QUANTUM DOT LABELED IMMUNOASSAY USING ZINC OXIDE NANOWIRES

Jung Kim,[1,2], Seyong Kwon[3], Je-Kyun Park[2,3] and Inkyu Park[1,2]*
[1] Department of Mechanical Engineering, KAIST, Daejeon, REPUBLIC OF KOREA
[2] KI for the NanoCentrury, KAIST, Daejeon, REPUBLIC OF KOREA
[3] Department of Bio and Brain Engineering, KAIST, Daejeon, REPUBLIC OF KOREA

ABSTRACT

In this paper, we proposed a novel and efficient quantum dot (QD)-based immunoassay method on the zinc oxide (ZnO) nanowire substrate. The ZnO nanowire substrate increases the immobilization sites for biomolecules, which results in the enhancement of fluorescence signal in QD-labeled immunoassay. However, energy transfer happens between the ZnO nanowire and QDs when QD is introduced as a labeling material. To prevent this energy transfer, we applied biotin–streptavidin complex to prolong the distance between two nanomaterials. Triple layers of biotin–streptavidin complexes reduced the energy transfer successfully. This immunoassay system was applied to quantify the concentration of carcinoembryonic antigen (CEA).

KEYWORDS

Quantum dot, ZnO nanowire, immunoassay, carcinoembryonic antigen, fluorescence resonance energy transfer

INTRODUCTION

Nanomaterials have become promising elements for various biological assays due to their unique optical and structural characteristics over the conventional materials. As an example, attempts have been made to apply zinc oxide (ZnO) nanowires in bioassay as a substrate to utilize their large surface area and unique photonic properties [1, 2]. Another type of nanomaterial vigorously studied for bioengineering is quantum dot (QD). In particular, notable photonic properties of QDs, including size-tunable emission, strong luminescence, and photostability make them as a promising labeling material [3].

In previous researches, ZnO nanowires were utilized mainly due to their high aspect ratios for enhancing fluorophore signal intensity. The seed patterning and hydrothermal synthesis processes used in this research is relatively simple than other ZnO nanowire synthesis methods such as chemical vapor deposition (CVD). Also, to utilize the advantages of QDs, the energy transfer from QDs to ZnO nanowires was one of the biggest obstacles. The fluorescence resonance energy transfer (FRET) between QDs and nanowires has been reported but it is totally dependent on the material and size [4]. Therefore, the bioassay system needs to be designed based on the material of substrate and labeling substance to maximize the reaction.

In this research, we exploit the ZnO nanowire substrate for the QD-based highly-sensitive immunoassay by utilizing the advantages of QDs and enhanced surface area with ZnO nanowires (Figure 1a). To prevent the energy transfer from QDs to nanowires, we applied multi-layered biotin–streptavidin complexes which will help to maintain the QD fluorescence energy by extending the distance between two nanoscale molecules (Figure 1b). We also demonstrated the quantification of carcino embryonic antigen (CEA) protein on the nanowire substrates.

EXPERIMENTAL

Chemicals and chip fabrication

ZnO nanowires were fabricated by hydrothermal synthesis on a glass substrate with ZnO seed layer (Figure 2a) [5]. Immunoassay substrates were fabricated by photolithography and lift-off techniques with Cr (30 nm) and ZnO thin layer. Square patterns (3 mm × 3 mm) were fabricated by AZ9260 on the glass wafer. Cr layer was fabricated by e-beam evaporator for better adhesion of ZnO seed layer and the ZnO layer was coated by DC sputtering at 150 W for 3 min. ZnO nanowire precursor solution was prepared with zinc nitrate hexahydrate ($Zn(NO_3)2{\cdot}6H_2O$, 98%; Sigma–Aldrich), hexamethylene-tetramine (HMTA, $C_6H_{12}N_4$, 99+%; Sigma–Aldrich) and polyethylenimine (PEI, $(C_2H_5N)_n$, Sigma–Aldrich) in deionized water. Prepared substrate was immersed in the precursor solution and heated in the convection oven at 95 °C for 2.5 h. After synthesis, the substrate with ZnO nanowire was washed with deionized water and air dried. The ZnO thin film substrate was prepared by e-beam evaporated Cr adhesion layer (30 nm) and DC sputtered ZnO thin film (150W, 3 min) on the glass substrate.

