Biosensors and Bioelectronics 90 (2017) 146–152

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

One-step separation-free detection of carcinoembryonic antigen in whole serum: Combination of two-photon excitation fluorescence and optical trapping

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ARTICLE INFO

Keywords:
Optical trapping
Two-photon excitation
Carcinoembryonic antigen
Separation-free

ABSTRACT

Direct analysis of biomolecules in complex biological samples remains a major challenge for fluorescence-based approaches due to the interference of background signals. Herein, we report an analytical methodology by exploiting a single low-cost near-infrared sub-nanosecond pulse laser to synchronously actualize optical trapping and two-photon excitation fluorescence for senstive detection of carcinoembryonic antigen (CEA) in buffer solution and human whole serum with no separation steps. The assay is performed by simultaneously trapping and exciting the same immune-conjugated microsphere fabricated with a sandwich immunization strategy. Since the signal is strictly limited in the region of a three-dimensional focal volume where the microsphere is trapped, no obvious background signal is found to contribute the detected signals and thus high signal-to-background data are obtained. As a proof-of-concept study, the constructed platform exhibits good specificity for CEA and the detection limit reaches as low as 8 pg/mL (45 fM) with a wide linear range from 0.01 to 60 ng/mL in the both cases. To investigate the potential application of this platform in clinical diagnosis, 15 cases of serum samples were analyzed with satisfactory results, which further confirm the applicability of this method.

1. Introduction

To date, although suspension arrays based on encoded microspheres have become one of the most facile and efficient approaches for high-throughput detection (Leng et al., 2015), they still have some inevitable limitations. For instance, the beads are always read one by one on a flow-based detection instrument while flowing very quickly in the channel, so they cannot be detected using a sensitive imaging camera, which not only detects the signals from the beads but also obtains the images of the beads of interest. Therefore, a visual measurement for such a single bead is drawing more and more attention. Optical tweezers (OT), which can be referred to as single-beam gradient force trap (Ashok and Dholakia, 2012), due to its advantages of non-contact and non-invasion, has tremendous application prospects in various domains (Marago et al., 2013; Heller et al., 2014) based on its ability of long-time study of a Mie or a Rayleigh object. By making use of this powerful handle, many single microscopic object such as red blood cell (Zhong et al., 2013), carbon nanotube (Tan et al., 2004), dielectric nanoball (Pang and Gordon, 2011), mental nanoparticle (Bosanac et al., 2008) and semiconductor nanoparticle (Jauffred et al., 2014) can be investigated in detail. Different from magnetic tweezers (Gollnick et al., 2015) and atomic force microscopy (Prechtel et al., 2002), this emerging manipulation means derives from the generation of pico-Newton forces induced by the momentum exchange between laser beam and the particle (Piggee, 2009). However, little attention has been brought to this fantastic single-particle technology in the field of analytical sciences.

During the past two decades, owing to the higher signal-to-background ratio and less photodamage, two-photon excitation (TPE) phenomenon has been widely expanded to act as an effective imaging method (Hwang et al., 2011; Lv et al., 2015). Although this predominant technology is expected to pave a way for analytical scientists to overcome the assay bottleneck in complex biological samples, the demand for extremely expensive laser device such as femtosecond pulse laser greatly confines its advance. Besides, to realize this nonlinear absorption process, the size of two-photon absorption cross

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http://dx.doi.org/10.1016/j.bios.2016.11.031
Received 25 August 2016; Received in revised form 9 November 2016; Accepted 11 November 2016
Available online 19 November 2016
0956-5663/

section is a major issue, which will also vastly influence the excitation efficiency (Kim and Cho, 2015). Becasuse of the rapid development of material sciences, many available fluorescent tags, including organic dyes and nanoparticles, have been arisen to address this concern. On account of the extremely large two-photon absorption cross section of quantum dots (QDs), which can reach as high as almost 10^5 g (1 g represents 10^{-50} cm^4 s·$molecule^{-1}$ $photon^{-1}$) (Li et al., 2009), which is much larger than the available organic molecules (& $2lt;$10^3$ g), along with their excellent optical properties (Gao et al., 2004; Michalet et al., 2005), it is clear that these unique semiconductor nanomaterials can be a wise alternative for performing TPE to achieve more efficient fluoresence emission.

Carcinoembryonic antigen (CEA), which is a wide-spectrum tumor marker expressed by cancer cells, has been recognized to be one of the most important clinical diagnostic object among various stages of cancer patients (Su et al., 2012). Although much progress associating with fluorescence-based approaches have been made to sensitively detect it, the direct analysis of CEA in human whole serum is in high demand for practical applications. As is well-known, either conventional fluoresence sepectrophotometer or wide-field fluorescence imaging is limited in the assay of whole serum, due to the challenging truth that the strong autofluorescence and scattering interference from complex matrix in serum very likely mask the positive signals (Huang et al., 2010), thus forcing a majority of the existing fluorescent assays even employing upconversion fluoresence materials (Sedlmeier and Gorris, 2015) are only competent in diluted serum. Therefore, it is desirable to introduce some separation means such as magnetic separation (Wang et al., 2015) and centrifugal separation (Deng et al., 2013) to remove the impurities to reduce the background signals, but this additional step not only leads to a longer operating process in turn but also most probably increases the usage of reagents.

