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Detection of an uncharged steroid with a silicon nanowire field-effect transistor

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ABSTRACT

Among biosensors of various types, the silicon nanowire field-effect transistor (SiNW-FET) is believed to be the most sensitive and powerful device for bio-applications. The principle of sensing is based on the variation of conductivity resulting from a disturbance of charge on the surface of the SiNW-FET, but this detection is feasible predominantly for charged analytes, such as a protein, DNA, antibody, virus etc. The objective of our work was to overcome this intrinsic weakness of a SiNW-FET and to develop a platform to detect steroids. For this purpose, we designed an engineered protein, Δ^5-3-ketosteroid isomerase, to function as a steroid acceptor that was chemically modified with a carbon chain-linked 1,5-EDANS moiety, and further immobilized on the surface of a silicon nanowire. In the presence of a steroid, the negatively charged 1,5-EDANS moiety, which presumably occupies the steroid-binding site, is expelled and exposes to the nanowire surface. The electrical response produced from the 1,5-EDANS moiety is measured and the concentration is calculated accordingly. The sensitivity of this novel nano-bio-device can attain a femtomolar level.

1. Introduction

Steroids are lipid compounds. With the exception of cholesterol, steroids are natural hormones or hormone precursors. The determination of the levels of steroid hormones is an important issue for the inspection of endocrinological disorders related to adrenal or gonadal function. Among analytical methods used to determine the concentrations of steroid hormones or their precursors are immunoassays [1–3], fluorescence resonance energy transfer [4], SPR [5], GC/MS [6–8] and LC/MS [9–11]. Our interest is a sensitive assay for hormone detection that typically involves a mass spectrometer coupled with either a gas chromatograph (GC/MS) or a liquid chromatograph (LC/MS). For these hormones, like anabolic steroids, according to the differences in fragmentation caused by collisions of medium energy related to the structure, it is difficult to find a common product ion or neutral loss. Furthermore, not only do most ELISA-like assays lack the sensitivity required to determine >90% of hormone derivatives [12] but also these analytical procedures might introduce artefacts. The detection limits of the above methods range from ng/mL to pg/mL [13,14].

One-dimensional nanostructures such as carbon nanotubes (CNT) and silicon nanowires (SiNW), have been demonstrated to be sensitive chemical and biological sensors [15]. That detection results from the disturbance of charge on the surface of the functionalized nanostructure on which the target molecules are specifically recognized. For instance, the real-time detection of single viruses [16], various antigens [14,17], oligonucleotides [18,19], proteins [20,21] and charged small molecules [22] has been shown to be feasible on devices using nanowire or carbon-nanotube transistors as active transducer. The sensing mechanism in an electrically based biosensor relies on an altered conductance or threshold voltage (V_{th}) induced by the attachment of the charged analytes. In this work, we attempted to integrate protein engineering with the sensitive nature of a SiNW-FET in charge disturbance to overcome the intrinsic weakness of SiNW-FET in detecting uncharged analytes. We chose an uncharged steroid, 19-norandrostendione (19-NA), as the target analyte.

Δ^5-3-Ketosteroid isomerase (KSI) has served as a receptor for steroid recognition because of its well understood enzyme function [23–30]. The primary concept of the sensing mechanism is based on intramolecular binding of a charged ligand, functioning as a reporter, to mimic the binding of an analyte to a protein. The major driving force favoring this association is generally thought to be the hydrophobic effect that prompts the hydrophobic ligand to bind with the protein. The thermodynamics of protein-ligand binding can be altered by a favorable control of enthalpy and, particularly in this model, the characteristic 'entropy-driven' thermodynamic signature of the steroid. The analyte might replace the pre-situated ligand, which becomes thus exposed to the surface of the SiNW and

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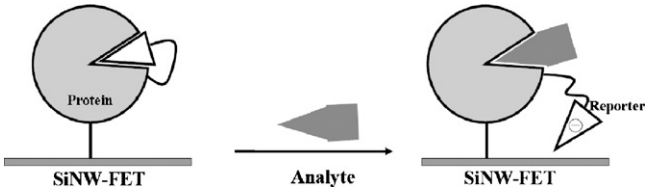

Fig. 1. Design of a SiNW-FET for the detection of an uncharged analyte.

perturbs the charge density and conductance of the nanostructure. Fig. 1 depicts the p-type SiNW-FET platform of this design.

