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# A nanoplasmonic biosensor for label-free multiplex detection of cancer biomarkers

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ABSTRACT

The development of highly sensitive, selective and multiplex sensors has become an important challenge for disease diagnosis. In this study, we describe a multiplex biosensor for the detection of cancer biomarkers based on unique plasmon response of single gold nanoparticles (AuNPs) and antibody–antigen binding activity. To demonstrate the ability of the plasmon biosensor to detect and quantify cancer biomarkers: a panel of biomarkers, including α-fetoprotein (AFP), carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) was used as a model analyte for multiple detection. A novel and sensitive multiplex biosensor was developed by immobilizing plasmonic nanoparticles in a site-specific manner and functionalized with monoclonal antibodies that recognize the target protein on hydrophilic–hydrophobic patterned glass slide. The proposed multi-analyte biosensor exhibited outstanding selectivity and sensitivity. The limit of detection was determined to be 91 fM, 94 fM and 10 fM for AFP, CEA and PSA from patient-mimicked serum, respectively. Finally, using this sensing strategy, this platform presents an excellent approach for versatile molecular diagnostics in both research and clinical medical fields.



## 1. Introduction

Cancer is a major cause of death worldwide. Approximately 14 million new cases and 8.2 million cancer-related death were reported in 2012, and annual number of cancer-related deaths continues to rise (Coleman, 2014). According to the report of World Health Organization, if case of cancer resulting in death were detected and treated at early stage, more than 30% of these death could be avoided (Ilbawi et al., 2015). Therefore, the detection of cancer biomarkers at the early stage has attracted considerable attention in the field of clinical diagnosis because it is useful for evaluating the extent of cancer, monitoring the response of cancers to therapy, and predicting recurrence (Maruvada et al., 2005).

In life science, immunoassay and immunosensor for a single cancer biomarker have yet to be developed (Wang et al., 2009; Jie et al., 2011). Approaches used in cancer diagnosis should be rapid, sensitive, highly reproducible, and reliable (Liu et al., 2009; Zhan et al., 2012; Xue et al., 2013; Zhao et al., 2013). Among the current methods for cancer biomarker detection, the plasmonic biosensor platform is one of powerful tools for clinical diagnostics and has recently attracted considerable attention, because it contributes many promising functions, such as high selectivity and, sensitivity, as well as real time and label-free detection (Haes et al., 2004; Rosi and Mirkin, 2005; Gish et al., 2007; Song et al., 2013). The plasmonic biosensor detects the incident photon frequency generated by the collective coherent oscillation of the free electrons surrounding nanometer-sized metallic nanoparticle (e.g., AuNPs) under the electromagnetic field of incoming light. This results in Rayleigh light scattering, which is also called localized surface plasmon resonance (LSPR) (McFarland and Van Duyne, 2003; Liu et al., 2006; Anker et al., 2008). The resonance wavelength at the surface of nanoparticle (LSPR) is dependent on the shape, size, and the dielectric environment surrounding the AuNPs (Truong et al., 2014). For this reason, nanoplasmonic biosensors have the potential to serve as platform for detecting biological molecules and biochemical binding events occurring on the nanoparticle surface due to changes in the local refractive index (Huang et al., 2009).

As reported in previous studies, our group has developed an individual nanoplasmonic platform for detecting cancer biomarkers (Cao and Sim, 2007; Truong et al., 2011; Hwang et al., 2012; Jun et al., 2014). Nanoplasmonic biosensors based on a single gold particle have a sensing concentration in the attomolar range, which is well suited for detection of a single biomarker. Moreover, the diagnostic power is increased when a panel of biomarkers can be monitored simultaneously in one device. However, the measurement of a single cancer biomarker is not sufficient for cancer diagnosis due to the heterogeneous nature of

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cancer genetics; most cancers are associated with more than one biomarker (Niklinski and Furman, 1995; Gregory Jr and Finlay, 1999). Panels of cancer biomarkers may provide abundant analytical and diagnostic information and improve diagnostic value by streamlining the procedure to one multiplex plasmonic sensor, thereby creating an urgent demand for enhanced detection throughput and multiple detection platforms (Zhan et al., 2012). Multiple detection platforms should be capable to rapid, sensitive, and simultaneous detection that also analyze multiple analytes in a single sample. In addition, it would result in ideally shorten analytical time, increased throughput detection and lower costs (Barbee et al., 2010).

In the present study, we propose a nanoplasmonic biosensor platform for multiple label-free detection of cancer biomarkers to achieve highly selective and sensitive detection of target biomarkers using site-selectively immobilized gold nanoparticles on the hydrophilic–hydrophobic patterned glass. The detection of target binding activity was monitored via spectral changes caused by a change in the local refractive index of vicinity of individual nanoparticles. As proof of concept, we used AFP, CEA, and PSA as model analytes in the proposed method to demonstrate good analytical performance. The detection limits of the platform were determined to be 91 fM, 94 fM and 10 fM for AFP, CEA and PSA from patient-mimicked serum. We anticipate that multiple label-free nanoplasmonic biosensor, as described herein, will be suitable for the detection of cancer biomarkers. This platform also provides a powerful tool that, when developed, will be a rapid, low-cost, high throughput bioprocess biosensor.

