Biosensors and Bioelectronics 96 (2017) 159–166

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Enhanced detection sensitivity of carcinoembryonic antigen on a plasmonic nanoimmunosensor by transmission grating-based total internal reflection scattering microscopy

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ARTICLE INFO

Keywords:
Carcinoembryonic antigen
Nanoimmunosensor
Single-particle detection
Transmission grating
Total internal reflection scattering microscopy

ABSTRACT

Carcinoembryonic antigen (CEA) is a glycoprotein associated with colorectal carcinomas and is commonly used as a clinical tumor marker. Enhanced detection sensitivity for the assay of CEA molecules was achieved on a plasmonic nanoimmunosensor by wavelength-dependent transmission grating (TG)-based total internal reflection scattering microscopy (TIRSM). The plasmonic nanoparticles were placed in an evanescent field layer on a glass nanoimmunosensor that produced evanescent wave scattering by the total internal reflection of light from two lasers. The light scattered by target protein (CEA)-bound 20-nm silver nanoparticles (plasmonic nanoprobes) was collected and spectrally isolated in first-order spectral images (n=+1) by a TG (70 grooves/mm). The combination of evanescent wave scattering and TG significantly enhanced the detection sensitivity and selectivity due to the minimized spectroscopic interference and background noise. The TG-TIRSM method detected the CEA molecules at concentrations down to 19.75 zM with a wide linear dynamic range of 19.75 zM-39.50 nM (correlation coefficient, R=0.9903), which was 45 to 1.25×10^{9} times lower than the detection limits and 2×10^{5} to 2×10^{11} times wider than the dynamic ranges of previous assay methods. In particular, by simply changing the antibody of the target molecule, this technique can be used to detect various disease-related protein biomarkers directly in human biological samples at the single-molecule level.

1. Introduction

The improvement of imaging equipment has permitted the application of highly sensitive and selective quantitative analysis at the single-molecule level (Harms et al., 2001; Moerner and Orrit, 1999). In multicomponent systems such as biomolecular screening assays, it is essential to distinguish the substrate and the probe signal. To date, different emission filter sets (Schütz et al., 1998) and excitation wavelengths (Caruge and Orrit, 2002; Schmidt et al., 1996) have been used to acquire the spectral image used to identify each signal. Moreover, a dichroic wedge was equipped to acquire dual-color information simultaneously with one charge-coupled device camera (Cognet et al., 2000).

Transmission grating (TG) has been used to disperse or separate light into its component wavelengths. As light is transmitted through the grating, the light is diffracted or dispersed, depending on its wavelength, by the grooves of the surface (Palmer, 2005). TG was first introduced to develop a high-speed and high-throughput single-molecule imaging technique for identifying molecules in free solution based on differences in their fluorescence emission spectra (Ma et al., 2000). TG was also used to track the spectra of dynamic quantum dots on a modified surface (Li et al., 2008). However, using these methods, it was not easy to distinguish the probe signal directly from the substrate because of the streak effect and photobleaching of the fluorescence-labelled probe. Recently, fluorescence-free microscopy with TG was introduced for single-particle spectral measurement (Xiong et al., 2013) and colorimetric detection (Liu et al., 2016) using dark field microscopy. However, dark field microscopy was still limited by the spectral image of the first-order due to a multichromatic light source (Lee et al., 2017). More recently, Kang et al. introduced a single-particle, dual-mode total internal reflection scattering method using TG for the direct quantitative screening of influenza A virus DNA (Lee et al., 2017).

