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Wide-range quantification of human thyroid-stimulating hormone using gold-nanopatterned single-molecule sandwich immunoassay chip

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ABSTRACT

We performed wide-range quantification of human thyroid-stimulating hormone (hTSH) using a gold nano-patterned sandwich immunoassay chip. Objective-type total internal reflection fluorescence microscopy (TIRFM) was used to detect hTSH at the single-molecule level. A gold spot with a diameter of 100 nm on a 10-mm square glass substrate was fabricated by electron beam nanolithography. When hTSH bound to antibodies conjugated to each 100-nm gold spot, there was an increase in the relative fluorescent intensity (RFI). The detection limit of this "TSH-nanoarray chip" was 360 zM (equivalent to five molecules), which demonstrated that a TSH-nanoarray chip could be used for detection at the single-molecule level. A linear response was observed over a wide dynamic range (from 360 zM to 36 pM, $R=0.9812$) without a fluorescence quenching effect. A significant enhancement in the sensitivity ($\sim$12,000-fold) was achieved with the 100-nm gold nano-patterned chip compared with results obtained using a traditional chemiluminescence immunoassay for the evaluation of TSH in human serum.

1. Introduction

Thyroid-stimulating hormone (TSH), also known as thyrotropin, is a 28 kDa glycoprotein hormone secreted by the pituitary gland. TSH stimulates the production and release of thyroid hormones (T4 and T3), and is a key protein in the control of thyroid function [1]. Measurement of TSH is useful for the detection of subclinical primary hypo- and hyperthyroidism. Patients suffering from hypothyroidism have elevated TSH levels, and more sensitive TSH assays would enable the diagnosis of subclasses of hyperthyroidism [2]. Approximately 85% of cases of congenital hypothyroidism are sporadic and 15% are hereditary [3]. Normal serum TSH concentrations range from 0.3–5.0 mIU/L (equivalent to 1.78 pM–28.6 pM), but over 95% of euthyroid subjects have TSH levels below 2.5 mIU/L (=14.3 pM) [4,5].

In the last few years, traditional analytical methods such as radioimmunoassay [6,7], immunoradiometric assay [8], immunoenzymetric assay [9], immunochemiluminescent assay [10], time-resolved fluorescence immunoassay [11], bioluminescent immunoassay [12,13], and homogeneous particle-based immunoassays [14] have been used to measure TSH. Available automated TSH immunoassays are third generation, meaning that they have a functional sensitivity of $\leq$0.02 mIU/L (118.7 fM) as recommended by the National Academy of Clinical Biochemistry [15]. Recently, Boemer et al. developed an enzyme-linked immunoabsorbent assay for quantification of TSH levels in dried blood spots from newborns [16]. William et al. determined the functional sensitivity of seven commercially available automated immunoassays and compared the performance of these assays at low TSH concentrations [17]. More recently, established time-resolved immunofluorometric assay [18], magnetic particle-based chemiluminescence enzyme immunoassay for a highly sensitive assay of TSH [19,20] as well as analyzed the TSH (detection limit, 0.09 mIU/L=534 fM) using 5 min incubation time and high-capacity surface [21]. However, these techniques used for the diagnosis of critical diseases are still not sensitive enough for early stage diagnostics, and TSH assay methods will need to be sufficiently sensitive to detect the low amounts of TSH seen in non-thyroidal illnesses and in hyperthyroidism.

Nanoarray techniques are currently the most promising alternative to DNA and protein microarrays, because they provide the possibility of detection of proteomic targets with very low limits of detection (i.e. femto- or atto-mole levels) using extremely small amounts of sample [22]. Since TSH level is widely used as a marker for thyroid disease, a highly sensitive assay is necessary for improved diagnosis. However, there have been no studies to date addressing the practical application and sensitivity of nanoarray chips.

The goal of our research was to demonstrate the feasibility of using a gold nanoarray chip as a key component of a biosensor platform for accurate and highly sensitive detection of TSH.

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A 100 nm gold array chip can make use of smaller sample volumes and can achieve higher sensitivities due to the small size of the entire array. Optimum conditions were successfully achieved using a sandwich immunoassay on a gold nano-patterned chip designed to detect human TSH (hTSH) using an objective-type total internal reflection microscopy (TIRFM) system. Finally, the hTSH biomarker was quantified over a wide dynamic range using TIRF intensities corrected by background subtraction.

