Research Article

Single-Molecule Sandwich Immunoassay for Quantification of Alpha-Fetoprotein Based on Evanescent Field-Enhanced Fluorescence Imaging

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Received 2 October 2012; Accepted 7 November 2012

Academic Editor: Haiyan Li

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A highly sensitive immunosensor based on a gold nanopatterned chip was developed for accurate determination of alpha-fetoprotein (AFP) *via* total internal reflection fluorescence microscopy (TIRFM). The surface of the gold nanopatterned chips was modified with dithiobis(succinimidyl propionate) and protein A/G for immobilization of the AFP antibody. The immunoassay created a sandwich of antigen between the AFP antibody on the chip that was modified with protein A/G, and the secondary antibody, a monoclonal anti-human-AFP labeled with biotin (biotin-labeled anti-AFP). AFP concentration was determined based on evanescent field fluorescence signal, which was generated by interaction between biotin-labeled anti-AFP and a streptavidin-labeled fluorescence dye. AFP concentration could be measured in a wide dynamic linear range of 720 zM–10 nM with a detection limit of 720 zM. A significant enhanced sensitivity ($\sim 40,000$ -fold) was achieved with the AFP-nanoarray chip compared to conventional chemiluminescence immunosensors. The immunoassay exhibited a wide detection range and high sensitivity and accuracy, qualities valuable for clinical assay of AFP.

1. Introduction

Since the introduction of sandwich assays using monoclonal antibodies, various immunoassays have been introduced with automated analysis and increased specificity [1]. However, many immunoassay methods such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and single radial immunodiffusion have disadvantages such as being time-consuming procedures using expensive instrumentation with complicated separation and labeling steps, and potential dangers (e.g., radiation hazards) or expensive materials [2]. New techniques such as electrochemistry [3], chemiluminescence [4], piezoelectricity [5], surface plasmon resonance (SPR) [6], and sandwich immunoassays based on nanoarray protein chips [7, 8] have attracted interests because of their characteristics. Specifically, nanoarray protein chips offer a sensitive, accurate, quantitative, and

simple alternative methodology for determination of tumor markers.

Alpha-fetoprotein (AFP) is a 70 kDa oncofetal glycoprotein of 591 amino acids. It contains a single asparagine-linked (Asn233) carbohydrate chain that is a known biomarker for hepatocellular carcinoma (HCC) [9–12]. The association between serum AFP and HCC has been extensively described [13–16]. AFP has been a diagnostic test for HCC since the 1970s, when most patients with HCC were diagnosed at an advanced stage with clinical symptoms [17]. AFP is suggested to function as a transport molecule for several different ligands and various drugs [18, 19] and to have immunosuppressive activity and a role in regulation of cell proliferation [20]. The first conditionally quantitative serum assays for AFP were introduced in 1971 [21]. Several approaches have attempted to enhance the techniques of SPR detection [22] such as bioluminescent sandwich

FIGURE 1: (a) DIC image of gold nanopatterned AFP chip with a spot diameter of 100 nm and (inner) SEM image of individual gold spots. (b) Schematic diagram of the AFP sandwich fluorescence immunoassay. (c) Schematic diagram showing the TIRFM detection system. L: laser; HL: halogen lamp; P: transmitted all-side polished dove-type prism; GNC: gold nanopatterned chip; OL: objective lens; BF: band-pass filter; C: charge-coupled device.

immunoassays [23], electrochemical immunoassays [24], and chemiluminescence resonance energy transfer [25]. However, the sensitivities for these assays are still poor [26].

In this study, a sandwich immunoassay using gold nanopatterned protein chips was developed for quantitative detection of tumor markers such as AFP in serum. A total internal reflection fluorescence microscopy (TIRFM) technique based on evanescent field fluorescence imaging was applied for trace analysis of AFP with a wide dynamic linear range to use in clinical diagnosis. The relationship between evanescent field-fluorescence signal and AFP concentration showed excellent and extensive linearity. The method was successfully applied to determine AFP in human serum.

