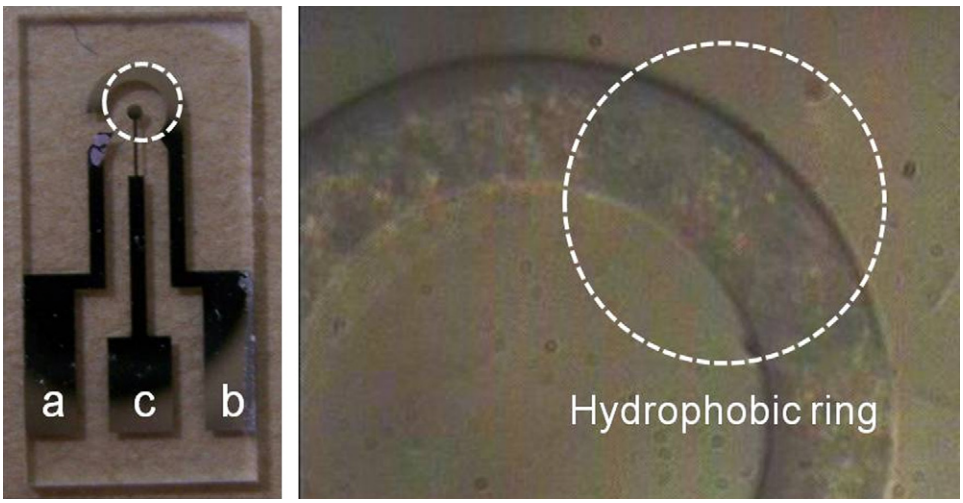

Fig. 3. The photograph of electrochemical biosensor with the proposed electrode strips. The 3-electorde is fabricated on the glass Pyrex 7740 wafer and has a hydrophobic ring: (a) reference electrode (RE); (b) counter electrode (CE); (c) working electrode (WE).

height of the CV voltammogram (I_p) to the analyte concentration (C):

$$I_p = (2.687 \times 10^5) n^{3/2} v^{1/2} D^{1/2} AC \quad (1)$$

where A is the electrode area, n is the number of electrons during the half-reaction, and D is the analyte diffusion coefficient [16]. Fig. 4b displays the Randles–Sevcik plots, in which the redox reactions of $K_3[Fe(CN)_6]$ were elicited by CV and the slope ratio was estimated as 1.85. According to Eq. (1), the sensitivity of the Ag-p8MMM phage-modified working electrode will increase by 1.85 times, since the slope ratio is directly related to the A_{eff} ratio.

Fig. 4a shows the CV plots of the modified bacteriophage electrode at scan rates of 50–200 mV s^{-1}. The high current response of the modified Ag-p8MMM phage electrode is attributed to the accelerated electron transfer and large electrochemical A_{eff} when highly dispersed bio-nanowires were spread on the working electrode. In particular, the peak-to-peak potential separation (ΔE_p) was affected by the electrode type and related to the electron transfer kinetics. Thus, the electrode with the lowest ΔE_p has the highest electron transfer ability. The Ag-phage-modified electrode had a more reversible electron transfer capability. The anodic and cathode currents of the electrode were linearly proportional to the scan rate, and the stability of the modified electrode was very good over the course of 100 cycles when a CV applied voltage range of −0.4 to +0.8 V was used.

3.2. Effects of pH and temperature on the Ag-p8MMM phage-modified biosensor

The pH of the Ag-p8MMM phage-modified electrode affected the current response of the reactants. The pH dependence of the sensor response was investigated using a phosphate buffer solution with a pH range of 6.0–9.0 (Fig. 5a). The maximum relative current response of the electrode to glucose was significant from pH 6.5 to 8.5, which agreed well with the results without Ag-p8MMM phages. Thus, the assembly of Ag-p8MMM phages as a working electrode did not change any bioanalytical reaction. We chose a pH of 7.4, based on the electroanalytical characteristics of the Ag-p8MMM phage-modified electrode.

The biosensor current response to glucose was measured at temperatures of 20–50 °C. Increased temperature enhanced the sensor sensitivity, but the current response decreased when the temperature was >35 °C, indicating that partial enzyme denaturation coincided with a sensitivity decrease. The growth temperature of the bacterium (25 °C) was selected based on the lifetime and response characteristics, since the biosensor response was similar between 25 and 35 °C (Fig. 5b).

