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Two-Dimensional Array of ZnSe-Ferritin Nanodots as a Sensor Media for Gamma-Aminobutyric Acid

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Quantum dots have been attractive especially in the area of biosensors due to their peculiar optical properties. In this context, less toxic Zinc selenide (ZnSe) quantum dots were synthesized in the cavity of the apoferritin from horse spleen (HsAFr), and the two-dimensional ZnSe-ferritin nanodots were prepared on modified silicon surface. For utilizing the array as a biosensor, the photoluminescence (PL) spectrum change was investigated by accompanying its conjugation reaction with gamma-aminobutyric acid (GABA) and glutamic acid, where GABA is a major inhibitory neurotransmitter in the central nervous system and glutamic acid is its physiological precursor. The results revealed that the fluorescence intensity of ZnSe quantum dots in ferritin core is dependent on the concentration of GABA and the enhancement factor was a linear function of the GABA concentration in the range of 0.03 to 0.18 μM despite the presence of glutamic acid. Accordingly, the ZnSe-ferritin nanodot arrays can be employed as a useful sensing media for even very tiny concentration of neurotransmitter GABA.

Keywords Biosensor; ferritin; GABA; neurotransmitter; photoluminescence; quantum dot; ZnSe

Introduction

Semiconductor nanoparticle quantum dots are luminescent inorganic fluorophores which can be excited with a single light source for multicolor light emission. In addition, their size and shape can be controlled by the reaction time, temperature, and ligand molecules used for their synthesis. Since quantum dots are quasi zero-dimensional, they have a sharper electronic density of states than that for materials

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with higher dimensional structure. As a result, they have superior transport and optical properties, and are being investigated for use in transistors, solar cells, LEDs, diode lasers, amplifiers, medical imaging and biological sensors.

Meanwhile, extensive attention has been paid to the preparation and characterization of selenide in the quantum confinement region in recent years mainly due to their various optoelectronic advantages over the commonly used organic fluorophores. These advantages are not only related to their size tunable optical properties but also to their active surfaces and low photodegradation rates [1]. CdSe is the most studied nanocrystal, however, regarding the inherent toxicity of this system that may hinder a safe use *in vivo* application. Accordingly, it is natural to seek for substituting cadmium ions and producing less toxic labeling materials. To obtain the biocompatibility, some recent reports presented ZnSe crystals in the quantum confinement region as potential materials in biolabeling procedures [2–5].

In the last two decades, ordered nanostructures with microscale featured size on the solid surface have generated considerable interests owing to their unique electronic, optical, and biological characteristics [6]. There have been numerous applications of these techniques in electronics and bio-chemical researches such as specific detection of biomolecular interaction which is most important for future drug and diagnostic development [7,8].

In this work, we prepared two-dimensional ZnSe-ferritin nanodots by simple touch method with modification of silicon wafer surface and the PL intensity of the QDs array was studied accompanying its conjugation reaction with gamma-aminobutyric acid (GABA) in order to utilize this array as a biosensor. GABA is a major inhibitory neurotransmitter in the central nervous system and GABA acts at inhibitory synapses in the brain and spinal cord. In the present work, GABA was conjugated to QDs array surface by an active ester coupling reaction. In addition, the same PL study was performed on its physiological precursor glutamic acid and the mixture of both GABA and glutamic acid to confirm the selectivity of the ZnSe-ferritin nanodot array for GABA.

Experimental

Materials and Reagents

Horse spleen apoferritin (HsAFr), zinc acetate, ammonium acetate, ammonia water, selenourea, Bradford reagent, BSA standard solution, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), γgamma-amino-n-butyric acid (GABA) and glutamic acid were purchased from Sigma-Aldrich with no further purification.

Glucose, 3-(N-Morpholino)propanesulfonic acid (MOPS), sodium chloride, and cadmium sulfate were purchased from Sigma-Aldrich of the highest obtainable purity and HPLC water (J.T.Baker) was also used.

Polished Si wafers (Sigma-Aldrich) cut along the 111 plane were cut into 5×5 mm pieces. Si substrates were washed in an ultrasonic bath, first in acetone and then in ethanol for 20 min, respectively. The substrates were dried with dry nitrogen gas. The Si substrates were cleaned by plasma stripper, and treated in the 1,1,1,3,3,3 hexamethyl disilazane (HMDS, Sigma-Aldrich) atmosphere in vacuum for 2 h to make the surface hydrophobic prior to the transformation of the array of protein molecules.

