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Determination of cyclic GMP concentration using a gold nanoparticle-modified optical fiber

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ABSTRACT

Guanosine 3′,5′-cyclic monophosphate (cGMP) produced from guanosine triphosphate (GTP) via soluble or particulate guanylyl cyclase (GC) is an important second messenger for signal transduction within cells. The currently used methods of measuring cGMP including radioimmunoassay and enzyme immunoassay involve the use of radioisotopes or tracers. To develop a cGMP assay that is label-free, non-radioactive, able to detect cGMP directly, and highly specific and sensitive, we used a gold nanoparticle-modified optical fiber (GNOF) conjugated with anti-cGMP antiserum to determine cGMP concentration based on localized surface plasmon resonance (LSPR). The optimal antisera dilution and incubation time for preparing the probe are 1:250 and 2 h, respectively. The sensor shows detection ranges of 0.0025–0.1 and 0.1–100 pmol/ml for acetylated cGMP and cGMP, respectively. The sensitivity for measuring acetylated cGMP is three orders of magnitude higher than that for cGMP. In addition, the biosensor can be stored at 4 °C up to 4 week without losing sensitivity significantly.

1. Introduction

Guanosine 3′,5′-cyclic monophosphate (cGMP) is an intracellular second messenger which is produced via activation of soluble and particulate guanylyl cyclase (GC) by nitric oxide (NO) and natriuretic peptides, respectively (Hofmann et al., 2006). Once activated, both these GCs become able to convert guanosine triphosphate to cGMP. The principal targets of cGMP are cGMP-regulated phosphodiesterases (Surapisitchat et al., 2007), cGMP-dependent protein kinases (PKGs) (Lohmann and Walter, 2005), and cGMP-gated cation channels (Boassa and Yool, 2002; Luo et al., 2008). The signal pathways of cGMP are involved in a variety of physiological functions such as vascular smooth muscle relaxation, platelet aggregation inhibition, phototransduction in the eye, and intestinal fluid regulation (Hofmann et al., 2006; Luo et al., 2008). Therefore, the measurement of the cGMP concentration could help to elucidate the role of GC in health and disease and indentify opportunities for therapeutic interventions.

cGMP can be measured by radioimmunoassay (RIA) (Steiner et al., 1969; Steiner et al., 1972), enzyme immunoassay (EIA) (Horton et al., 1992, Pradelles et al., 1989), high performance liquid chromatography (HPLC) (Lorenzetti et al., 2007), scintillation proximity assay (SPA) (Horton and Baxendale, 1995), and luminescent oxygen channeling assay (LOCI) (Peppard et al., 2003; Schmidt, 2009). Although with high sensitivity, the use of ^{3}H or ^{125}I-cGMP as the tracer in RIA and SPA has the drawbacks of a reduced shelf life and the safety risks for the radioisotopes. Lorenzetti et al. (2007) combined HPLC with mass spectrometry to increase the sensitivity of the cGMP detection from picomolar to femtomolar. However, the serial processing of samples limits the HPLC method. EIA is increasingly preferred over RIA because of its non-radioactive approach and same range of sensitivity as RIA. Nevertheless, EIA is not suited to determination of kinetic parameters due to its use of endpoint measurements. LOCI is capable of determining kinetic parameters and detecting femtomolar amounts of cGMP in crude cell lysates (Schmidt, 2009) but the existence of antioxidants or metal ions in the sample might affect release of the singlet oxygen from the photosensitizer beads resulting in misinterpretation of the data.

Recently, Chau et al. (2006) have developed a colloidal gold modified optical fiber as a label-free sensing platform. The mechanism of the sensor measurement is based on the following. When the incident photon frequency is resonant with the collective oscillation of the conduction electrons, it results in an absorption band of gold nanoparticle, which is known as the localized surface plasmon resonance (LSPR). The resonance frequency of the LSPR highly depends upon the local environment of the nanoparticle such as the refractive index (RI) of the surrounding solvent and the binding events to the nanoparticles (Nath and Chilkoti, 2002). The absorbance change due to the environmental change of the nanoparticle can be enhanced by about two orders of magnitude by this fiber-optic sensor as compared to that by a film-type sensor. For such LSPR fiber-optic biosensors, when interacted with the analytes, the immobilized gold nanoparticles act like chromophores, resulting in colorimetric changes. Here, we developed a new cGMP assay based

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on the LSPR fiber-optic sensing platform. This biosensor system is label-free, non-radioactive, able to detect cGMP directly, and highly specific and sensitive.

