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Nanoimprinted thrombin aptasensor with picomolar sensitivity based on plasmon excited quantum dots

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ABSTRACT

To achieve sensitive biomolecular detection, a gold nanohole array sensing chip was designed by plasmonic simulation and fabricated by high fidelity nanoimprinting technology. The gold nanohole array on a glass substrate serves as an optical antenna array, to produce localized surface plasmon resonance (LSPR) of 10–100 times stronger than the illumination light for quantum dot (QD) excitation. In this work, a robust sandwich bioassay of primary aptamer/thrombin/secondary aptamer with QD655 was established on the chip, to detect thrombin at a limit of detection (LOD) of 1 ng/ml (27 pM), with low non-specific binding even at the presence of 1000 times more concentrated bovine serum albumin (BSA). The conformation and QD enhancement mechanism of the sandwich assay were characterized by atom force microscopy (AFM) and dual polarization interferometry (DPI). This is the first time that DNA is surface functionalized on gold nanostructures for protein detection by plasmon enhanced QD emission. Considering the low-cost, easy regeneration and long shelf-life of the capturing aptamer, low non-specific binding and high sensitivity of the bioassay, as well as the inexpensive mass fabrication of the high quality chips, this novel aptasensor platform is particularly useful for a point-of-care system on medical diagnostics.

1. Introduction

Thrombin (coagulation factor II) is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin, and it is involved in the blood coagulation related process [1]. The concentration of thrombin is one of the physiological and pathological indicators of human body, and the elevation of thrombin is related with blood vessel narrowing, cardiovascular diseases, Alzheimer's disease, and tumors like pulmonary metastasis [2]. Thus the development of a specific and simple to use thrombin biosensor is pivotal for clinical diagnostics.

Aptamer is a single-stranded nucleic acid or a peptide that binds to a specific target, e.g. protein, small molecule, DNA or cell [3,4]. It has been demonstrated that aptamers are efficient substitutes for antibodies. In fact, aptamers are superior to antibodies because of their high binding affinity, small size, robustness [5–7], easy of being synthesized or incorporated with labeling reporters, additional functional linkers for immobilization [8,9], resistance to denaturalization, and long shelf-life. Therefore, aptamers are much suitable for biosensors, with high repeatability and feasible surface regeneration due to the aptamer's durability and reproducibility.

Thrombin is suitable for sandwich detection, because it has two aptamer binding sites: the fibrinogen recognition exosite (FRE) for a 15-mer oligonucleotide (APT_{15}) binding [10] and the heparin binding site [11] for a 29-mer oligonucleotide (APT_{29}) binding. The two binding sites locating at opposite positions of thrombin enable the construction of sandwich assay for sensitive and selective thrombin detection by optical or electrochemical methods. Thrombin binding changes the aptamer conformation from single-stranded oligonuclotide to quadruplex structure, and sensors based on the conformation change induced fluorescent signal has been reported [12,13]. Traditional signal transducers are organic dyes, however, they quenches quickly and possess very close Stokes shift (i.e., the proximity of the excitation and emission wavelengths). Due to the brightness and long life time, quantum dots (QD) were introduced as fluorophores for thrombin sensing. QD based aptamer beacon sensor was first developed by Levy, in which thrombin was quantitatively detected with 19 times of increase in fluorescence [14]. Sandwiched DNA electrochemiluminescent method using $Ru(phen)_3^{2+}$ functionalized hollow gold nanoparticles as signal-amplifying tags could detect thrombin at 5 fM, but the process was complicated [15]. Sandwiched detections of thrombin with CdS [16], CdSe [17], and PbS [18] QD labels were reported,

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but they used potentiometers to test, and the detection destroyed the whole biofunctional layer by dissolving the QDs in solution and making the chip unreusable.

Nanohole array in gold films had been widely used in optical detection of chemical and biological molecules adsorption based on the enhanced light transmission [19–21]. The peak shift was measured to calculate the adsorbed analytes. However, there is strict nanohole shape and size requirement, therefore, it is challenging to fabricate large area of uniform nanohole for biosensing application. In our group, gold nanohole array (140 nm × 140 nm, 400 nm pitch) was mass fabricated on a glass wafer using nanoimprint lithography technology [22,23]. The fabrication technology is convenient and cost effective, and high quality nanoarray chips were obtained in millimeter scale. The nanohole array had been used for sensitive biomarker detection, e.g. human cardiac troponin I (cTnI), prostate specific antigen (PSA) [24,25]. In this paper, a novel QD aptasensor based on the QD light emission excited by LSPR from nanohole array was developed for thrombin detection. Since the LSPR generated by the gold nanoholes can enhance the QD emission 10–100 times, a limit of detection (LOD) of 1 ng/ml (with 100 ms of optical integration time) for thrombin detection was achieved.

Two challenges were encountered in this work, one is to establish the high selectivity and specificity of the sandwich assay on the chip, the other is to control the distance (10–20 nm is preferred) between the gold nanostructure and the QD, as it was reported that the QD emission will be quenched by LSPR when the QD is too close to the gold surface (<5 nm); or unaffected when the QD is far away (>30 nm). We solved these difficulties by optimizing the sandwich assay, and cross characterizing the functional surface using atom force microscopy (AFM) to investigate the three dimensional morphology and dual-polarization interferometry (DPI) to measure the real time surface coverage, thickness, density and refractive index of the adsorbents [26]. The AFM images confirm that the aptamer links and the sandwich assay are successfully established on the nanochip. The bioassay on DPI glass chip indicates a resolved thickness of 17 nm [12 nm (DPI measurement of streptavidin/aptamer/thrombin) + 5 nm (radius of QD)] from the gold surface to the center of the QD, which proves that our QD sandwich assay renders an optimal LSPR electromagnetic field for QD excitation.