2. Experiments

2.1. Design of an engineered protein, overexpression and purification

The structural design of a KSI was based on the understanding of a KSI from previous work undertaken by many groups [28,31]. To eliminate the possibility of multi-labeling and the complicated orientation evolved from ligand conjugation and further protein immobilization, we constructed a KSI mutant gene by PCR amplification with eight mutated sites (Y55F, K60R, F86C, F88G, K92R, K108R, K119R, and A125K) in a single protein molecule. The resulting KSI mutant, designated Art_KSI, contains only one cysteine residue (Cys-86) and one lysine residue at the C-terminus (Lys-125), which serve for further chemical conjugation of the reporter and for the immobilization of protein on SiNW, respectively.

The engineered protein, named Art_KSI, was constructed from a pRSET A vector and further expressed in *Escherichia coli* BL21 (DE3) at 28 °C for 16 h. A bacterial culture (1 L) was collected on centrifugation and further resuspended in phosphate buffer (15 mL, 20 mM, pH 7.5). Cells were disrupted by ultra-sonication. A precipitant containing crude enzyme was obtained from the supernatant on treating with ammonium sulfate (up to 50% saturation). The crude enzyme was further resuspended in phosphate buffer (10 mL, 20 mM, pH 7.5). After desalting, the sample solution (10 mL) was loaded onto a HiTrap Q column (30 mL, Pharmacia) for chromatographic separation. The column was eluted with phosphate buffer (20 mM, pH 7.5) at a flow rate 1 mL/min and a linear gradient 2.5 mM/min of NaCl. KSI was eluted in a range 50–75 mM of NaCl. The protocol for protein purification is appropriate also for other KSI mutants. The quality of the purified proteins was examined by both SDS-PAGE and LC/MS.

2.2. Bioconjugation of Art_KSI with fluorophore (mA51)

The bioconjugate reaction was performed at 4 °C, for 12 h in Tris–HCl buffer (50 mM, pH 7.5) containing enzyme (0.1 mM) and labeling reagent (1 mM). The excess labeling reagent was removed with dialysis or ultra-filtration. The efficiency of the labeling reaction was evaluated with LC/MS. The modified protein is named Art_KSI/mA51.

2.3. Fabrication of a SiNW device

This p-type SiNW-FET was fabricated on a 6 in. silicon-on-insulator wafer which top silicon layer with boron-doped of 10^{15} cm^{-3}. The thicknesses of the top Si layer and the buried oxide layer were 50 and 150 nm, respectively. The silicon nanowires (SiNWs) were defined by electron-beam lithography and followed by plasma etching. A SiO_2 film (thickness 10 nm) was thermally grown as a screening oxide. The SiNWs were doped by boron implantation with dose of 5×10^{13} cm^{-2} at 15 keV. After thermal activation at 950 °C for 30 min, the screening oxide was removed with HF solution. After defining the contact pad patterns, a stack of Ti (10 nm) and Au (100 nm) was then evaporated with a thermal evaporator and lifted off to create the contacts to the SiNW. The p-type SiNW devices were sintered in nitrogen gas at 400 °C for 10 min to ensure a good ohmic contact. The electric parameter of SiNW-FET was measured using a semiconductor parameter analyzer (HP 4155B) in the ambient.

2.4. Immobilization of the SiNW surface

Before immobilization of the Art_KSI/mA51 onto the SiNW, these SiNW were pretreated with an UV/ozone plasma (Samco model UV-1). The SiNW were further treated with 3-aminopropyltriethoxysilane (APTES, Merck) on adding droplets of APTES solution (2.2 mM) onto the top of the nanostructure for 10 min. After reaction for 10 min, the chip was rinsed with absolute ethanol three times and dried at 120 °C for 30 min. The amine-derivatized nanowires were immersed in the *Bis* (sulfosuccinimidyl) suberate (BS3, 5 mg/mL, Sigma, 20 min, 23 °C), and then dried (37 °C, 15 min). Art_KSI/mA51 (0.01 mM in 1 mM sodium phosphate buffer, pH 7.0) coupled with the chemically activated SiNW for 3 h at 23 °C. Tris (1 mM, pH 7.5) was then used to block the remaining N-hydroxysulfosuccinimide groups [32].