2. Materials and methods

2.1. Reagent preparation

Dithiobis(succinimidyl propionate) (DSP) and Protein A/G were purchased from Pierce (Rockford, IL, USA). Dimethyl sulfoxide (DMSO), glycine and Alexa Fluor® 647 donkey anti-mouse IgG were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Tris(base) was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). StabilGuard was purchased from Surmodics (Eden Prairie, MN, USA). Monoclonal mouse anti-human thyroid stimulating hormone (TSH-antibody, 2TS11-10C7 and 2TS11-5E8) was purchased from HyTest (Turku, Finland) and thyroid stimulating hormone protein (TSH-antigen, 30-AT09) was purchased from Fitzgerald (North Acton, MA, USA). Normal human serum samples were isolated from blood by centrifugation at 2,000 rpm for 15 min at 2 °C. To mimic clinical conditions, standard TSH protein was spiked into $1/10^5$ diluted normal human serum. Prior to use, 1 × PBS buffer solution (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) was filtered through a 0.2-μm membrane filter and photobleached overnight using a UV-B lamp (G15TE, 280–315 nm, Philips, The Netherlands).

2.2. Preparation of gold nanoarray chips

The 4 × 5 gold nano-patterned substrate was fabricated on a 10 mm^2 glass substrate using an electron beam evaporator. The individual Au/Cr (20/5 nm thickness) spots were 10 μm in pitch, and 100 nm in diameter (National Nanofab Center, Daejeon, Korea) (Fig. 1).

All chips were prewashed prior to linker deposition. The chips were immersed in acetone (99.5% purity) for 30 s, isopropyl alcohol (99.9% purity) for 30 s, then piranha solution (1:1 = H_2SO_4:30% H_2O_2) for 30 min. Between each wash step, the chips

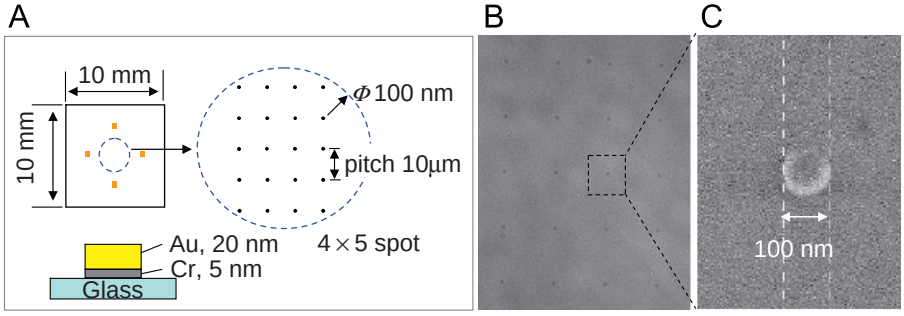

Fig. 1. (A) hTSH chip design using 4 × 5 nanoarray gold patterning with a spot diameter of 100 nm on a glass substrate. (B) DIC image and (C) SEM image of individual gold spots. * The chip was patterned on a 5 nm layer of chromium and 20 nm layer of gold.

