Applied Surface Science 332 (2015) 229–236

Contents lists available at ScienceDirect

Applied Surface Science

journal homepage: www.elsevier.com/locate/apsusc

Surface plasmon resonance for detecting clenbuterol: Influence of monolayer structure

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ARTICLE INFO

Article history:
Received 22 July 2014
Received in revised form 13 January 2015
Accepted 22 January 2015
Available online 30 January 2015

Keywords:
Surface plasmon resonance
Self-assembled monolayer
Clenbuterol detection
Indirect competitive inhibition immunoassay

ABSTRACT

Surface plasmon resonance sensor equipped with a fabricated immunosensor chip is used for detecting clenbuterol in this study. Since clenbuterol is a small analyte, indirect competitive inhibition immunoassay is employed. For fabricating the immunosurface, the Au-chip was functionalized by succinimidyl-terminated alkanethiol, and the terminal *N*-hydroxysuccinimide group of the self-assembled monolayer was either replaced with clenbuterol or blocked with ethanolamine. Scanning tunneling microscope experiments and electrochemical measurements depicted the domain structures of the succinimide group of succinimidyl-terminated propanethiol monolayer. The surface concentration and the orientation of succinimide group was significantly dependent on the concentration of dithiobis(succinimidyl) propionate (DSP) used in fabricating the monolayer. Furthermore, the structure of monolayer significantly influenced both the surface concentration and the orientation of clenbuterol on the sensor surface. Consequently, high coverage and standing-up configuration of clenbuterol showed high affinity for clenbuterol antibody. However, high affinity constant exhibited by the sensor surface was coupled with a low sensitivity. By contrast, lowest concentration of DSP solution (0.1 mM) used in fabricating the immunosurface showed a detection sensitivity of 3 ppt – the highest reported sensitivity for clenbuterol. For regeneration the immunosurface, 0.1 M NaOH was used and the same sensor surface could be reused for performing >100 rapid immunoreaction.

1. Introduction

In recent decades, surface plasmon resonance (SPR) sensors have been developed for numerous applications in fields, such as drug discovery, environmental monitoring, food safety, and security [1–6]. SPR is an optical transducer based on surface plasmon phenomena. SPR can measure the dielectric constant changes of the interface caused by the binding of target analytes with biointerfacial materials. In our SPR system, the sensitivity can be reached up to 30 pg/cm^2 (0.3 RU) in case of protein adsorption. In order to add high selectivity, biochemical recognition elements, such as antibodies, enzymes, proteins, DNA and cells are immobilized on the solid surface of the SPR sensor [7–10]. Biochemical recognition elements can be immobilized by physical adsorption [11], by embedding in polymers or membranes [12,13], by trapping in sol–gels [14,15], and by using functionalized alkanethiol or functionalized alkylsilane self-assembled monolayer (SAM) [16,17]. Each immobilization approach has advantages. For example, the fabrication of protein-conjugate physically adsorbed on the sensor chip (protein-conjugate/sensor chip) is performed in a single step. With a capacity to store large quantities of the biochemical recognition elements, polymer, membrane, and sol–gel/sensor chips show a large signal response to mass change. Although SAM has the disadvantage of low signal response of mass transducer because immobilization on a flat surface limits the number of accessible biorecognition elements, its sensitivity and stability are remarkably high.

The preparation of SAM is straightforward; the immersion of an appropriate substrate into a solution containing a functionalized alkanethiol or a functionalized alkylsilane leads to the spontaneous formation of a mololayer [18,19]. The monolayer structure is considered to influence the sensitivity and stability; therefore,

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http://dx.doi.org/10.1016/j.apsusc.2015.01.169
0169-4332/

researchers direct their efforts to form an ideal monolayer structure that is close-packed and defect-free. However, analyses by the scanning probe microscope reveal that monolayers consist of ordered and disordered domains [20–24]. Moreover, pinholes and defects are often observed at domain boundaries; analytes and impurities can be adsorbed onto these vacant sites and the mass change due to nonspecific adsorption can significantly affect the sensitivity and selectivity.

Since SH-group of alkanethiol covalently binds to metal, the domain structure of alkanethiol monolayer partially reflected to the surface structure of underlying substrate, such as terrace, step, plane indices, and crystalline structure. Several academic researchers use a single crystalline metal substrate; however, since polycrystalline metal substrates are used in sensor chips with practical applications, the monolayer structure on polycrystalline metal substrate will be discussed here.

Monolayer formation is very sensitive to the cleanliness of the substrate surface. If the surface is partially covered with contaminants, the surface coverage of SAM is low. Thus, the metal substrate requires sufficient cleaning prior to the fabrication of the SAM. In this study, the surface of the metal substrate is cleaned or ashed by irradiation with an excimer light with oxygen, a method that is widely used for cleaning semiconducting wafers. Ozone produced by exciting oxygen using excimer light ($\lambda = 172$ nm) oxidizes the contaminants on the metal, thereby cleaning the surface without damaging it.