3. Results and discussion

The Art_KSI was successfully expressed in *E. coli* and further purified on an anion-exchange column. The quality of the purified protein was confirmed with gel electrophoresis (sodium dodecyl sulfate-polyacrylamide) to have homogeneity >95% (data not shown). The precise molecular mass determined by LC/MS analysis showed m/z = 13,402 Da ($M+H^+$), consistent with the molecular mass calculated from the amino-acid composition of Art_KSI. Art_KSI possesses substantial activity with a value $k_{cat}/K_m = 1.12 \times 10^7$ (M^{-1} s^{-1}) when Δ^5-androstene-3,17-dione is used as substrate for assay. The result of enzymatic catalysis confirmed the protein folding of Art_KSI is maintained.

To convert the action of steroid binding into an electrical signal, a negatively charged ligand (the reporter) must be covalently labeled at the appropriate position of Art_KSI. The precursor of the reporter, named mA51-mA51 (shown in Fig. 2), was synthesized on coupling two molecules of 5-(2-aminoethylamino)-1-naphthalenesulfonic acid (1,5-EDANS) with one 4,4′-dithiodibutyric acid. The Cys-86 residue, located at the rim of the steroid-binding site, was designed to react with the mA51-mA51 through the thiol substitution to form a new disulfide bond between the protein and the reporter (mA51). The modified protein is designated Art_KSI/mA51; the success of chemical conjugation of Art_KSI was confirmed on LC/MS analysis as shown in Fig. 2. The Art_KSI/mA51 was further immobilized on the SiNW through the Lys-125 residue (C-terminal residue) or the amino group of the N-terminus. Based on an inspection of the protein structure, we predict that either method immobilization does not cause steric hindrance for steroid binding. In principle, the reporter molecule can be expelled from the binding site and expose to the surface of SiNW when a steroid is present. To ensure the feasibility of this system, the binding affinity of the reporter should be taken into account. If the reporter binds strongly to protein, it is unlikely to be replaced by a steroid. In contrast, a weakly binding reporter cannot promise the application as most reporters are outside the steroid-binding site. We chose 1,5-EDANS as the candidate reporter for its specific, but moderate, binding affinity towards Art_KSI (K_d = 0.35 mM). The mA51 moiety on the Art_KSI/mA51 is hence presumably able to fit into the steroid-binding site.

Immobilization of the protein was evaluated on examining the observed density of gold nanoparticles (AuNPs) on a Si sample with

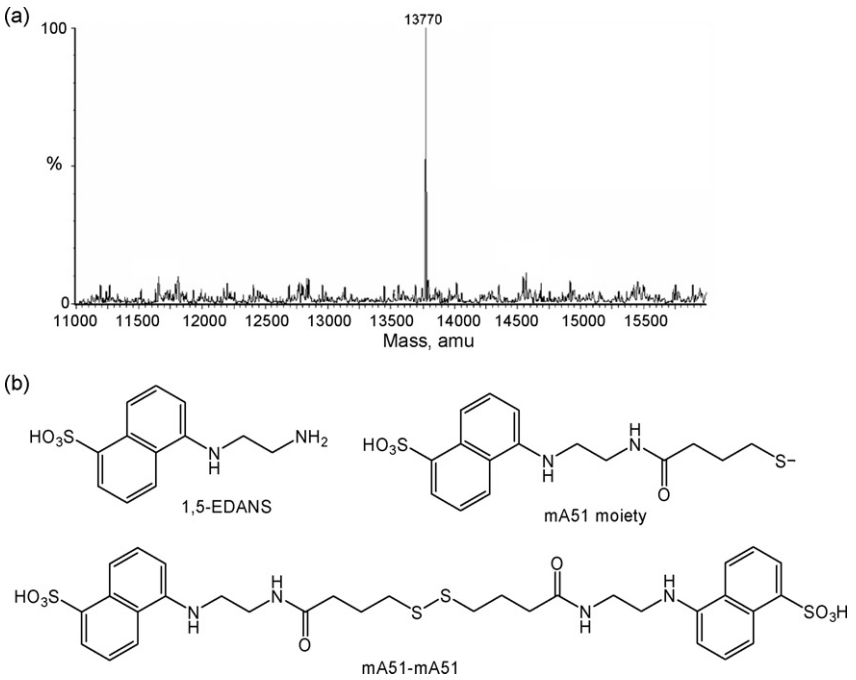

Fig. 2. Mass-spectrometric analysis of modified KSI and the chemical structures of reporter and its precursor. (a) Mass spectrum of Art_KSI conjugated with the reporter. The measured molecular mass of Art_KSI/mA51 is 13,770 ± 2 Da, consistent with the calculated value 13,768 Da (13402 Da for Art_KSI and 366 Da for mA51 moiety). (b) The structures of 1,5-EDANS, mA51 moiety and mA51-mA51.