Surface modification

The bare ZnO nanowire does not have chemical groups apt to protein or biomolecule immobilization. Therefore, surface modification is necessary for immunoassay on the ZnO nanowire surface. In this work, we applied 3-aminopropyltrietoxysilane (3-APTES) and glutaraldehyde to form an aldehyde group for protein immobilization [6]. Glass substrate with ZnO nanowire patterns was washed with deionized water and dried with air. To eliminate PEI residue on the surface of the ZnO nanowire, oxygen plasma treatment was performed for about 30 s. Dried substrate was placed in 3-APTES in ethanol solution (v/v, 4/100) at room temperature for 4 h. After silanization with 3-APTES, the substrate was washed with ethanol and deionized water and air drying. Silanized ZnO nanowire substrate was treated in 2% glutaraldehyde in phosphate buffered saline (PBS) buffer solution at 4 °C overnight. The modified ZnO nanowire substrate was thoroughly rinsed with deionized water and dried by air. The ZnO thin film substrate was also modified with the same procedure of ZnO nanowire substrate for surface modification.

Immunoassay method

The schematic of the ZnO–QD immunoassay is depicted in Figure 1. To form anti-FRET layer with biotin and streptavidin layer, 10 µL of 0.01 mg/mL biotin (Sigma–Aldrich) in PBS buffer solution was dropped on the ZnO nanowire grown pattern and incubated at room temperature for 1 h. After biotin incubation step, 10 µL of 0.01 mg/mL streptavidin (Sigma–Aldrich) in PBS buffer solution was also incubated for 1 h each. These steps were repeated until desired length of biotin–streptavidin layers was obtained. At the last step of making biotin–streptavidin complexes, a streptavidin conjugated antibody from 0.01 mg/mL mouse monoclonal antibody to CEA (Abcam) was reacted with EasyLink Streptavidin Conjugation Kit (Abcam) for 1 h at room temperature. CEA antigen (Human CEA protein; Abcam) and antibody (Rabbit polyclonal antibody to CEA; Abcam) incubation steps were followed in series. Finally, secondary antibody conjugated QD605 (Invitrogen) was incubated for 1 h.

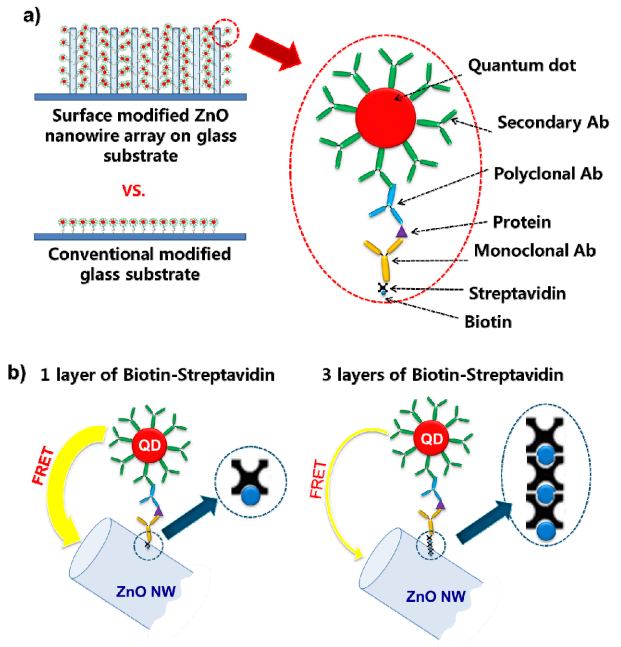

Figure 1. Schematic diagram of (a) ZnO nanowire-based QD immunoassay system for QD fluorescence signal amplification and (b) multiple biotin–streptavidin complexes for fluorescence resonance energy transfer (FRET) reduction.