Here, dithiobis(succinimidyl) propionate (DSP) is used for immobilizing an antigen onto a Au biosensor chip. DSP – a widely used commercially available reagent – has a short alkyl chain, a thiol and a succinimide at its terminal. Thiol terminal of the DSP binds to the Au surface and a biochemical recognition element with an NH_2-terminal can be instantly immobilized, since succinimide group of DSP is replaced by amines in a neutral buffer in a single step. However, since alkanethiols with short alkyl chains are often shown to adopt a lying-down phase [25,26], the orientation of biochemical recognition element can significantly affect the sensitivity of the biosensor. We will, therefore, discuss the relationship between the sensitivity of the sensor and the lying-down configuration of the molecules in domain structure.

The analyte chosen for this study is clenbuterol, an illegal ingredient in meat from food producing animals. Conventional analyses of clenbuterol including chromatography method, ELISA, electrophoresis, and electrochemistry involve complex time-consuming steps (such as incubation and repeated washings) can take up to 72 h for a single sample, with sensitivity detection at ppb levels [27–32]; thus, there is an urgent need for a highly sensitive sensor to detect clenbuterol to ensure food safety. However, the detection of small molecule clenbuterol (Mw = 277) by a mass transducer using the direct immunoassay is challenging. In this study, we employ the indirect competitive inhibition immunoassay for detecting the small analyte [6,33–36]. This method ensures that the signal response of the mass transducer is large, because the antibody, rather than the antigen, is being measured. Thus, reveals relatively higher sensitivity at ppt level. Results from previous reports [10,17] predict that the orientation of immobilized antigen on the sensor surface influences the sensitivity of the detection. Therefore, this study will also discuss the molecular scale structure in the context of sensing performance.

2. Experimental

2.1. Materials

Potassium hydroxide and ethanol were obtained from WAKO, Japan, while Sodium hydroxide was from Junsei chemical Co., Ltd., Japan. DSP and phosphate buffer saline (PBS) were purchased from Sigma–Aldrich, USA. Methanol was obtained from Dojindo, Japan. Clenbuterol hydrochloride and monoclonal mouse IgG antibody of clenbuterol (Ab) were ordered from LKT laboratories, Inc., USA and Novus Biologicals, USA, respectively. Refractive index matching fluid (refractive index = 1.518) was obtained from Cargille Labs., USA. All chemicals were of reagent or higher grade, and water (18.2 MΩ cm) from a Millipore system was used in all experiments.

2.2. Preparation of Au substrates

Glass substrates (BK7 type, 20 mm × 13 mm × 0.7 mm^t from Matsunami Glass Ind., Ltd., Japan) were sonicated in soap water (10% Contrad 70 detergent from Fisher). After rinsing with sufficient water, the glass substrates were dried with pure-nitrogen gas. In order to make the surface hydrophilic, the glass substrates were cleaned using plasma at 15 W under 2.0–2.5 Pa. Subsequently Au (50 nm) was sputtered onto the glass chips under 2.0–2.5 Pa. The prepared Au-chips were loaded into the SPR sensing system immediately after excimer ashing pretreatment (172 nm of incident light) for 2 min.

Reported surface densities of monolayers were corrected for the surface roughness of Au-chip ($\delta = 1.50$), which was calculated from the reduction charge density of Au–O in the cyclic voltammogram in 0.1 M sulfuric acid.

2.3. Electrochemistry

Electrochemical measurements were performed in an electrochemical cell holding using HZ-5000 Automatic Polarization System (Hokuto Denko, Japan). Experiments were conducted with a conventional three-electrode system with the Au-chip substrate, a platinum wire, and Ag/AgCl (saturated KCl) as the working electrode, the counter electrode and the reference electrode, respectively. Aqueous potassium hydroxide (0.1 M), deaerated with 5 N Ar in a glove box, was used as the electrolyte solution.

2.4. Scanning tunneling microscopy (STM)

STM experiments were conducted using a NanoScope STM III (Digital Instruments, USA) operating in the constant current mode (a few hundred picoampere) with the bias voltage typically between 0.5 and 1 V. For imaging, Pt/Ir (80/20) mechanically cut tips (diameter = 0.25 mm, Bruker, USA) were used to scan the surface.

2.5. X-ray photoelectron spectroscopy (XPS)

XPS experiments were conducted using a Rigakudenki model XPS-7000 X-ray photoelectron spectrometer. Monochromatic Mg Kα radiation is operated at 300 W. The angle of take-off is 90°. For determining the binding energy of elements, Au4f7/2 emission is used as an internal reference.

2.6. Surface plasmon resonance (SPR) sensing

SPR experiments were performed on SPR-670 (Nippon Laser Electronics, Japan) equipped with a fully automated flow system consisting of a plunger pump and an injector. The Au-chip was mounted on the semi-cylindrical prism with a refractive index matching liquid. Red light (670 nm) emitted from Ni–Cd laser was reflected at the Au-coated glass plate at attenuated total reflection angles, and the reflected light intensity was recorded using CCD camera. The reflectance angle, at which the light intensity was minimum (SPR angle), was recorded with time. All the experiments were conducted in an air-conditioned room (25 °C).