a film of SiO_2 (thickness 30 nm) and surface modification at varied stages, such as a treatment with APTES and also with BS3 and protein as shown in Fig. 3. Fig. 3 also presents SEM images of AuNP on derivatized surfaces. As AuNPs were synthesized through citrate reduction [33], the negatively charged AuNPs are expected to bind to the amine-derivatized surface effectively via an electrostatic interaction [34]. After coupling with BS3, the amine-derivatized SiO_2 surface was presumably converted into a sulfonated surface.

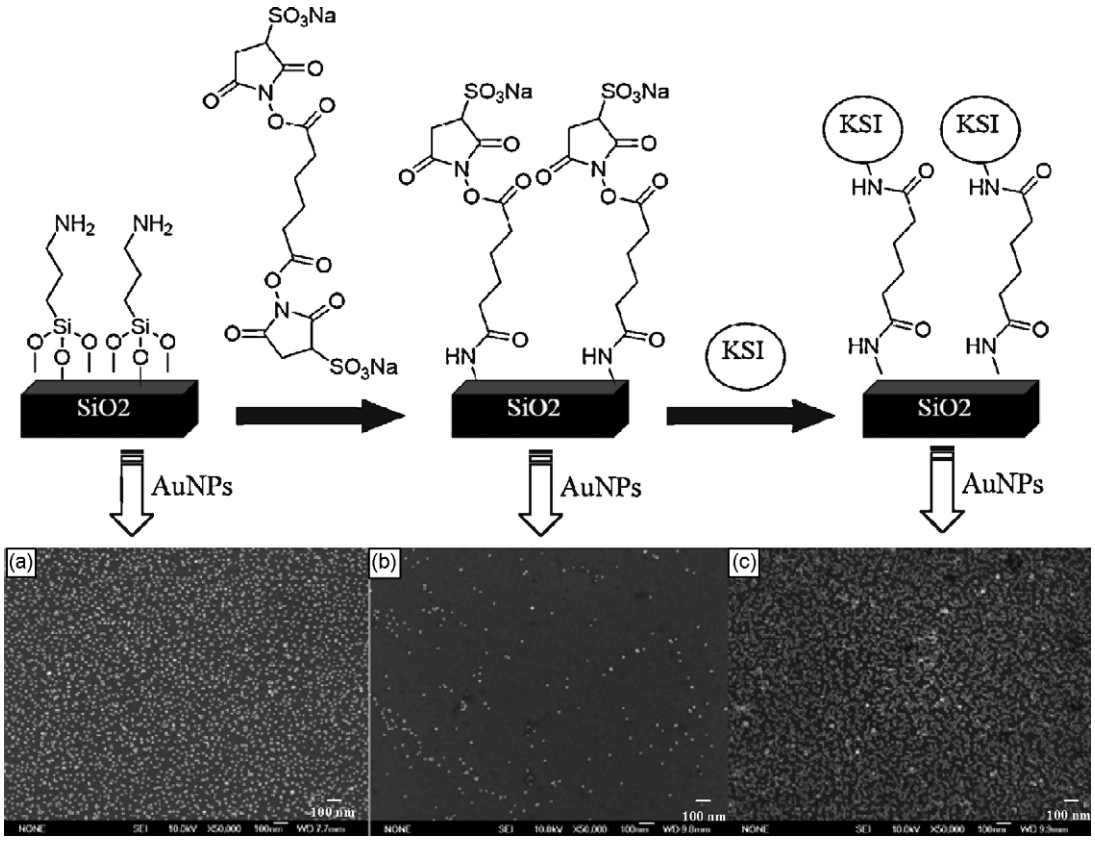

Fig. 3. Various stages of modification of a SiO_2 substrate and corresponding SEM images after treatment with AuNPs. The SEM images reveal the existence of AuNP on the surface of the substrate with modification by (a) APTES, (b) BS3 and (c) KSI_126C.