In this work, we bring forward an exceptional analytical technique relating to near-infrared optical tweezers and two-photon excitation to one-step fluorescence quantitative detection of CEA without a separation procedure. The detection principle of this single-microsphere based approach is illustrated in Fig. 1A. Carboxylated polystyrene microspheres (CPS) are conjugated with capture antibody (Ab1) to prepare immunomicrospheres and amine-modified 605 nm QDs are bound with detection antibody (Ab2) to obtain QD probes. The final immune-conjugated microspheres enriched with CEA are fabricated through one-step immune recognition. A single target-enriched microsphere can be optically trapped by the strong gradient force originated from a tightly focused near-infrared laser beam, followed by real-time visual detection. Besides, it is worth noting that the difficulty in accurately adjusting focal plane in conventional imaging method is avioded by introducing OT, which ensures the same excitation condition for individual measurements. For quantitative detection, the mean fluorescence intensity (quantified by gray-level) of 50 microspheres in each concentration group is determined. Furthermore, taking advantages of the extremely low background signals upon TPE, this method can also be applied to straightforwardly analyze complex clinical samples such as human whole serum. To the best of our knowledge, this method may offer a meaningful application for optical tweezers in clinical analysis.

2. Experimental

2.1. Materials and reagents

Carboxylated polystyrene microspheres (3.03 μm, coefficient variation 1.5%) were obtained from Huge Biotechnol. Co. Ltd. (China). Amine-modified 605 nm CdSe/ZnS QDs (Fig. S1A , diameter 7.4 ± 0.2 nm , quantum yield 85%) was supplied by Wuhan Jiayuan Quantum Dots Co. (China). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 99%), N-hydroxy succinimide sulfur generation (Sulfo-NHS, 98%), Cy3 labelled sheep anti-mouse IgG and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (USA). Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, 99%) and DL-Dithiothreitoal (DTT, 99%) were purchased from Thermo Fisher Scientific Inc. (USA). CEA, prostate specific antigen (PSA), alpha fetoprotein (AFP), and pairing monoclonal mouse antibodies against CEA (Fig. S1B, purity over 95%) were obtained from Zhengzhou Biocell Biotechol. Co. Ltd. (China).

2.2. Real samples

Real samples including 12 cases of positive samples and 3 cases of random negative samples were supplied by Hubei Cancer Hospital (China) and Wuhan Zhongnan Hospital (China). The study of human serum was approved by the Wuhan University's Institutional Review Board for the use of human serum samples.

2.3. Preparation of immunomicrospheres

250 μg of CPS microspheres were first washed three times with 100 μL of MES buffer solution (100 mM, pH 5.5). Next, a mixture solution containing 10 μL of 50 mg/mL EDC and 10 μLof 50 mg/mL Sulfo-NHS was added into the above microspheres to activate the surface carboxyl functional groups. After reacting for 40 min at room temperature, the activated microspheres were washed three times with 100 μL of PBS buffer solution (pH 7.4) and continued to incubate with 10 μg of Ab1 for 4 h at 37 °C with gentle shaking. Subsequently, the conjugated microspheres were washed three times with 100 μL of PBST buffer solution (pH 7.4, 0.05% Tween-20). The final immunomicrospheres were obtained by using 0.5% BSA to block other binding sites on the surface of CPS and stored at 200 μL of PBS buffer solution (pH 7.4) for further use.

2.4. Quantification of the conjugating efficiency between CPS and Ab1

First, immunomicrospheres conjugated with various amounts of Ab1 were prepared by reacting various masses (0, 1, 2, 5, 8, 10 μg) of Ab1 with 250 μg of CPS. Afterwards, each group of immunomicrospheres was incubated with 2 μL of 1 mg/mL Cy3 labelled anti-mouse IgG for 1 h at 37 °C with gentle shaking to acquire the immunomicrospheres tagged with Cy3 and then the corresponding fluorescence intensities of the Cy3-labelled microspheres were measured by flow cytometry, which the excitation laser wavelength was selected as 488 nm and the collecting channel was set at 575 ± 25 nm.

2.5. Preparation of QD probes

100 μL of 4 μM amine-modified 605 nm QDs were first activated by mixing with 11 μL of 10 mM Sulfo-SMCC for 1.5 h at room temperature and then the excessive Sulfo-SMCC was removed through size-exclusion column (NAP-5, GE). Simultaneously, 300 μg of Ab2 was reduced with 6.1 μL of 1 mol/L DTT for 30 min at room temperature to expose its free sulfhydryl groups and the same separation approach was used to purify. Subsequently , the as-activated QDs and the as-reduced Ab2 were incubated for 2 h at 37 °C to obtain the QD probes after separating the QD probes from free Ab2 by gel filtration (Superdex™ 200, GE). The final product was concentrated with spin filtration to keep its concentration to 2 μM and stored at borate buffer solution (BR, pH 8.4) for further use.

2.6. Characterization of QD probes

5 μL of QD probes containing 0.5 μL of glycerin (experimental group) and the same amount of pure QDs (control group) were loaded in two lanes of 1% agarose gel and then soaked in 0.5× TAE buffer solution. After running 20 min under the voltage of 120 V, the mobility bands were illuminated by a UV lamp and the corresponding digital