2. Experimental Details

2.1. Reagent Preparation. Dimethyl sulfoxide (DMSO) and glycine were from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dithiobis(succinimidyl propionate) (DSP) and protein A/G were from Pierce (Rockford, IL, USA). Tris (base) was from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). StabilGuard was from SurModics (Eden Prairie, MN, USA). Alexa Fluor 488 streptavidin was obtained from Molecular Probes (Eugene, OR, USA). Human AFP antigen, monoclonal antibody to human AFP (5H7), and monoclonal antibody to human AFP labeled with biotin (biotin-labeled anti-AFP, 4A3), were from Biodesign International (ME, 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, a standard AFP sample of 20 fM was spiked into 1:10⁴ diluted normal human serum at a three-to-one ratio. Before use, 1× PBS buffer solution (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) 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. Gold Nanopatterned Chips. A gold nanopatterned substrate was designed as shown in Figure 1(a) and fabricated by the National Nanofab Center (Daejeon, Republic of Korea). Four-inch soda-lime glass wafers from Winwin Tech (Bucheon, Republic of Korea) were used to make 4 × 5 nanoarrays with 100 nm diameter spots (SEM in Figure 1(a)) with a 10 μm pitch. Gold spots were deposited on the glass substrate by an electron beam evaporator. Substrates were coated with a 5 nm adhesive layer of chromium (99.997% purity) at a rate of 0.1 nm/s, followed by deposition of a 20 nm layer of gold (99.997% purity) at a rate of 0.1 nm/s. Before linker deposition, chips were immersed in acetone (99.5% purity) for 30 s, followed by isopropyl alcohol (99.9% purity) for 30 s. Gold nanopatterned chips were exposed to piranha solution (1:1 = H₂SO₄:30% H₂O₂) for 30 min, rinsed with deionized water, and dried under a stream of nitrogen. Before use, chips were stored in a desiccator.

2.3. AFP Single-Molecule Sandwich Immunoassay on Gold Nanopatterned Chips. The analytical procedure for sandwich immunoassay of AFP on a gold substrate is schematically depicted in Figure 1(b). Gold patterned chips were immersed in 4 mg/mL DSP in DMSO for 30 min, then rinsed with DMSO and deionized water. Addition of 0.1 mg/mL of protein A/G, which binds to the heavy chains of the antibody Fc region, was used to uniformly orient the antibodies for 1 h. Unreacted succinimide groups were blocked with 10 mM Tris (pH 7.5) and 1 M glycine for 30 min. Chips were incubated with StabilGuard for 30 min to stabilize bound proteins, then rinsed briefly with a few drops of deionized water. Chips were incubated with 20 μL of 2 μg/mL monoclonal antibody to human AFP (5H7) in PBS (pH 7.4) for 1 h. After washing, AFP standard protein that was diluted to various concentrations, or normal or spiked clinical samples were incubated on chips for 1 h. The incubation time of the sample with 720 zM AFP was increased to 5 h to allow sufficient time for Brownian motion. Reaction with

TABLE 1: Comparison of detection limits between AFP-nanoarray chips and other methods.

Method	Dynamic range (ng/mL)	LOD (ng/mL)	References
SPR	1.0–200 (14 pM–2.8 nM)	0.65 (9.1 pM)	[22]
BL immunoassay I	0.01–100 (140 fM–1.4 nM)	0.01 (140 fM)	[23]
BL immunoassay II	0.02–200 (280 fM–2.8 nM)	0.02 (280 fM)	[28, 29]
CRET	5–70 (70 pM–980 pM)	2.50 (35 pM)	[25]
FRET inhibition assay	0.8–45 (11.2 pM–0.63 nM)	0.41 (5.74 pM)	[30]
Pz immunoassay	20–640 (280 pM–8.96 nM)	20 (280 pM)	[31]
EC immunoassay I	0.5–80 (7 pM–1.12 nM)	0.25 (3.5 pM)	[32]
EC immunoassay II	0.1–30 (1.4 pM–420 pM)	0.018 (252 fM)	[33]
EC immunoassay III	0.01–200 (140 fM–2.8 nM)	0.004 (56 fM)	[34]
EC immunoassay IV	1.0–10 (14 pM–140 pM)	0.70 (9.8 pM)	[35]
ECL immunoassay	0.002–32 (28 fM–448 pM)	0.002 (28 fM)	[36]
CL immunoassay	0.01–0.5 (140 fM–7 pM)	0.005 (70 fM)	[37]
AFP-nanoarray chip	50×10^{-9} –714.3 (720 zM–10 nM)	50×10^{-9} (720 zM)	This work