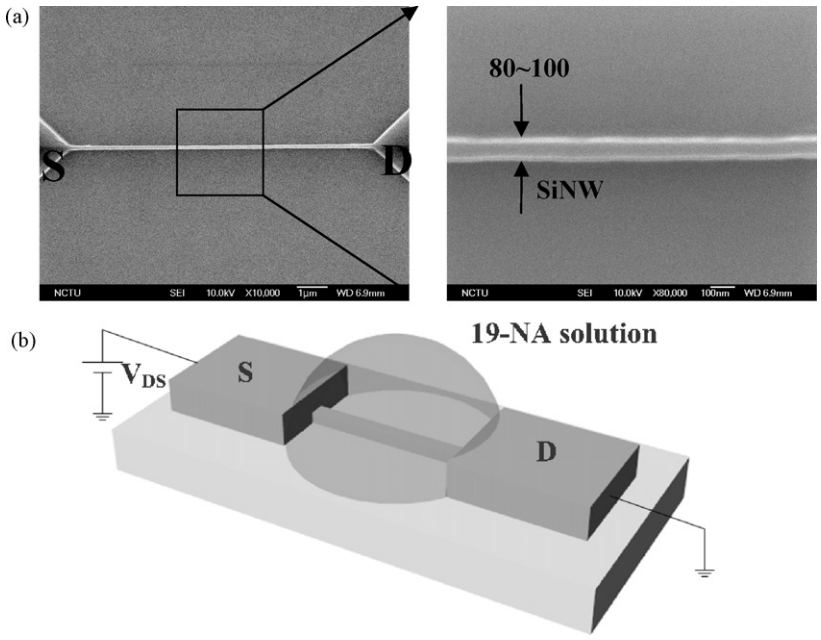

Fig. 4. Structure of the SiNW-FET device. (a) Scanning electron-microscope images of a SiNW (width 90 nm, height 40 nm) on silicon-on-insulator. (b) Diagram of the device employed for sensing on adding 19-NA solution over the SiNW.

The deposition of AuNP hence became rare because of the repulsion force, as shown in Fig. 3. KSI_126C, a mutant with an extra cysteine residue added at the C-terminus, was further immobilized on this substrate through the reaction of lysine residue with the BS3-activated surface. The resulting substrate was treated with AuNP. If the immobilization of KSI_126C is effective, the deposition of AuNP becomes much increased, presumably through the formation of an Au–S bond or an electrostatic interaction between the two substances. The highly dense AuNP layout [Fig. 3(c)] clearly demonstrates the efficiency of KSI immobilization under the conditions employed in the case of SiNW.

The dimensions of SiNW were determined from measurements with a scanning electron microscope [Fig. 4(a)] with a line width about 80–100 nm. A measurement of the conductance of SiNW was performed on adding the analyte between the source and drain electrodes, as schematically demonstrated in Fig. 4(b); the distance between the two electrodes is approximately 50 μm. The typical output and transfer curve of SiNW-FETs were observed. When the drain bias was set at 10 mV, the leakage current between the source and drain electrodes was typically with the scale of 1 pA. The noise level of SiNW-FET sensors was around 0.1–1 nS, and was often observed with the scale of 0.1 nS. The conductance of the Art_KSI/mA51-labeled SiNW-FET becomes modulated when the charge state of the surface is altered. As no existing reference is available to justify the influence of a steroid in the present system, we ensured that the observed signals were derived from the binding of steroid to Art_KSI/mA51 by comparing the responses of SiNW-FET modified by BS3 and further by Art_KSI. The effects of 19-NA on those devices are shown in Fig. 5. The electrical response of SiNW-FET was measured in Tris buffer (0.1 mM). Typical data for the time dependence were obtained from the output of the Art_KSI/mA51-labeled SiNW-FET after introducing 19-NA at varied concentrations. The conductance of BS3- and Art_KSI-labeled SiNW-FET remained constant on the addition of 19-NA up to 0.3 pM, indicating that the background disturbance of 19-NA is insignificant [Fig. 5(a); data for BS3-modified devices are not shown]. Upon addition of 19-NA at various concentrations, the conductance of Art_KSI/mA51-labeled SiNW-FET rapidly increased to a constant value [Fig. 5(b)]. 19-NA at a greater concentration resulted in a greater conductance, indicating that 19-NA competed with mA51 for the steroid-binding site in Art_KSI/mA51. The negatively charged mA51, of which the charge can be compensated by a protein when it is bound, is expelled to expose to the solution near SiNW. The correlation between the increased conductance and the applied 19-NA is shown in Fig. 6. A satisfactorily linear correlation was found for 19-NA at a concentration greater than 0.6 fM. In summary, the variation of conductance observed for Art_KSI/mA51-labeled SiNW-FET corresponds to the specific binding of 19-NA to Art_KSI/mA51. The sensitivity of 19-NA detection can attain a level of femtomolar. According to this first successful demonstration, a SiNW-FET is usable for sensing an uncharged analyte by integration with protein engineering. It is