Carcinoembryonic antigen (CEA) is a glycoprotein associated with

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http://dx.doi.org/10.1016/j.bios.2017.05.009
Received 23 February 2017; Received in revised form 28 April 2017; Accepted 4 May 2017
Available online 04 May 2017
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colorectal carcinomas and is commonly used as a clinical tumor marker for the clinical diagnoses of breast tumors, colon tumors, ovarian carcinomas, and lung cancer (Naghibalhossaini and Ebadi, 2006; Sun et al., 2013). The levels of CEA are significantly lower in the colon tissues of adults, at about 12.5 pM (2.5 ng/mL) (Zamcheck and Martin, 2006) (Table S1). The CEA level in serum is also related to the state of tumor progression. The proportion of patients that above the 2.5 ng/mL of CEA concentrations was reported as follows: 28% in Dukes' A (stage 1), 45% in Dukes' B (stage 2), 75% in Dukes' C (stage 3), and 84% in Dukes' D (stage 4) (Duffy, 2001). Thus, the determination of the CEA level provides useful diagnostic information (He et al., 2008; Kau et al., 1999; Lechner et al., 2000). CEA level is also useful for monitoring colon cancer after surgery. Monitoring CEA level pre- and post-surgery indicates the surgical success and the prognosis of patient's recovery (Hasanzadeh et al., 2017). Therefore, it is important to monitor CEA level periodically for the patient who is known to have a malignancy. Therefore, it is desirable to develop a highly sensitive, non-invasive, and simple detection method for CEA levels.

Previous methods for CEA detection include radioimmunoassay (Szturmowicz et al., 1995), enzyme immunoassays (Tang et al., 2008), fluoroimmunoassays (Yuan et al., 2001), and piezoelectric immunosensors (Zhang et al., 2007). Despite the many advantages of these methods, some of them suffer from the drawbacks of limited sensitivity, elevated background signals, radiation hazards, or long analysis times (Darain et al., 2003). Compared to these methods, wide-field microscopy has some advantages, including the ability to obtain the spatial information of the localized target molecules and the high-throughput tracking of multiple molecular species presented at very low concentrations (Table 1). However, a multicomponent biosystem could not easily distinguish the emission signals from single molecules labelled with different probes in a sample mixture. Therefore, the additional spectral information of each individual molecule should be helpful for studying molecular interactions and monitoring the dynamic processes of the biomolecules (Han et al., 2008).

Herein, we describe a novel enhanced detection method for CEA using nanoimmunosensor by wavelength-dependent TG-based total internal reflection scattering microscopy (TIRSM). Plasmonic 20-nm silver nanoparticles (SNP) bound with target protein molecules were placed in an evanescent field layer (EFL) on a nanoimmunosensor produced by the total internal reflection of incident light with a specific wavelength in a trapezoidal prism. The light scattered by the individual particles in the EFL was collected and spectrally isolated using a TG beam splitter. The TIRSM equipped with TG separated the scattering signal in a single shot to decrease spectroscopic interference as well as background noise. Under appropriate experimental conditions, the quantification of CEA protein molecules could be performed on the calibration curve obtained based on an immunoreaction. The wavelength-dependent TG-TIRSM method enabled us to detect other disease-related protein biomarkers in human biological samples with ultra-high selectivity and sensitivity at the single-molecule level.

Table 1
Comparison of various methods for the detection of CEA molecules.

Method	Dynamic linear range (M)	LOD (M)	Ref.
ELISA	2.5×10^{-11}–4.0×10^{-10}	2.5×10^{-11}	Qiumei et al. (2015)
CLI	1.0×10^{-13}–3.5×10^{-10}	2.5×10^{-11}	Wang et al. (2012)
ECI	5.0×10^{-17}–5.0×10^{-10}	9.0×10^{-19}	Wang et al. (2016)
ECISI	2.5×10^{-13}–4.0×10^{-10}	5.0×10^{-15}	Zeng et al. (2015)
SAWI	5.0×10^{-12}–8.0×10^{-11}	5.0×10^{-12}	Zhang et al. (2015b)
FRETI	1.3×10^{-12}–6.5×10^{-11}	2.5×10^{-14}	Zhou et al. (2014)
FQI	1.0×10^{-13}–1.0×10^{-12}	5.0×10^{-14}	Zhu et al. (2016)
ECLI	1.0×10^{-16}–5.0×10^{-12}	3.4×10^{-17}	Zhuo et al. (2014)