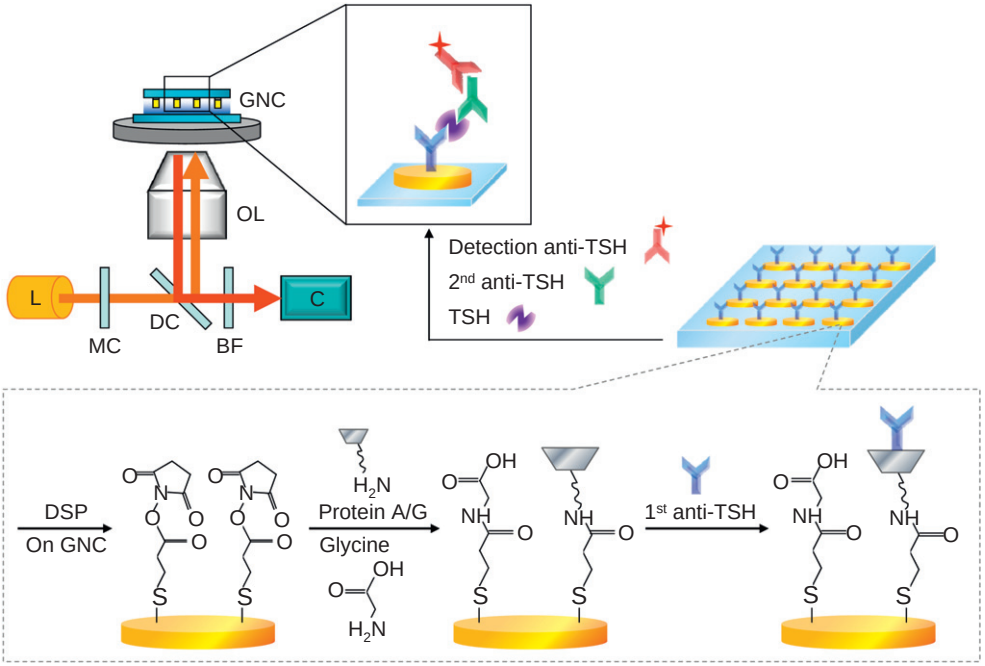

Fig. 2. Schematic diagram of the mechanical setup and the sandwich fluorescence immunoassay on the gold nano-patterned hTSH chip. The following acronyms are used: L, laser; MS, mechanical shutter; DC, dichroic mirror; BF, band-pass filter; C, charge-coupled device; OL, objective lens; GNC, gold nano-patterned chip.

were rinsed thoroughly with distilled water. The cleaned chips were dried under a nitrogen stream and stored in a desiccator.

2.3. The TSH sandwich immunoassay on gold nano-patterned chips

The basic processing of the sandwich immunoassay was performed as previously described [23]. Briefly, the gold-patterned chip was immersed in 4 mg/mL DSP in DMSO for 30 min, then rinsed with DMSO and distilled water. Protein A/G was added at a concentration of 0.1 mg/mL to the activated gold surface for 1 h. Unreacted succinimide groups were blocked with 10 mM Tris (pH 7.5) and 1 M glycine for 30 min. The chips were incubated with StabilGuard for 30 min to stabilize them and then rinsed briefly with a few drops of distilled water. The chips were incubated with 20 μL of 2 μg/mL TSH-antibody (10C7) in PBS pH 7.4 for 1 h. After washing, 20 μL TSH standard protein (30-AT09) diluted to various concentrations, normal and spiked clinical samples were incubated on a chip for 1 h. However, the incubation time of 360 zM TSH concentration was increased to 4 h considering that there are only 5 molecules and enough time is required for Brownian motion. 20 μL of 2 μg/mL TSH-antibody (5E8) was reacted for 1 h. Finally, 20 μL of 2 μg/mL Alexa Fluor® 647 donkey anti-mouse IgG was reacted for 30 min to measure the TIRFM fluorescence intensity. The chip was washed by dipping in 100 mL of 1 × PBS for 2 min at each step. All of the reactions were carried out at room temperature with agitation (Fig. 2).

2.4. The objective-type TIRFM system

Fig. 2 depicts the optical and mechanical setup of the objective-type TIRFM. An inverted model IX-71 microscope (Olympus, Tokyo, Japan) was equipped with a high-numerical aperture 100 × (1.65 N.A.) oil-immersion objective (Apo, Olympus). A 680/30 nm band-pass filter (Semrock, Inc., NY, USA) was coordinated with the use of 633 nm laser excitation during imaging. Fluorescence images were captured by a charge-coupled device (CCD) camera (Cascade 512B, Photometrics, Tucson, AZ, USA) equipped with a Uniblitz mechanical shutter (Vincent Associates, Rochester, NY, USA) and a driver (Model VCM-D1, Vincent Associates). The exposure time of the CCD camera was 100 ms. The quantitative analysis from the images of the objective-type TIRFM system was as following: We selected signal regions and background regions with the same area. And then, sum of TIRF intensities of occupied pixels per one spot corrected by background subtraction was calculated. All quantitative analysis of data and image acquisition were used MetaMorph 7.1 software (Universal Imaging Co., Downing Town, PA, USA).