2. Materials and methods

2.1. Reagents and materials

Multimode plastic-clad silica optical fiber (model F-MBC) was purchased from Newport (Irvine, CA) with core and cladding diameters of 400 and 430 μm, respectively. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), except as specifically stated otherwise.

2.2. Immobilization of the anti-cGMP antiserum onto a gold nanoparticle-modified optical fiber

A gold nanoparticle-modified optical fiber (GNOF) was prepared as described previously (Lin et al., 2006; Lin and Chung, 2009). The modified fiber was then incubated with 0.02 M cystamine dihydrochloride (pH 7.4 in Dulbecco's phosphate buffered saline, DPBS) for 2 h to form a self-assembled monolayer that contains amine functional groups. The cystamine-modified gold nanoparticles fibers were then immersed at 4 °C for 0.5–4 h in the mixture of 5 wt% N-hydroxysuccinimide (NHS)/5 wt% 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) in DPBS and the anti-cGMP antiserum in DPBS containing 0.1% BSA at a volume ratio 1:10. Various dilutions of the anti-cGMP antiserum with DPBS from 1:2000 to 1:100 were used to find the optimal condition for immobilization. For evaluating the stability, the biosensor was dried by purging with nitrogen, sealed with Parafilm, and stored at 4 °C for 1, 2, and 4 weeks. At a specified time point, the preserved biosensors were then used to measure cGMP.

2.3. Instrumentation

The optical configuration for the GNOF biosensor is a reflection-based fiber-optic configuration as shown in Fig. 1. Basically, the system consists of a laser (Hitachi HL6320G laser diode, 635 nm, 10 mW; Thorlabs LDC500 laser diode controller; Thorlabs TEC2000 temperature controller; Thorlabs TCLDM9 laser mount), a chopper (Stanford Research SR540), a fiber coupler, a beam splitter, a sensing fiber, a liquid cell (1 ml), a photoreceiver (Thorlabs PDA55), and a lock-in amplifier (Stanford Research SR830) as shown in Fig. 1. The photoreceiver was used to convert light signals into a voltage. A silver film was coated at the terminal end of the unclad portion of the probe and the light reflected by the sensing material was collected at the proximal end of the fiber probe. For the detailed description of the biosensor system, please refer to Chau et al. (2006). The temperature of the liquid testing cell was maintained by a circulatory water bath (Wisdom, model LC-06) within ±0.05 °C. The sample was filled in the liquid testing cell, in which the biosensor was installed horizontally.

2.4. Cell culture

Rat aortic smooth muscle cells (SMCs) A7r5 (ATCC CRL-1444) were cultured as described previously (Huang et al., 2005). Briefly, the SMCs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% cosmic calf serum containing iron supplement and growth-promoting factors (Hyclone), 2 mM glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 μg/ml gentamicin at 37 °C under 5% CO_2 humidified atmosphere. When the cell confluence reached 70–80%, the serum concentration was reduced to 1% for 1 day, and then the cells were trypsinized and subcultured at 40% of cell confluence. One day after the passaging, the serum concentration was further reduced to 0.3% by exchanging the medium. The investigations were carried out 18 h after the last adjustment of serum concentration. The SMCs used in the experiments underwent 5–7 passages.