Characterization of material and immunoassay reaction

The structure characteristics of ZnO nanowire and biomolecule binding were observed by scanning electron microscope with platinum sputtering (SEM, Sirion by FEI, Netherlands). Fluorescence image was taken by fluorescence microscope (Olympus IX72; Japan). To quantify the fluorescence intensity, we utilized photospectrometer (QE65000; Ocean Optics). Imaging and photospectrum obtaining processes were performed in wet condition by placing PDMS well with deionized water on the each experiment spot.

RESULTS AND DISCUSSION

ZnO nanowire structure and biomolecule binding

Synthesized ZnO nanowires are shown in Figure 2a. Average diameter of ZnO nanowire was around 30 nm and length was 2–3 µm. The binding of biomolecules and QD on the ZnO nanowire substrate was confirmed by SEM images in Figure 2b. Compared to the bare ZnO nanowire, clusters were found on the surface of ZnO nanowires.

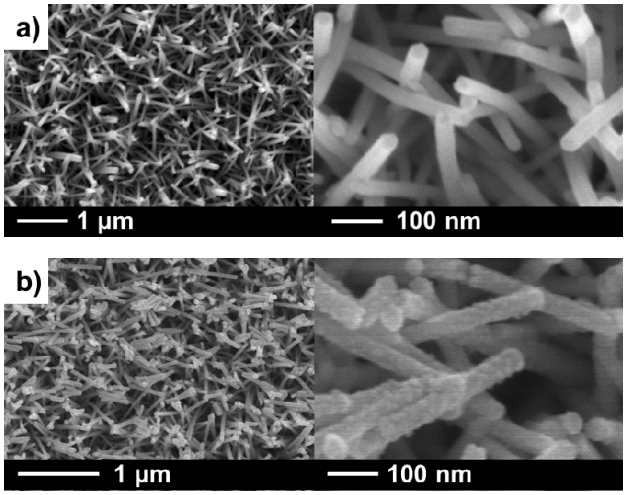

Figure 2. SEM images of ZnO nanowires (a) after synthesis and (b) after the CEA immunoreaction.

Effect of the large surface area of ZnO nanowire

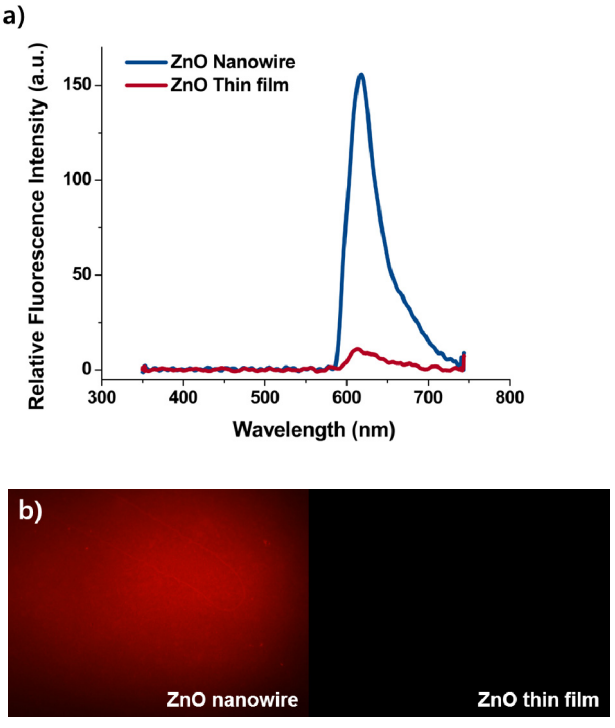

Figure 3. (a) Fluorescence intensities of Texas-Red tagged streptavidin on biotin immobilized (red line) ZnO thin film and (blue line) ZnO nanowire substrate. (b) Microscopic images of Texas-Red tagged streptavidin immobilized ZnO nanowire and ZnO thin film.