3. Results and discussion

3.1. Specificity of the TSH sandwich immunoassay

TSH is a glycoprotein with two subunits (α and β). The α subunit of TSH included three other glycoprotein such as follicle stimulating hormone, luteinizing hormone, and human chorionic gonadotropin. Monoclonal mouse anti-human thyroid stimulating hormone (TSH-antibody, 10C7, and 5E8) purchased from HyTest Ltd. specifically recognized the whole molecule of TSH. The TSH-antibodies 10C7 and 5E8 were confirmed by the producer that there was no cross-react with three glycoprotein hormones [24]. Furthermore, Lin et al. showed the satisfactory specificity of the developed method due to the use of paired monoclonal antibodies for cross-reactivity with three other glycoprotein hormones [25]. Therefore, the effect of interferential antigen in TSH sandwich immunoassay using infinitesimal concentration was ignored.

3.2. Sensitivity of the sandwich immunoassay for detecting standard TSH

The assay sensitivity, or the lowest limit of detection, is largely determined by the specific activities of the antigen and the antibody, the extent of non-specific binding, and by other assay conditions such as target concentrations and reaction time. A gold nanoarray chip immunoassay with enhanced capturing capacity and optimal orientation of the antibodies could thus provide further possibilities in the improvement of detection limits and the accuracy of immunoassays. Protein A/G binding to the heavy chains of the antibody Fc region was used to uniformly orient the antibodies. In addition, the chips were incubated with Glycine/Tris (pH 7.5) and StabilGuard to remove unreacted succinimide groups and to stabilize the chip, respectively (Fig. 2).

Size reduction and site-specific labeling of antibodies to create a surface with high functional capacity increase sensitivity of an immunoassay [26]. Use of 100-nm gold array chips reduces the required sample volume and increases sensitivity due to the small spot size. It is well known that the use of nanospheres (GNSs) or gold nanorods (GNRs) leads to fluorescence quenching. However, there was no quenching of fluorescence dyes by the 100-nm gold spot on the chip since the spot has a smaller surface area and lower surface energy compared with GNSs and GNRs. The sensitivity of the TSH assay was calculated using the limit of detection method.

The positive standard-TSH was diluted serially and assayed. The linear range of the TSH-nanoarray chip was 360 zM-36 pM

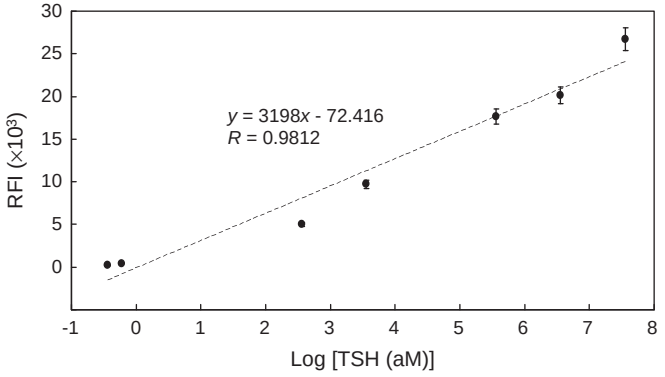

Fig. 3. The calibration curve of TSH standard concentrations over a wide dynamic range produced by serial dilutions from 360 zM to 36 pM (n=5). The relative fluorescence intensities were corrected by background subtraction.

Table 1
Comparison of detection limits between the TSH-nanoarray chip and other methods.

Method	LOD (mIU/L)
CL immunoassay I [17]	0.006 (35.6 fM)
CL immunoassay II [27]	0.010 (5.93 fM)
CL immunoassay III [27]	0.000 7 (4.15 fM)
CL immunoassay IV [27]	0.003 (17.8 fM)
CL immunoassay V [28]	0.010 (5.93 fM)
ECL immunoassays [29]	0.100 (593 fM)
NP label immunoassays [30]	0.001 2 (7.12 fM)
TSH-nanoarray chip	6.07×10^{-8} (360 zM)

Indication: CL, chemiluminescence; ECL, electrochemiluminescence; NP, nanoparticle; LOD, limit of detection.