2.5. Measurement of intracellular cGMP concentrations

The SMCs cultured in 35-mm dishes were pretreated with 0.2 mM 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) 30 min before addition of 12 μM spermine NONOate (Cayman) and 16 μM oxyhemoglobin (Huang et al., 2005, Yin et al., 2007). At the time points of 15, 30, and 60 min after the addition of the NO donor, the cells were lysed with 0.1 M HCl, centrifuged at 800 × *g* for 10 min, and the supernatants were dried on a new dish. Intracellular cGMP content recovered by incubation the dish with 1 ml of 20 mM pH 7.4 Tris buffer for 2 h was then measured using the GNOF biosensor or the cGMP EIA kit (Assay Designs) following the manufacturer's protocol. The biosensor response was expressed as I/I_0, where I_0 and I are the averaged light intensity measured in the absence and presence of cGMP, respectively. The limit of detection (LOD) was defined as the biosensor response at a cGMP concentration that yielded a signal to noise ratio of 3. The noise was measured as the standard deviation of the intensity for three repetitive measurements.

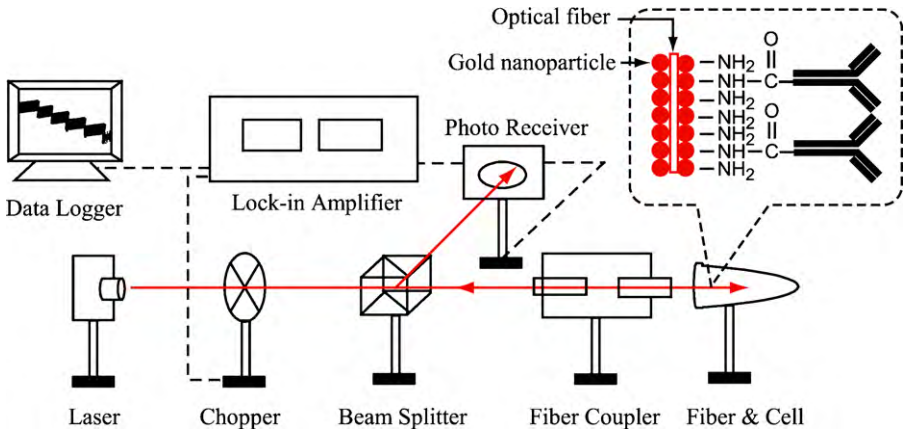

Fig. 1. Exprimental setup for the optical fiber biosensor system.

3. Results and discussion

3.1. Optimal conditions for anti-cGMP antiserum immobilization

The use of glutaraldehyde solution as the crosslinker between antisera and the cystamine-modified gold nanoparticles probably produces a wide range of high molecular weight complexes, resulting in reduction of the average antigen-binding activity for each antibody molecule. This drawback caused by using glutaraldehyde in the immobilizing procedure can be avoided by using the mixture of NHS and EDC instead, which crosslink the amine group of the cystamine-modified gold nanoparticles with the carboxyl group of the antibody. The antigen recognition region of the antibody is at the N-terminus. Batalla et al. (2008) showed that there is a high density of Asp and Glu along the Fc region of the antibody, but not the Fab region. Therefore, the immobilization using the carboxyl groups of the antibody could have a less effect on the Fab and achieve a greater antigen-binding capacity as compared to using amine groups.

The binding of cGMP to the immobilized anti-cGMP antisera caused a decrease in light intensity of the GNOF biosensor due to an increase in the local RI. Fig. 2 shows an example of serial responding signals from the anti-cGMP antiserum-modified GNOF biosensor immersed in a 20 mM Tris buffer solution after sequential addition of cGMP to a final concentration of 50 pmol/ml. The responding light intensity decreases with increasing cGMP concentration at room temperature. The voltage change of the biosensor is about 4 mV in the presence of 50 pmol/ml and the LOD of the biosensor is about 0.1 pmol/ml.

The parameter determining the time at which the steady state response with respect to mass transfer is established for a confluent colony without flow is $p = 2\sqrt{Dk_{off}}/k_{on}R_0$, where R_0 is the total surface concentration of binding sites, k_{on} and k_{off} are the association and dissociation rate constants respectively, and D is the diffusion coefficient of the antigen (Model and Omann, 1995). The response time is decreasing with the increase of the value of p. The diffusion coefficients for protein and cGMP are in the range of 10^{-7} and 10^{-6} cm^2/s (Sugawara et al., 2004, Dworkin and Keller, 1977), respectively. Therefore, if the antigen is a protein, the time for reaching a steady state response is about tens of minutes. However, it takes only about few minutes for cGMP. This may explain why the quick response of the sensor in our system.