Indication: SPR: surface plasmon resonance; BL: bioluminescent; CRET: chemiluminescence resonance energy transfer; FRET: fluorescence resonance energy transfer; Pz: piezoelectric; EC: electrochemical; ECL: electrogenerated chemiluminescence; CL: chemiluminescence; LOD: limit of detection.

20 μ L of 2 μ g/mL biotin-labeled anti-AFP (4A3) was for 1 h. To detect biotin-labeled anti-AFP, 20 μ L of Alexa Fluor 488 streptavidin (2 μ g/mL) was added to chips and incubated for 30 min. Chips were washed in 100 mL 1 $\times$ PBS for 2 min and rinsed briefly with deionized water at each step. All reactions were carried out at room temperature with agitation.

2.4. Total Internal Reflection Microscopic System. A schematic diagram of the TIRFM system is in Figure 1(c). An upright Olympus BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with an Olympus 100 $\times$ UPLFL objective lens (oil type, 1.3 N.A., W.D. 0.1 mm) was used. A 520/10 nm band-pass filter from Semrock (Rochester, NY, USA) was coordinated with the use of 473 nm laser excitation during imaging. Fluorescence images were captured by an electron-multiplying, cooled charge-coupled device (EM-CCD) camera (QuantEM 512SC, Photometrics, AZ, USA) equipped with a Uniblitz mechanical shutter (Vincent Associates, Rochester, NY, USA) with an exposure time of 100 ms. All quantitative analysis of data and image acquisition used MetaMorph 7.1 software (Universal Imaging Co., Downing Town, PA, USA).

3. Results and Discussion

A single-molecule sandwich immunoassay for sensitive detection of AFP by evanescent field-enhanced fluorescence was designed. The sensitivity of the AFP assay could calculate the detection limit of 720 zM using 100 nm gold nano-patterned chips. Size reduction and site-specific labeling of antibodies to create a surface with high functional capacity increases the sensitivity of an immunoassay [27]. Furthermore, the 100 nm gold array chips showed no quenching of fluorescence dyes and had increased sensitivity. Under optimal conditions, the sandwich immunoassay had high sensitivity and a wide dynamic range for monitoring on a single-molecule level.

FIGURE 2: Calibration curve with linear range of standard AFP concentrations produced by serial dilutions from 720 zM to 10 nM. Relative fluorescence intensity (RFI) was corrected by background subtraction.

The increase in relative fluorescence intensity (RFI) was proportional to AFP concentration (Figure 2), and the linear response range was 720 zM to 10 nM (linear regression equation, $y = 884.12x + 836.76$, $R = 0.9932$) with a low detection limit of 720 zM and a signal-to-noise ratio (S/N) of 3 (Table 1). The AFP-nanoarray chip method showed 40,000 times higher sensitivity than other methods (i.e., surface plasmon resonance, bioluminescent immunoassay, electrochemical immunoassay, and chemiluminescence immunoassay). These results showed that the proposed method was highly sensitive, especially for ultralow levels of AFP.

In addition, the wide quantification range (720 zM to 10 nM) would be useful for healthy human serum, which has unestablished normal ranges for AFP. The normal range of AFP for adults and children is variously reported as under 50 ng/mL [38], under 10 ng/mL [39], and under 5 ng/mL [40]. Bader et al., Wang and Xu reported that the average concentration of AFP is about 25 ng/mL in healthy human

FIGURE 3: (a) Peak of fluorescence intensity of serially diluted AFP standard antigen reacted with a chip as “a” to “b” horizontal line in (b). (i) 14.3 aM; (ii) 7.2 fM; (iii) 14.3 fM; (iv) 7.2 pM; (v) 14.3 pM; (vi) 143 pM. RFI: relative fluorescence intensity.