## 2. Materials and methods

### 2.1. Materials

Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot 3H_2O$), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), phosphate buffer saline pH 7.4 (PBS buffer), 3-aminopropyltriethoxysilane (APTES), bovine serum albumin (BSA), trichloro(octadecyl) silane (OTS), sulfuric acid ($H_2SO_4$), hydrogen peroxide ($H_2O_2$), nitric acid ($HNO_3$), 2-aminoethanethiol and human serum were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Sodium borohydride ($NaBH_4$) was purchased from Fluka Analytical (Missouri, USA). AFP monoclonal antibody (AFP-mAb), CEA monoclonal antibody (CEA-mAb), PSA–ACT complex monoclonal antibody (PSA-mAb), AFP protein, CEA protein, and PSA–ACT complex protein were supplied by Fitzgerald Industries International, Inc. (Acton, MA, USA). Acetone, ethanol, methanol, and n-hexane were purchased from Samchun Chemical Co. (Gyeonggi-Do, Korea). Coverslip slides ($22 \times 40 \times 0.1$ mm$^3$) were purchased from Deckglaser (Germany). Other essential reagents were supplied by Sigma Aldrich Inc. The sylgard 184 silicone elastomer kit was purchased from Dow Corning (Michigan, USA) and was used to fabricate poly(dimethylsiloxane) (PDMS) stamps. Ultra-pure water (18.2 m$\Omega$ cm$^{-1}$ ), purified by reverse-osmosis, was used to prepare all chemical solutions. All glassware used in the experiments was cleaned in freshly prepared aqua regia solution and rinsed thoroughly in ultra-pure water before use.

### 2.2. Amino-modified gold nanoparticles synthesis

Amino-modified gold nanoparticles were synthesized in aqueous solution by sodium borohydride reduction, following a previously published protocol with some modifications (Niidome et al., 2004; Wang et al., 2012). An HAuCl4 solution (290 μL, 100 mM) was diluted with water (79.71 mL), and a 2-aminoethanethiol solution (170 μL, 213 mM) was added to the gold solution. After stirring for 20 min at room temperature, $NaBH_4$ solution (4.2 μL, 18 mM) was added, and the mixture was stirred for more than 10 h in the dark. Then, the solution was filtered through a 0.2 μm filter to remove any aggregated particles and was characterized using a UV/vis spectrometer (Shimadzu UV-3600, Kyoto, Japan). The colloidal solution was subsequently centrifuged at 7000 rpm for 15 min to separate unbound 2-aminoethanethiol. The sediment was re-suspended in ultra-pure water and stored at 4 °C until further use. The size and the homogeneity of AuNPs were determined by high-resolution transmission electron microscopy (HR-TEM) conducted on a JEM3010 instrument operating at a voltage of 300 kV. The final solution was stored at 4–8 °C in refrigerator.

### 2.3. Preparation of immuno-gold nanoparticles

For preparation of immuno-gold nanoparticles, 500 μL of each target protein monoclonal antibody (mAb) (1 mg mL$^{-1}$) was mixed with EDC/NHS (1 M, 10 μL) to convert the carboxyl groups of the C-terminal Fc region of antibodies to NHS esters in order to prepare the immuno-gold colloids. After 15 min, 1 mL of the stabilized AuNPs ($A_{535} \approx 2$) was added to activate each target protein. The solution was mixed well and incubated at room temperature for 1 h. Then, the immuno-gold nanoparticles were centrifuged at 5000 rpm for 15 min, the supernatant was discarded, and the sediment was re-dispersed in 0.01 M PBS buffer (pH 7.4) and stored at 4 °C until further experimentation.

### 2.4. Fabrication of PDMS stamp

PDMS stamps with 3 defined holes (2 mm in diameter) were designed with AutoCAD software (Autodesk, USA) and printed on transparency photomask film (Han & All Technology, Korea). Briefly, silicone elastomer base and curing agent were thoroughly mixed in a 10:1 ratio and degassed for 1 h before pouring into a pre-casted mold. Curing was carried out at 80 °C for 4 h. The cured PDMS sheet with a thickness of 2 cm was carefully detached from the mold after cooling to room temperature and cut into pre-designed patterns.

### 2.5. Preparation of hydrophilic–hydrophobic patterned glass slides and immuno-AuNPs immobilization

Microscopic glass slides ($22 \times 40 \times 0.1$ mm$^3$) from Warner Instruments were cleaned overnight using freshly prepared aqua regia solution and then thoroughly rinsed with ultrapure water. Briefly, the cleaned glass slides were dipped in 1% (v/v) trichloro (octadecyl)silane (OTS) in n-hexane for 15 min. To remove excess OTS, glass slides were rinsed with ethanol three times before drying at 60 °C for another 4 h. To fabricate hydrophilic–hydrophobic patterned glass slides, a pre-designed PDMS stamp was compressed onto OTS-treated glass and exposed to oxygen plasma at 80 W for 9 s to locally convert the terminal groups of the OTS in exposed regions to hydroxyl groups (Lin et al., 2009). The site-selectively modified glass slide was dipped in 5% (v/v) APTES in 99.9% ethanol for 15 min. Then, the glass slides were sonicated in ultrapure water three times for 5 min. Therefore, the hydrophilic regions treated with $O_2$ plasma were functionalized by APTES to expose amino groups, while the other regions maintained the hydrophobic characteristics of OTS. After the washing step, silanization was completed by drying the slides at 120 °C for 2 h. Immuno-AuNPs were randomly immobilized onto a silanized glass slide by drop-coating 10 μL of the diluted AuNP solutions ($OD_{520} \approx 0.04$) onto the center of the predesigned holes and incubating for 1 min to determine the optical response of individual