To optimize the sensitivity of a fiber-optic biosensor, we altered the anti-cGMP antiserum concentration and its incubation period to maximize the total antigen-binding activity of the immobilized antibody. In Fig. 3, the working range of cGMP concentration for the GNOF biosensor is at least three orders of magnitude from 0.1 to 100 pmol/ml. This wide range of cGMP detection will reduce the chance of sample dilution requirements. In Fig. 3(a), the dilution of anti-cGMP antisera is 1:250 and the incubation periods of anti-cGMP antisera with the cystamine-modified gold nanoparticles are from 0.5 to 4 h. The response of the GNOF biosensor is enhanced by increasing the incubation period and reaches saturation at 2 h. To investigate whether the response of the GNOF biosensor could be further increased by altering the antiserum concentration, various dilutions of anti-cGMP antisera from 1:2000 to 1:100 were used in the immobilization procedure. Fig. 3(b) shows that in the range of dilution ratio from 1:2000 to 1:250, the lower the dilution ratio of anti-cGMP antisera, the greater the response of the GNOF biosensor. The response of the GNOF biosensor becomes saturated at a 1:250 dilution of anti-cGMP antisera so further increasing the concentration of antisera at a 1:100 dilution does not enhance or reduce the response of the GNOF biosensor.

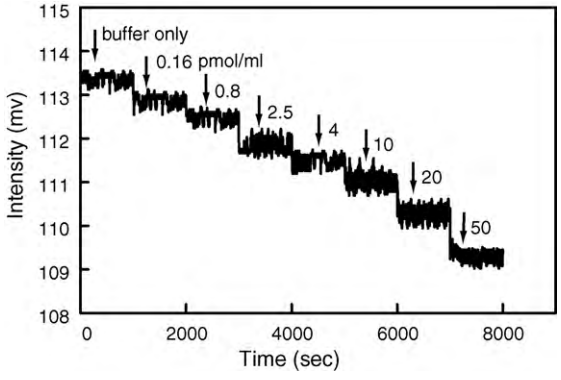

Fig. 2. Representative of serial cGMP response in the range of 0.16–50 pmol/ml by the biosensor. The antiserum dilution and incubation time for the probe are 1:250 and 2 h, respectively.

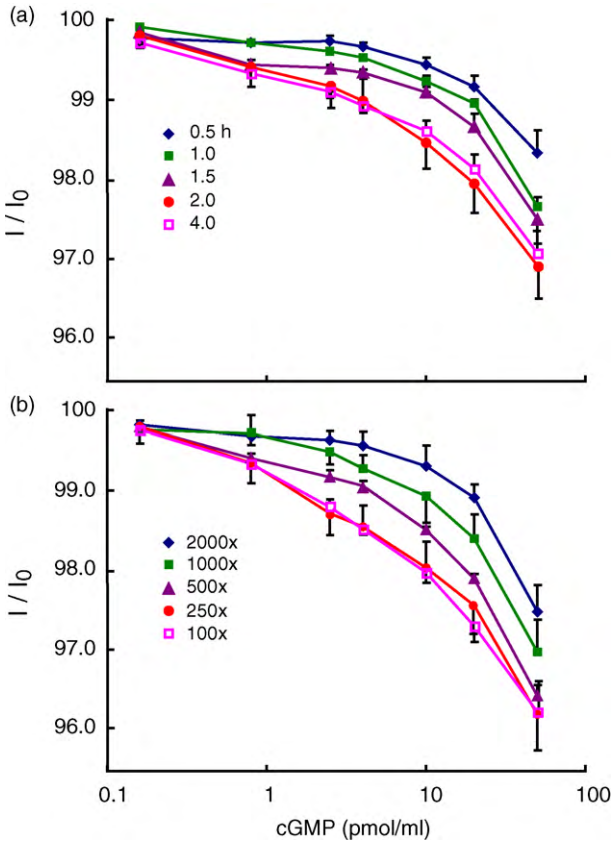

Fig. 3. Effect of (a) incubation time and (b) antiserum dilution on the sensor response to 0.16–50 pmol/ml cGMP in 20 mM pH 7.4 Tris buffer.