FIGURE 4: Evanescent field-enhanced fluorescence images *via* prism-type TIRFM of (a) standard AFP (AFP-positive sample), (b) normal (nonpathologic) human serum, and (c) AFP-spiked human serum on gold nanopatterned chip.

serum [41, 42]. Ju et al. also reported a low average value of 3.4 ng/mL [43]. However, a level above 500 ng/mL of AFP in adults can be indicative of HCC, germ cell tumors, and metastatic liver cancer.

The assay method for quantitative analysis was based on evanescent field-enhanced fluorescence imaging *via* prism-type TIRFM. First, we selected signal regions and background regions with the same area. The sum of TIRF intensities of occupied pixels per single spot was corrected by background subtraction and RFI was calculated. Figure 3(a) shows the peak of fluorescence intensity of serial diluted AFP

standard antigen reacted on chip. The peak width was greater than the spot diameter of 100 nm because of fluorescence imaging. The peak shows a moderate increase with increasing AFP concentration from 14.3 aM to 143 pM.

In this study, samples including standard AFP antigen, normal human serum, and human serum with added standard AFP antigen were evaluated using TIRFM and single-molecule sandwich immunoassay chips. The AFP standard (AFP, positive sample) (Figure 4(a), 1.42 pg/mL = 20 fM), normal (nonpathologic) human serum (Figure 4(b), 0.13 pg/mL = 1.8 fM), and the sample of human serum

spiked with AFP (Figure 4(c), 1.16 pg/mL = 16.3 fM; theoretical, 1.1 pg/mL = 15.4 fM) were analyzed using gold nanopatterned chips. The results indicated that the sandwich immunoassay using the gold nanopatterned chips gave the high accuracy and sensitivity required for the quantification of biomarkers in human serum samples.

Specificity is an important factor in practical use of immunoassays. AFP is closely related to albumin, both genetically and structurally. The amino acid sequences of AFP and albumin have extensive homology, and the genes coding for the proteins are localized to the same area of human chromosome 4 (4q11–q13) [44]. Since the 1980s, research on monoclonal antibodies with unique specificity for individual binding sites on antigens has been used to improve the sensitivity and specificity of AFP determination [45]. Ding et al. evaluated the selectivity of an immunosensor with four kinds of potential interferents, including L-glutamic acid, bovine serum albumin, hemoglobin, and D-glucose [32]. Chan et al. showed specificity satisfactory with paired monoclonal antibodies for AFP [46]. The monoclonal antibodies against human AFP from Biodesign International (5H7 and 4A3) that we used for the AFP sandwich assay specifically recognized the human AFP molecule [47]. This allowed us to ignore the negligible effects of interfering antigens in the AFP sandwich immunoassay.

4. Conclusions

We developed a single-molecule sandwich immunoassay on gold nanopatterned chips that was highly sensitive for AFP detection in human serum. The method has a wide range of quantitation and could be applied for testing healthy human serum with the normal range of AFP that has been reported as 47.6 pM–700 pM. The method could be used to diagnose AFP-negative or AFP-positive human serum from pathological clinical samples.

The linear response range of the assay for AFP concentration was 720 zM to 10 nM with a correlation coefficient of 0.9932. The detection limit of 50×10^{-9} ng/mL (720 zM) with a S/N of 3 was in linear range of the calibration curve, and much lower than LODs from 0.004 ng/mL to 20 ng/mL reported for other methods [28–31, 33–35]. For the AutoDELFIA hAFP immunoassay for the quantitative determination of hAFP, Mannings et al. established that falsely low AFP concentrations occur in 2.8% of samples with AFP concentrations <15 kU/L (13.8 ng/mL = 193 pM) due to immunoassay interference [48]. Our results showed that a sandwich immunoassay chip for quantitative analysis of AFP by evanescent field-enhanced fluorescence imaging was simple and sufficiently sensitive for determination of AFP in human serum samples. The assay had good precision and accuracy at the single-molecule level. The new immunoassay is expected to be widely useful for highly sensitive clinical analysis and other biotechnology applications.

Conflict of Interests

The authors have declared no conflict of interests.

Acknowledgment

This research was supported by a postdoctoral fellowship Grant from Kyung Hee University in 2011 (KHU-20110212).

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