ZnSe Synthesis in Apoferritin Cavity

The introduction of ZnSe into the apoferritin cavity was based on a method described by Ichiro Yamashita [9]. The 5 mL reaction mixture solution with a final concentration of 0.6 mg/mL HsAFr, 2 mM zinc acetate, 80 mM ammonium acetate, and 10 mM selenourea was prepared, and ammonia water was used to maintain the solution at pH 8.2. The addition of selenourea was the final step. Selenourea was dissolved in pure water with a minimal amount of ethanol just before use and added to the reaction mixture solution with a final concentration of 20 mM. The solution was left two days at room temperature to ensure that the reaction goes to completion. The solution after ZnSe quantum dot synthesis was centrifuged at low speed to remove the precipitates and concentrated by the centrifugal filter. The Sephadex-G25 gel filtration column and 20 nm filter were also applied to remove the impurities as necessary. The protein concentration of the supernatant was measured by the Bradford protein assay method.

Fabrication of the Two-Dimensional ZnSe-Ferritin Nanodot Array on Si Substrate

Two-dimensional arrays of ferritin molecules were obtained using the method developed by Nagayama *et al.* [10,11] with some modifications. A volume of 1.1 mL of the subphase; 10 mM MOPS, 10 mM $CdSO_4$, 2% glucose (w/v), pH 5.8 was filled into a homebuilt circular teflon trough (diameter 15 mm, depth 5 mm). Then the solution containing 3 μg ZnSe-cored ferritin was injected underneath the dense glucose subphase. Two-dimensional array of ZnSe-cored ferritin was obtained after an incubation period of more than 10 min. The array of ferritin molecules was transferred onto the hydrophobic Si surface by laying the Si substrate on the air-water interface, which is similar to the Langmuir-Blodgett method. The transferred array was rinsed with HPLC water and excess liquid on the substrate was removed by centrifugation at 5,000–10,000 g for 10 sec and then stored in a desiccator until use.

Bioconjugation and Detection of GABA with ZnSe-Ferritin Nanodots

The carboxyl group on the surface of ferritin can conjugate with biomaterials which have active amine group by an active ester coupling reaction using NHS and EDC. The activation buffer was prepared by adding 0.4 mg EDC (~2 mM) and 0.6 mg NHS (~5 mM) into 1 ml MOPS buffer solution at pH 5 and the reaction components were mixed well. GABA solutions of various concentrations were prepared in PBS buffer at pH 7.5. Then, the Si substrate with ZnSe-ferritin nanodot array was immersed in the activation buffer solution. After incubation for 15 minutes at room temperature, the Si substrate was rinsed with PBS buffer and immersed in 1 ml GABA solution and incubated for 2 hours at room temperature. The Si substrate was rinsed with PBS buffer and excess liquid on the substrate was removed by centrifugation at 5000–10,000 g for 10 s. Finally, the photoluminescence measurement was performed on ZnSe-ferritin nanodot array before and after bioconjugation reaction for evaluation of the array as biosensor for GABA. In order to investigate the selectivity of GABA detection, the same procedure was also performed on glutamic acid which is the physiological precursor of GABA and on the equimolar mixtures of GABA/glutamic acid. In Figure 1, the schematic of reacted GABA and ZnSe-ferritin nanodots on modified silicon surface is given.

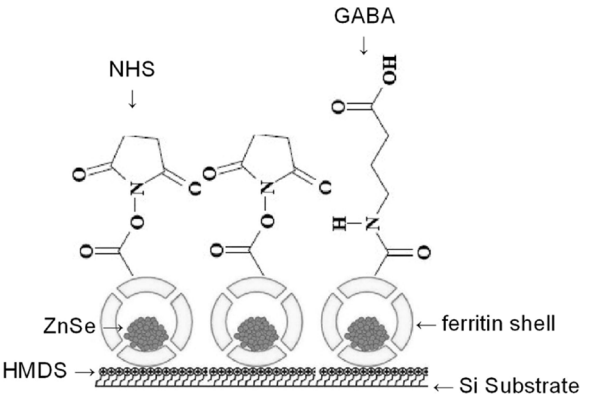

Figure 1. Schematic of reacted GABA and ZnSe-ferritin nanodots on modified silicon surface.