3.2. Sensitivity of the biosensor and cGMP acetylation

The antiserum used in this study is made to the cGMP-bovine serum albumin conjugate. The acetylated cGMP having its chemical structure more similar to the conjugate has greater affinity than cGMP to the antiserum. The sensitivity of cGMP detection for EIA and RIA can be increased by about 100-fold, as cGMP is acetylated (Pradelles et al., 1989). Fig. 4(a) shows that when the GNOF biosensor was used, it was able to measure the concentration of acetylated cGMP as low as 1 fmol/ml that is similar to that for EIA or RIA. To estimate the dissociation constants for non- and acetylated cGMPs, the relative change of the response of the biosensor

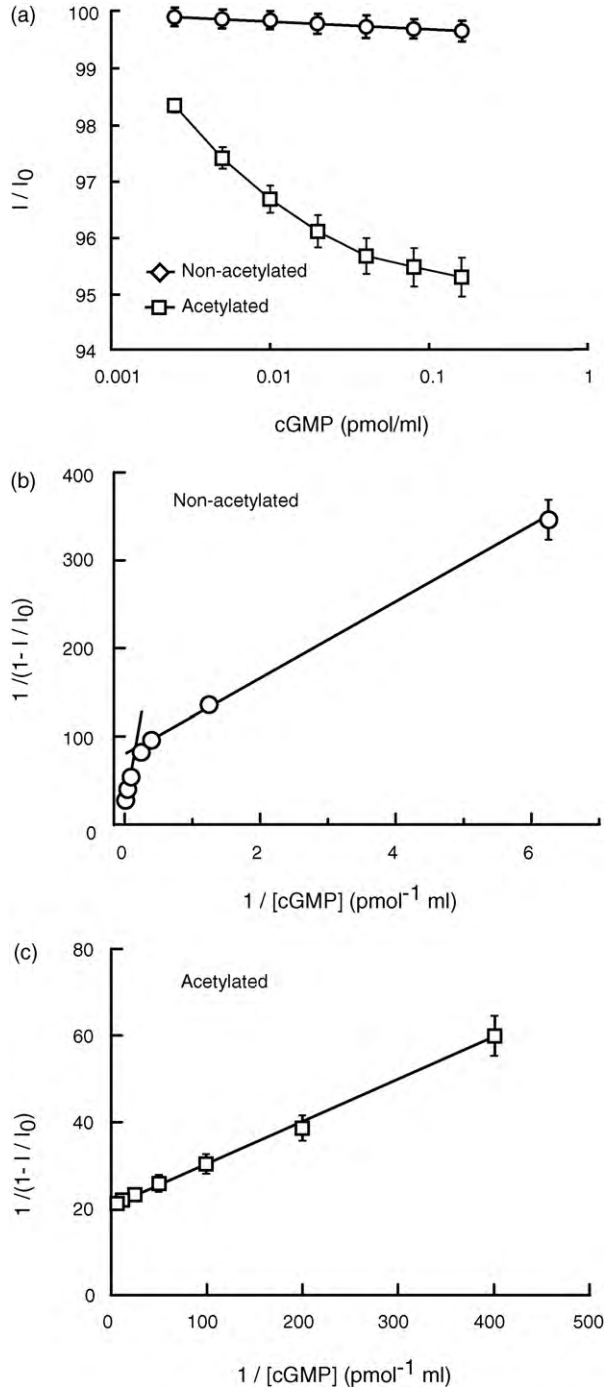

Fig. 4. (a) Comparison of the sensor responses between cGMP and acetylated cGMP. Determination of the dissociation constants between anti-cGMP antiserum and (b) cGMP or (c) acetylated cGMP.