CLI, chemiluminescence immunosensor; ECI, electrochemical immunosensor; ECISI, electrochemical impedance spectroscopy immunosensor; SAWI, surface acoustic wave immunosensor; FRETI, fluorescence resonance energy transfer immunosensor; FQI, fluorescence quenching immunosensor; ECLI, electrochemiluminescence immunosensor; LOD, limit of detection.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO, 99.5%), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC), 6-mercapto-1-hexanol (MCH, 97%), 2-(morphozlino)ethanesulfonic acid (MES), 11-mercaptoundecanoic acid (MUA, 95%), glycine, phosphate-buffered saline (PBS), and 20-nm (4.5×10^{11} particles/mL) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Dithiobis(succinimidyl propionate) (DSP) and protein A/G were obtained from Pierce (Rockford, IL, USA). *N*-hydroxysulfosuccinimide (NHSS) was acquired from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Tris-HCl was purchased from J.T. Baker Company (Phillipsburg, NJ, USA). StabilGuard was purchased from Surmodics (Eden Prairie, MN, USA). Sodium dodecylsulphate (SDS, 95%) was purchased from the China Medicine Group, Sinopharmic Chemical Reagent Company (Shanghai, China). Human CEA antigen and monoclonal human CEA antibody (057-10009 and TF3-H8-1–6) were acquired from Meridian Life Science, Inc. (Saco, ME, USA). Cancer antigen 125 (CA125) and monoclonal mouse anti CA125 antibodies pair (X306 and X75) were purchased from HyTest (Joukahaisenkatu, Turku, Finland). The human CEA enzyme-linked immunosorbent assay (ELISA) kit was purchased from Abcam Inc., (Cambridge, MA, USA).

2.2. Instruments

A scanning electron microscope (SEM) (Quanta FEG 650; FEI Co., Hillsboro, OR, USA) with an accelerating voltage of 30 kV was used to confirm the width of the gold nanoplates. The surface morphology of the SNP nanoprobes was observed using transmission electron microscopy (TEM) (JEM 2100F, JEOL Ltd., Tokyo, Japan). Samples for characterization by TEM were prepared by placing a drop of sample solution on a carbon-coated copper grid and drying it at 37 °C. TEM measurements were performed at an accelerating voltage of 200 kV. The surface topology of the gold nanoplates was obtained using an atomic force microscope (AFM) (NanoScope microscope, Digital Instruments, Santa Barbara, CA, USA) equipped with a type J scanner (scan size, 100-µm) and operating in tapping mode for air imaging. The resonance frequency of the silicon tips (Olympus Co. Ltd., Tokyo, Japan) was 300 kHz. These 150–160 µm short cantilevers had a nominal force constant (k) of 42 N/m and a tip with a radius curvature of < 10-nm. The AFM images were obtained by means of NanoScope software (Version 8.10, Digital Instruments, Santa Barbara, CA, USA).

2.3. Fabrication of the gold nanoplate for plasmonic nanoimmunosensor

The gold nanoplate fabrication process was performed according to a previously reported procedure (Lee et al., 2013). A glass wafer was cleaned using piranha solution (H_2SO_4:30% H_2O_2=3:1, v/v). The glass wafer was then coated with a 100-nm-thick layer of ZEP520A (Zeon Co., Tokyo, Japan) as an electron beam resist. Electron-beam exposure was carried out using JBX-9300FS (JEOL Ltd., Tokyo, Japan) equipment with a beam current of 100 pA at an accelerating voltage of 100 keV. Next, Au/Cr (20/5-nm thickness) was deposited by thermal evaporation, and the resist was removed by dimethylacetamide to form array patterns with a pitch of 5-µm and diameters of 100-nm. The pattern consisted of 4×4 gold nanoplates on a 10-mm^2 glass wafer (Fig. 1a; National Nanofab Center, Daejeon, Korea).

2.4. Preparation of SNP-antibody complex

The bioconjugation of CEA antibodies on the 20-nm SNP nanop-

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