Instruments

The image of ZnSe-ferritin nanodot array on silicon surface was observed by SEM (Hitachi S-4700). The AFM image was acquired in non-contact mode using a PSIA Xe-150 (Park systems, Korea). Silicon cantilevers were used and the AFM scanner and position sensors were calibrated using standard samples from Mikromash. Photoluminescence measurement was performed on ZnSe-ferritin nanodot array before and after bioconjugation reaction by employing a photofluorometer (PTI QuantaMasterTM).

Result and Discussion

Formation of ZnSe Quantum Dots and 2-D Array of ZnSe-Ferritin Nanodots

In the previous study at our laboratory, ZnSe quantum dot was successfully synthesized in the core of ferritin and two-dimensional array of ZnSe-ferritin nanodot was prepared [12]. However, in the present work, the denser array of the ZnSe-ferritin could be obtained on the surface of silicon wafer by optimizing the concentration of ZnSe-ferritin nanodot on the air-water interface in a circular teflon trough. As shown in Figure 2, a uniform array and the nanodots of near 14 nm in diameter could be observed. Meanwhile, the AFM image in Figure 3 provided the topography for ZnSe-ferritin nanodots on the silicon surface. As shown in the figure, the uniform nanodots were obtained and the measurement result revealed that the average height of the dots were about 12 nm (the length of peak to valley was 11.8 nm). In reality, the size of the ferritin is about 11–12 nm and the core of the ferritin is near 6–7 nm [13–15]. Accordingly, it could be concluded that the array of ferritin-ZnSe nanodots on the silicon wafer surface was monolayer since the average size of nanoparticles in SEM image and the average height of the nanodots in AFM image were almost same with the diameter of a ferritin.

Fluorescence Measurement

As shown in Figure 4, it was obvious that significant enhancement of the fluorescence occurred due to the conjugation of ZnSe-ferritin nanodot array with GABA.

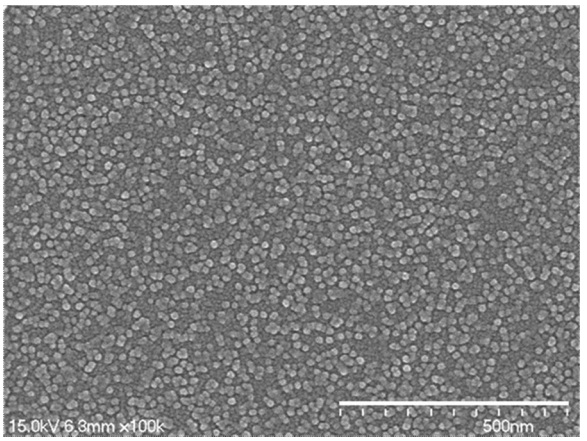

Figure 2. SEM image of two-dimensional array of ZnSe-ferritin nanodots on modified silicon surface.

The intensity of the ZnSe characteristic peak at 408 nm increased with increasing GABA concentrations. Accordingly, the array can be employed as a biosensor for neurotransmitter GABA. However, since glutamic acid is the physiological precursor of GABA, it is important to check the PL intensity response for glutamic acid especially when the array is utilized for *in vivo* assay of GABA. In Figure 5, the PL spectra for ZnSe-ferritin nanodot arrays as a function of glutamic acid concentrations are given. With the increase of glutamic acid concentration, no enhancement of the fluorescence was observed and there was no PL intensity response to its conjugation with ZnSe-ferritin nanodot array. In reality, the intensity of fluorescence was slightly reduced with the addition of glutamic acid.

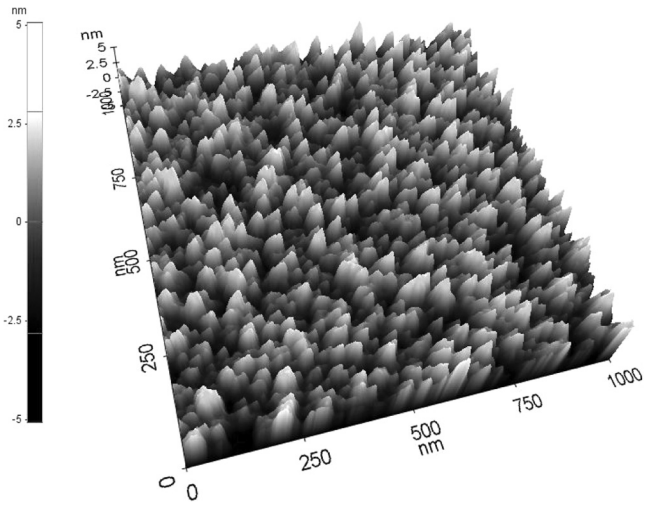

Figure 3. AFM image of two-dimensional array of ZnSe-ferritin nanodots on modified silicon surface.