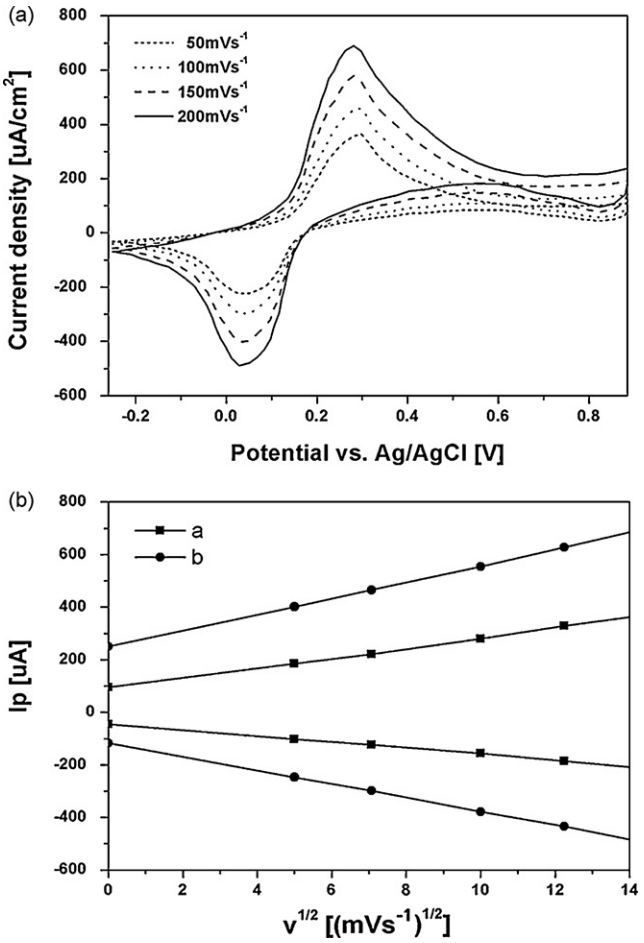

Fig. 4. Cyclic voltammograms of working electrode at different scan rate from 50 to 200 mV s^{-1}: (a) Ag-phages modified electrode; (b) plot of I_p vs. $v^{1/2}$ of each working electrode. A black square (■, (a) or circle (●) (b) indicates the value of the Au nanoparticles as working electrode or Ag-phage-modified electrode, respectively.

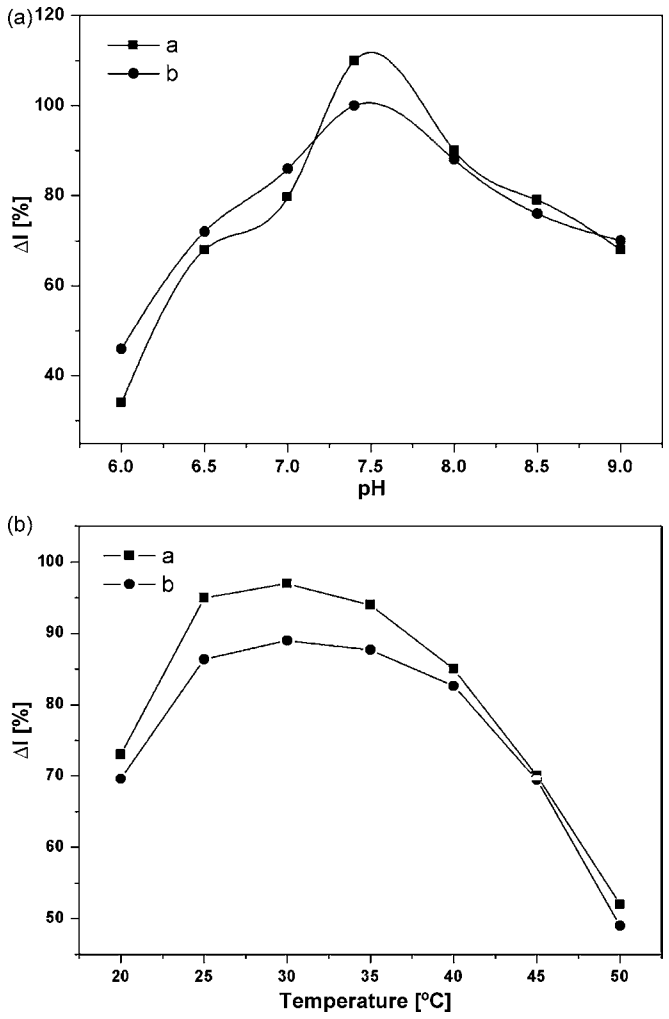

Fig. 5. Biosensor response of each working electrode. (a) Effect of pH; (b) effect of temperature. The black squares (■, (a) or circles (●) (b) indicate the electrode values with or without Ag-p8MMM phages, respectively.