To exam the effect of surface area, Texas-Red conjugated streptavidin immobilization was performed both on the biotin coated ZnO thin film and biotin coated ZnO nanowire substrates. As shown in Figure 3, the fluorescence intensity peak for ZnO nanowire substrate was more than 15 times larger than that for ZnO thin film substrate. It is also cleared shown in the fluorescence microscopic images. When the Texas-Red was immobilized on the ZnO thin film surface, it did not exhibit significant intensity difference with the glass substrate as shown in Figure 3b, while the Texas-Red immobilized on the ZnO nanowire pattern showed a bright illumination under same exposure time. From this result, we can confirm the fact that ZnO nanowires enhanced the reaction sites for immunoassay and resulted in high fluorescence intensity.

Effect of the biotin–streptavidin complex length

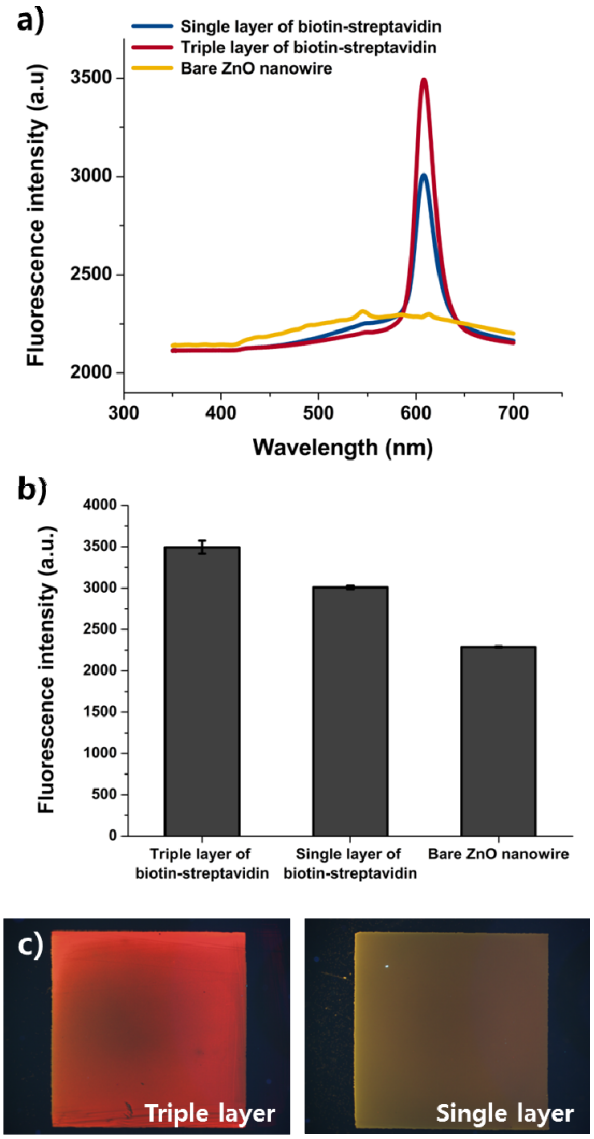

Figure 4. (a) Fluorescence intensity spectrum of triple layers, single layer, and zero layers of biotin–streptavidin complexes. (b) Fluorescence peak intensity at 608 nm wavelength at different length of complexes. (c)Microscopic images of triple layers and single layer of biotin–streptavidin complexes.

The FRET phenomenon between QDs and ZnO nanowires was observed by the QD signal after the reaction. To figure out the effect of biotin–streptavidin complexes on the energy transfer between QDs and ZnO, we examined zero, single, and triple layers of biotin–streptavidin complexes. As shown in Figure 4, the assay using triple layers of biotin–streptavidin complexes shows much stronger red fluorescence light intensity than single layer complexes. To confirm the relationship between the fluorescence intensity and the length of the biotin–streptavidin complexes, we measured the fluorescence intensity by using a photospectrometer. When only single layer was utilized, the fluorescence intensity was decreased to 3008 a.u. while it was 3493 a.u. at triple layer was utilized. An anti-FRET phenomenon was more clearly observed by comparing with triple layer and zero layers of biotin–streptavidin complexes. The fluorescence intensity was decreased to 2288 a.u. when no biotin–streptavidin complex was used. The energy transfer from QDs to nanowires depends on the distance between two nanomaterials [7]. As the length of the biotin–streptavidin complex was shrunk, the fluorescence signal was faded. This result reflects the energy transfer from QDs to ZnO nanowires. It was also obviously observed in the microscopic images (Figure 4c).