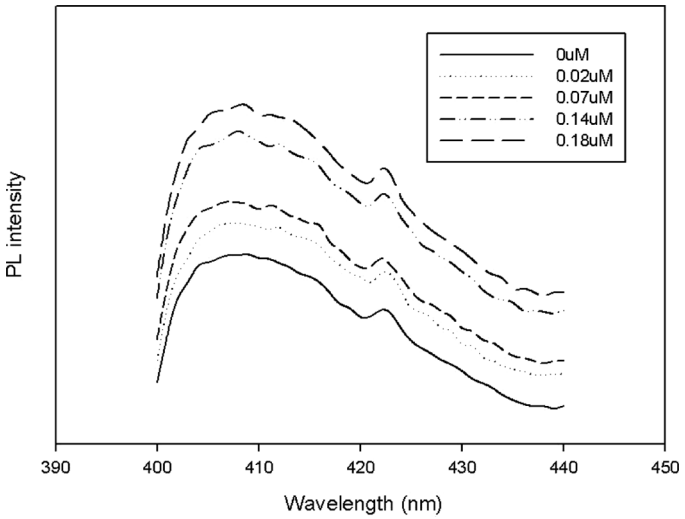

Figure 4. PL spectra for ZnSe-ferritin nanodot arrays as a function of GABA concentrations.

In general, most amino acids could not emit fluorescent light. However, some amino acid zwitter ions such as GABA can transform fluorescent derivatives . Thus the derivatives formation can cause useful fluorescence enhancement with negligible change in the emission band shape and wavelength for a fluorescent sensor. In the case of glutamic acid, the no PL intensity response to its conjugation with ZnSe-ferritin nanodot array can be probably ascribed to the attenuation of the charge density of the ammonium unit by α-carboxylate anion [16].

Although glutamic acid provides poor performance in the enhancement of PL intensity with ZnSe-ferritin nanodot array, it should be checked the effect of

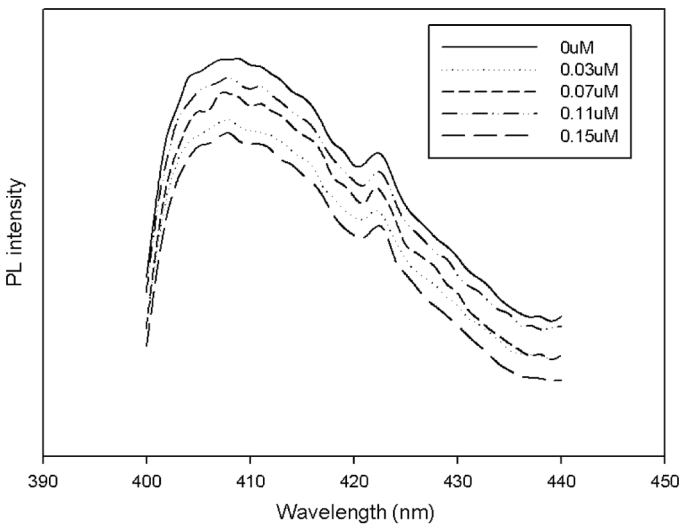

Figure 5. PL spectra for ZnSe-ferritin nanodot arrays as a function of glutamic acid concentrations.

glutamic acid on the PL intensity for GABA. Accordingly, the dependence of fluorescence intensity on the concentrations of GABA/glutamic acid equimolar mixture was investigated and the results are shown in Figure 6. It was observed that enhancement of the fluorescence occurred despite the presence of glutamic acid in the solutions when the GABA concentrations were increased.

In order to evaluate this array as a sensor media, the fluorescence enhancement factors were obtained and given in Figure 7. The fluorescence enhancement factor (FE) is defined as the ratio of the fluorescence intensity of the ZnSe-ferritin nanodot array after conjugation to the intensity before conjugation. As shown in Figure 7, the fluorescence enhancement factor was a linear function of the GABA concentration in the range of 0.02 to 0.18 μM with R^2 of 0.9592. In addition, the enhancement factor for glutamic acid was less than 1 and no PL intensity response to its conjugation with ZnSe-ferritin nanodot array was obtained. On the other hand, the fluorescence enhancement factor for the GABA/glutamic acid mixture was reduced when the factor was compared with the values for GABA itself. However, the figure still shows the linear relationship with R^2 of 0.9658 between GABA concentrations and PL intensity. Thus, it was concluded that the ZnSe-ferritin nanodot array could be successfully employed for GABA detection without losing its accuracy despite the presence of glutamic acid.