3.3. Response of the Ag-p8MMM phage-modified biosensor

The electroanalytical properties of the designed biosensor using surface-modified bio-nanowires were determined from the relationship between the response current and glucose concentration when the background current was stable. The Ag-p8MMM phage-modified working electrode was electrochemically activated to easily allow the Ag-p8MMM phage to conjugate with glucose, as shown Fig. 1b. An enzyme solution was prepared with GOx at room temperature. The proposed biosensor determines the quantity of glucose based on the amperometric detection of hydrogen peroxide, which is generated with gluconic acid when GOx catalyzes glucose oxidation in the presence of dissolved oxygen. The p8MMM phage is thought to act as a scaffold for electrocatalytic support through the interaction between the GOx and the amino acid carbonyl functional groups within phages. The p8MMM phages also have unique electrical conductivity, and conjugation with Ag nanoparticles improves electrocatalytic formation of hydrogen peroxide at the working electrode.

Fig. 6 shows the current responses of each tri-electrode biosensor to the glucose concentration by amperometric detection method. Glucose oxidase covalently attached to the Ag-phage bio-nanowire matrix surface was compared with the control solution to ensure that any detectable reaction was due to immobilized GOx. The calibration graph of the current response was linearly proportional to the glucose concentration in the range of 10^{-7} to 10^{-4} M, the applied potential was 0.3 V vs. Ag/AgCl in stirred buffered (pH 7.0) at 95% reaction time. The slopes for Fig. 6a and b were 19.97 and 10.21 $\mu A\,cm^2\,mM^{-1}$, respectively. The glucose current response of the Ag-p8MMM phage-modified electrode was much higher than the other electrode. These results demonstrate that the nanostructured bacteriophage could affect the electrochemical reaction properties of biomolecules and promote electron transfer through Ag nanoparticles bonded to surface p8MMM phages. Furthermore, it can be bioaffinity devices based on the enhanced detection using filamentous phage as reaction material of working electrode such as implantable and in vivo systems. We conclude that the proposed electrochemical sensor using Ag-p8MMM phage-conjugated bio-nanowires has a higher sensitivity, stability, and linear calibration range of detection.

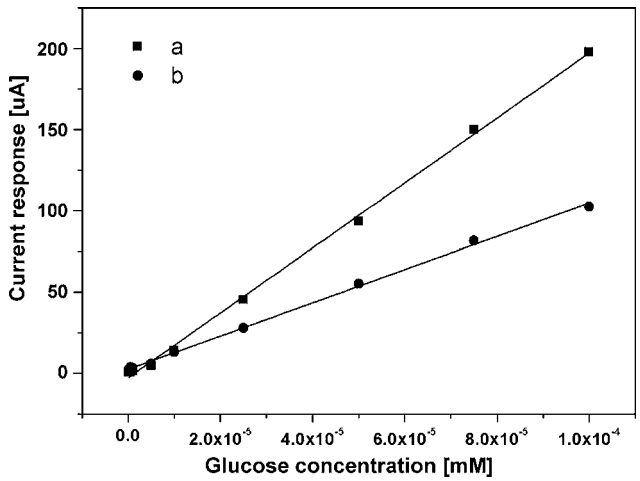

Fig. 6. Calibration curves correspond to the currents generated about increasing glucose concentration at an applied potential of +0.3 V (vs. Ag/AgCl) through amperometric measurement is shown. A black circle (■) (a) and square (●) (b) indicate the values of the modified electrode with the Ag-p8MMM or p8MMM phage bio-nanowire matrix, respectively.

4. Conclusions

The biological sensors generally display insufficient stability or defective enzyme reactivity during the immobilization of target-sensing materials onto the sensor. Therefore, careful consideration of the electrode material used is fundamental for maintaining electroanalytical activity. Here we report a novel, fabricated nanocomposite biosensor with networked Ag-binding phage-generated bio-nanowire matrix for electrode optimization. The electrochemical biosensor was constructed by combining an Ag/AgCl reference electrode, a Pt thin film counter electrode, and a working electrode coated with Ag-binding phages. The electroanalytical properties of the phage-based biosensor were evaluated with CV and microscopy, and revealed that assembly between the biomaterials and the nanoscale metallic particles on the working electrode improved enzyme detection parameters, including the linear data range, stability, and sensitivity. The possibility of influencing reactions through electrode surface modification with specific binding materials should encourage the development of useful sensors for electrochemical detection research. The results of our use of nanobio complexes constituted with genetically modified bacteriophages to an enzymatic biosensor may be extended to label-free immunodetection and other diagnostic or life science applications. Also, the physical and biological features using nanoengineered networks will offer convenient multifunctional integration as having variable potential for biomedical applications.

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