Although we demonstrated the detection of the plasmonic chips with a microscope, it can be further incorporated into a microfluidic device [27] for better flow control of the biosample injections, and the sensing device can also be viewed under a smartphone camera for its extra advantage of rapid signal transmission for point-of-care detections [28],

2. Materials and methods

2.1. Gold nanohole chip fabrication

The gold nanoholes were fabricated by nanoimprinting using a nickel mold. To fabricate a nickel mold, the nanopatterns were first written by e-beam lithography on a 4″ silicon wafer. Following the resist development, a seed metal layer was coated on the gold surface, and a nickel layer up to 300 μm in thickness was electroplated on the silicon wafer. The 4″ nickel mold was obtained by the separation of the wafer and the nickel layer. The nickel mold was UV nanoimprinted on a 4″ glass wafer coated with UV curable photoresist, and a gold film (5 nm of chromium as the adhesive layer and 50 nm of gold for plasmonic generation) was deposited on the nanoimprinted photoresist thereafter. Only the gold nanoholes remained after photoresist lift-off, and the glass wafer was diced into chips. The fabricated gold nanohole array is with a pitch of 400 nm and size of 140 nm × 140 nm. Each gold nanohole chip is 1 cm × 1 cm, with its central 1.8 mm × 1.8 mm covered by gold nanoholes and the rest covered with gold film, which is a good reference for the QD emission enhancement effect of the gold nanoholes.

2.2. Bioassay on the gold nanohole array

2.2.1. Chemicals and biological materials

Human-α-thrombin, bovine serum albumin (BSA), streptavidin were purchased from Sigma–Aldrich (Singapore). Prostate specific antigen (PSA) was purchased from BBI Solutions (Sittingbourne, UK). Thiolated 15-mer thrombin aptamer (APT_{15}: 5′-HS-C6-TTTTTTTTTT-**GGTTGGTGTGGTTGG**-3′) and biotinylated 29-mer thrombin aptamer (APT_{29}: 5′-biotin-**AGTCCGTGGTAGGGCAGGTTGGGGTGACT**-3′) were synthesized by Sigma® Life Science. Qdot® 655 streptavidin conjugate (QD655) and NHS-PEG_4-Biotin were purchased from Life Technologies (Singapore). PEG750 (α-methoxy-ω-mercapto PEG, MW 750 Dalton) was purchased from Rapp Polymere GmbH (Germany). The surface blocking peptide ligand HS-Cysteine-Valine-Valine-Valine-Threoninol (HS-CV_3T-ol) and the mixed ligand of HS-CV_3T and 4× ethylene glycol (HS-CV_3T-EG_4) were purchased from Peptide Protein Research Ltd. (Peptidesynthetics, UK).

Tris buffer 1 (pH 7.4, 50 mM Tris–HCl, 100 mM NaCl, 5 mM KCl, prepared) was used as binding buffer for thrombin/aptamer binding, and Tris buffer 2 (pH 7.4, 50 mM Tris–HCl, 0.1% Tween 20) was used as washing buffer.

2.2.2. Aptamer sequence and sandwich assay

Scheme 1 shows a schematic drawing of our bioassay constructed on a gold nanohole chip with four steps: (1) Gold nanostructure on glass chip was functionalized with 5′-thiol 15-mer primary aptamer (APT_{15}); (2) the surface was blocked by thiolated poly(ethylene glycol) (Mw 750, PEG750) and peptide-PEG750 mixed matrix (mixture of CVVVT-EG4-ol and PEG750); (3) thrombin was applied for detection; and (4) the bound thrombin was stained by the mixture of QD655 and biotinylated 29-mer secondary aptamer (APT_{29}), and the fluorescent signal was read by a fluorescent microscope.

2.2.3. Pre-treatment of APT_{15}-SH with TCEP

The purchased APT_{15}-SH was disulfide protected, and the dithiol protecting group was cleft before use. 20 μL of aptamer (100 μM) was diluted to 100 μL by Milli-Q water, treated with 9 μL of tris(2-carboxyethyl) phosphine (TCEP, 6 mM) for 30 min, and purified using a 1 mL G-25 column. The APT_{15}-SH was diluted to 1 μM with Milli-Q water before use.

2.2.4. Prepare APT_{29}-QD655

2 μL of QD655–streptavidin conjugate (1 μM) was added into 100 μL of Tris buffer 1. APT_{29}-biotin (100 μM) was diluted to 100 nM by the same buffer and 120 μL of the diluted aptamer was mixed with 100 μL of the above diluted QD655. The mixture was kept at least for 10 min before use.

2.2.5. Sensing chip biofunctionalization

The fabricated gold nanohole chip was treated by UV/O_3 for 8 min. The freshly cleaned chip was rinsed with water and treated with a drop (70 μL) of APT_{15}-SH (1 μM) for 15 h. The chip was thoroughly cleaned with water, then immersed in PEG750 solution (10 mM) for 10 min and cleaned with water. Next, the chip was soaked in a mixture of 20% CV_3T-EG_4–80% PEG750 in H_2O (1 μM) for 30 min and rinsed with water, after which the chip is ready for use.