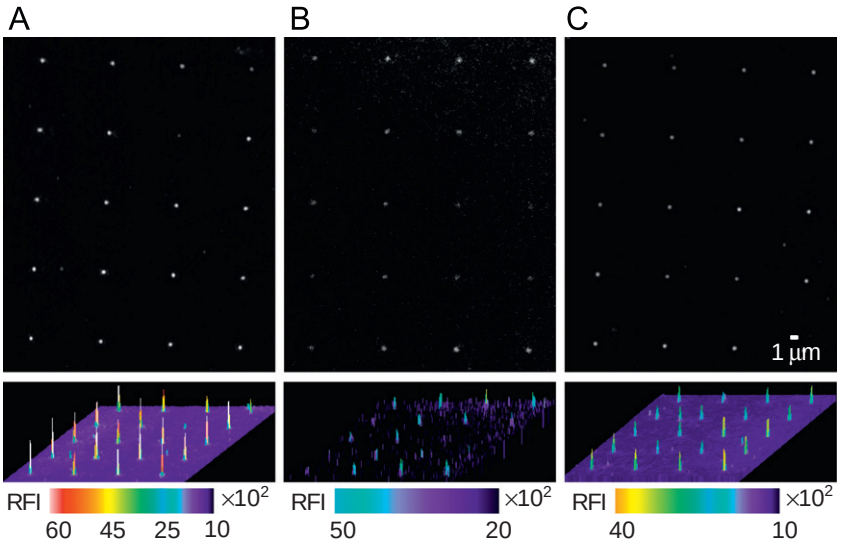

Fig. 4. Objective-type TIRFM images of (A) standard TSH (TSH-positive sample), (B) normal (nonpathologic) human serum and (C) spiked normal human serum on hTSH-nanoarray chips.

(correlation coefficient, $R=0.9812$). The lowest limit of detection was 6.07×10^{-8} mIU/L (360 zM) as calculated from the linear slope value (Fig. 3). At a TSH concentration of 360 zM, the incubation time required to attain a sufficient binding probability of individual TSH molecules to the antibody molecules was extended to 4 h because the theoretical number of TSH molecules is only five at 360 zM (500 μL). These results demonstrate that the TSH-nanoarray chip can be used at the single-molecule level.

The functional sensitivity of seven automated TSH immunoassays was reported by Owen et al. [17]. In addition, Ognibene et al. evaluated TSH quantitation on commercial immunoassay analyzers, and reported the limits of detection for TSH to be 0.0007 mU/L (Architect), 0.003 mU/L (Immulite) and 0.010 mU/L (ACS-180) [27]. Table 1 summarizes the LODs of the different TSH immunoassays. The sensitivity of the TSH-nanoarray chip based on a sandwich immunoassay using TIRFM was 12×10^{3} times higher than that obtained using the chemiluminescence immunoassays technique (>0.0007 mIU/L, 4.15 fM) (Table 1). Also, the limit of detection using the TSH-nanoarray chip immunoassay was 3×10^{6} times lower than the sensitivity of ≤0.02 mIU/L (118.7 fM) recommended by the National Academy of Clinical Biochemistry [15].

3.3. Application to clinical samples on TSH-nanoarray chip

The standard TSH (TSH-positive sample) (Fig. 4A, 6.07 mIU/L= 36 pM), normal (nonpathologic) human serum (Fig. 4B, 11.95×10^{-6} mIU/L, 71 aM), and spiked normal human serum (Fig. 4C, 3.44 ± 0.57 mIU/L, 20.4 ± 3.4 pM; theoretical, 3.03 mIU/L, 18 pM) were quantified using the gold nanoarray chip. This result indicates that the TSH-nanoarray chip assay could be used for quantitative analysis of serum samples with high accuracy and sensitivity.

4. Conclusions

We developed a TSH-nanoarray chip for a single-molecule sandwich immunoassay based on the measurement of a fluorescence signal on 100-nm gold spots by TIRFM. hTSH was chosen as a model target protein molecule because it is a widely used marker for thyroid disease and requires high detection sensitivity. The TIRFM system enables quantification of the biomarker protein (hTSH) over a wide dynamic range from the zM to the pM level (10^{-21} M-10^{-12} M) without an outstanding fluorescence quenching effect by measuring the relative fluorescence intensity emitted from a fluorescent dye on a TSH-nanoarray chip compared with the range of automated TSH immunoassays (10^{-15} M to 10^{-12} M) [17,27]. The TSH-nanoarray chip method based on a single-molecule sandwich immunoassay produced an improved detection sensitivity of TSH of 360 zM (equivalent to five molecules) and demonstrated the possibility of this assay's use for diagnosing disease.

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