Triple layer of biotin–streptavidin complexes utilized ZnO nanowire substrate showed clear red color which is the color of QD 605 while single layer utilized substrate showed yellowish color.

Quantification of CEA protein concentration

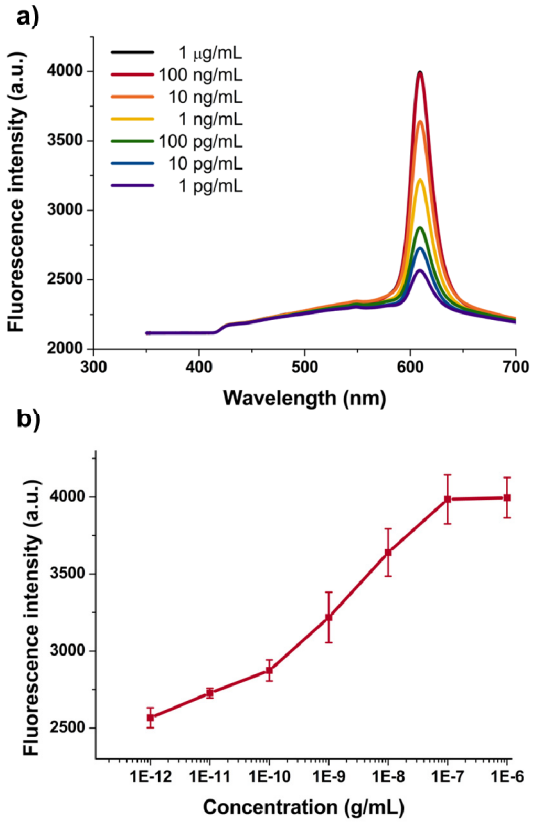

Figure 5. Fluorescence intensities for different CEA protein concentrations; (a) spectral intensity curves and (b) peak intensity vs. concentration.

We applied the QD labeling immunoassay platform based on ZnO nanowire substrate to quantify the concentration of CEA protein. CEA protein was diluted in PBS buffer solution with different concentrations (from 1 pg/mL to 1 μg/mL, from 5 nM to 5 fM) was spotted on ZnO nanowire substrates conjugated with CEA antibody. Triple layers of biotin–streptavidin complexes were applied to maximize the fluorescence intensity. Figure 5a shows the fluorescence spectra for different CEA concentrations. The spectra showed common peaks at λ = 605 nm and decreasing intensities at lower CEA concentration. The plot of peak intensity vs. CEA concentration shows that CEA molecules can be detected in the range of 1 pg/mL ~ 100 ng/mL. Also, the detection limit appears to be lower than 1 pg/mL and the detection signal becomes saturated at CEA concentrations higher than 100 ng/mL. This detection capability is appropriate for the clinically important range of CEA protein molecules as a cancer biomarker.

CONCLUSION

In this study, we developed a novel QD immunoassay platform based on the ZnO nanowire substrate. The high surface to volume ratio enabled larger biomolecule immobilization sites. Therefore, more antigens were attached on the surface of the nanowire and more QDs emitted their fluorescence signals. In this process, minimization of FRET between QDs and ZnO nanowires is an essential step. We suggested multiple biotin–streptavidin layers to extend the distance between QDs and ZnO nanowires. This technology enabled a very sensitive detection of CEA biomarker in clinically meaningful concentration range. This platform will be very useful for highly sensitive detection of biomolecules and disease diagnosis.

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CONTACT

*I. Park, tel.: +82-42-350-3240; inkyu@kaist.ac.kr