In reality, in our previous work, fluorescence resonance energy transfer (FRET) process was employed for GABA detection [17]. In the work, the dependence of FRET efficiency on GABA concentration showed that QDs combined with fluorescent dyes can be used for the detection of GABA. However, the GABA concentration was in the range of 10–120 μM which is almost two orders of magnitude higher than that of the ZnSe-ferritin nanodot array system. Besides, Liesenfeld *et al.* [18] have been prepared GABA sensor where the sensor's detection concentration limit was 0.93 μM GABA by employing enzyme immobilization on optical fiber probe surfaces. In addition, Niwa *et al.* [19] developed a GABA sensor by using

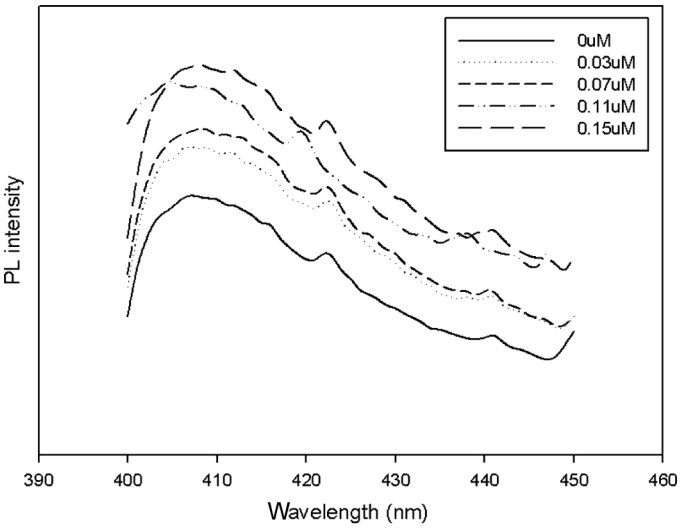

Figure 6. PL spectra for ZnSe-ferritin nanodot arrays as a function of GABA/glutamic acid equimolar mixture concentrations.

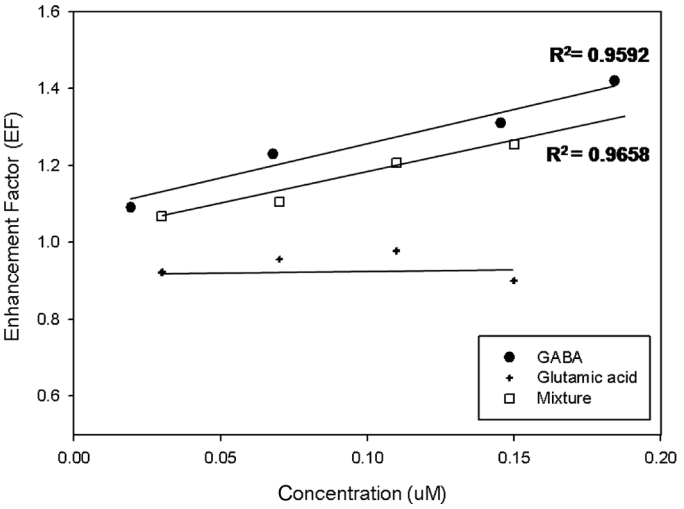

Figure 7. Dependence of fluorescence enhancement factor on concentrations of GABA, glutamic acid, and GABA/glutamic acid equimolar mixture.

an electrochemically pretreated carbon electrode and they revealed that carbon electrode based-GABA sensor provides lower detection limits of 0.03 μM for GABA than any previously reported GABA sensors. The present ZnSe-ferritin nanodots array can be utilized for the detection of GABA in the range of 0.02 to 0.18 μM, which indicated that the array could be a potential sensor media for GABA detection which is comparable to their carbon electrode based-GABA sensor.

Conclusions

ZnSe quantum dots were efficiently synthesized in the cavity of the cage-shaped apoferritin and a compact two-dimensional array of ZnSe-ferritin nanodot was prepared on a hydrophobic Si surface. When the array was conjugated with GABA, a significant enhancement of the fluorescence occurred and the enhancement factor was a linear function of the GABA concentration in the range of 0.03 to 0.18 μM despite the presence of glutamic acid. In reality, it was found that the two-dimensional ZnSe-ferritin nanodot array could be a potential sensor media for GABA detection.

Acknowledgments

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