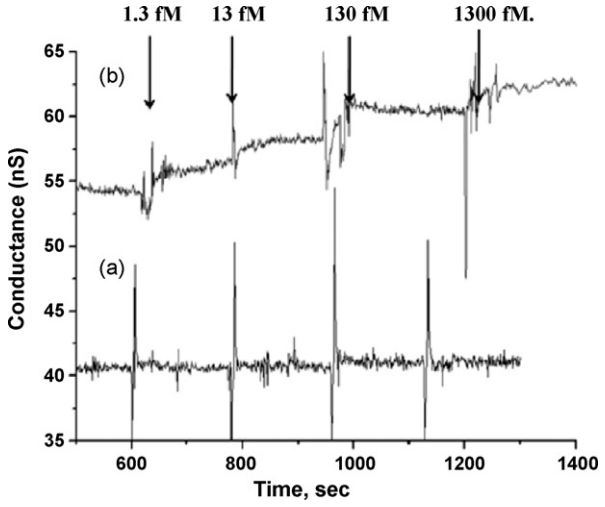

Fig. 5. Response of conductance of a SiNW-FET in the presence of 19-NA with Vds = 10 mV, Vgs = 0 V. Arrows indicate the point of addition of 19-NA to SiNW-FET labeled with (a) Art_KSI and (b) Art_KSI/mA51 at concentrations (1) 1.3 fM, (2) 13 fM, (3) 130 fM, and (4) 1300 fM. Note that the measurement of the conductance change was performed by adding 10 μL of Tris buffer (0.1 mM, pH 7.5), as a background conductance, between the source and drain electrodes of SiNW [as schematically demonstrated in Fig. 4(b)] and further directly and subsequently added 5 μL of the various concentration of 19-NA stock solution on top of the existed sample solution.

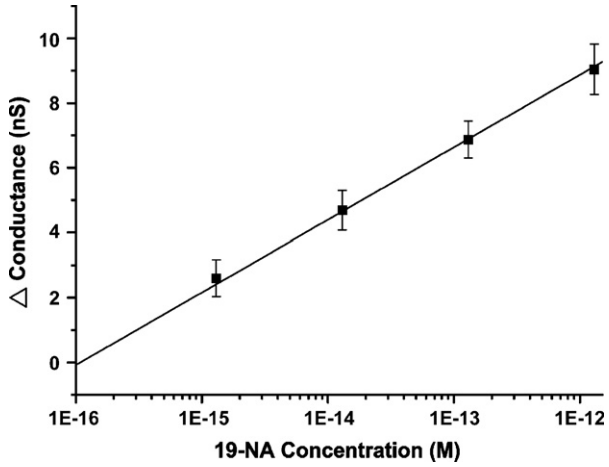

Fig. 6. Linear correlation of the variation of conductance for an Art_KSI/mA51-labeled SiNW-FET with respect to the applied concentration of 19-NA. The abscissa shows the 19-NA concentration on a logarithmic scale in mol unit. Each data point is the average of 130 times of measurements. G_0 = conductance at 0.13 fM 19-NA, G = conductance at varied concentration of 19-NA, $\Delta G = G - G_0$.

worth to note that the conductance of SiNW showed a signal spike at the point when analyte (19-NA) was added. This phenomenon was commonly observed in literatures with similar studies [35–37]. Although the mechanism of the appearance of signal spike is not clear, it is assumed that the spike is resulted from the disturbance or the redistribution of the double layer when analyte was added.

4. Conclusion

A silicon-based nanobiosensor, SiNW-FET, for the detection of steroid was fabricated. An engineered steroid-binding protein, Δ^5-3-ketosteroid isomerase, was chemically modified with a charged reporter molecule containing 1,5-EDANS moiety, and further immobilized on the surface of a silicon nanowire. In the presence of a steroid, the negatively charged 1,5-EDANS moiety is expelled and exposes to the nanowire surface and consequently produces electrical response. According to the change of conductivity, the concentration of 19-NA is calculated. The sensitivity of this novel nano-bio-device can attain a femtomolar level.

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Biographies

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