can be expressed:

relative change of response

$$= \frac{1 - I/I_0}{1 - I_{min}/I_0} = \frac{[Ab - cGMP]}{[Ab]_t} = \frac{[cGMP]}{K_d + [cGMP]} \quad (1)$$

where I_{min} is the biosensor response when the cGMP concentration reaches infinity. $[Ab]_t$ and [Ab–cGMP] are on the biosensor the concentrations of the total antibody and cGMP–antibody complex, respectively. [cGMP] is the unbound cGMP concentration and K_d is the dissociation constant between cGMP and its antibody. Eq. (1)

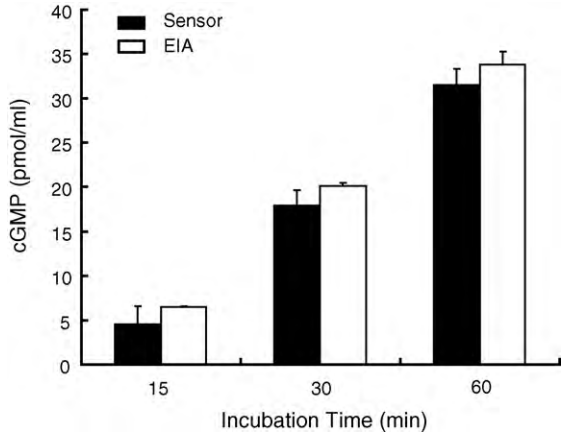

Fig. 5. Measurements of intracellular cGMP concentrations by the biosensor and by EIA after treatment of rat SMCs A7r5 with spermine NONOate and oxyhemoglobin.

can be linearized in double-reciprocal form:

$$\frac{1}{1 - I/I_0} = \frac{K_d}{1 - I_{min}/I_0}\frac{1}{[cGMP]} + \frac{1}{1 - I_{min}/I_0} \quad (2)$$

Fig. 4(b) shows the plot of $1/(1–I/I_0)$ vs. 1/[cGMP] for non-acetylated sample. The data can be fitted by two straight lines. In the low cGMP concentration range from 0.16 to 2.5 pmol/ml, the slope and intercept of the line are respectively 42.46 ± 0.62 pmol/ml and 80.7 ± 2.3, corresponding to $I_{min}/I_0 = 0.988$ and $K_d = 0.526$ pmol/ml. In the high cGMP concentration range from 10 to 50 pmol/ml, the slope and intercept of the line are respectively 326 ± 35 pmol/ml and 22.1 ± 2.3, corresponding to $I_{min}/I_0 = 0.955$ and $K_d = 14.8$ pmol/ml. These results indicate the existence of the antibody heterogeneity and about one fifth of the antibody having 30-fold higher affinity to cGMP as compared to the rest. For the acetylated samples as shown in Fig. 4(c), the slope and intercept of the line are respectively 20.51 ± 0.36 pmol/ml and 0.096 ± 0.002, corresponding to $I_{min}/I_0 = 0.951$ and $K_d = 0.0047$ pmol/ml. The affinity of the antibody to the acetylated cGMP is about three orders of magnitude higher than that to cGMP.

3.3. Validation and stability of the biosensor

To confirm whether the biosensor system can be applied in measuring the intracellular cGMP concentration, the production of intracellular cGMP in SMCs was induced by addition of spermine NONOate and then the measurements of intracellular cGMP levels by the biosensor and EIA were compared. Fig. 5 shows that the determined cGMP concentrations at 15, 30, and 60 min by the biosensor and EIA are similar and are increased with time. This comparison validates the cGMP measurement by the biosensor system.

To demonstrate the stability of our biosensors, the biosensors stored at 4 °C for up to 4 weeks were used to measure the cGMP concentration. Fig. 6 shows that the intensity change of the biosensor due to the presence of 50 pmol/ml cGMP decreases by about 25% after one week of storage and by about 50% after 4 weeks of storage. The reduction of the biosensor response may be due to denaturation of the immobilized antibody after a long-term storage. However, the biosensor can still be used as long as the residual activity remains 50% of the original value.

Mitsui et al. (2004) immobilized the gold nanoparticles onto the end face of their fiber. Although there may be only several points of internal reflection at the end face of the fiber where the light beam interacted with an analyte, the use of a photomultiplier tube made their resolution as high as 2×10^{-5} RI. In the future, we may also

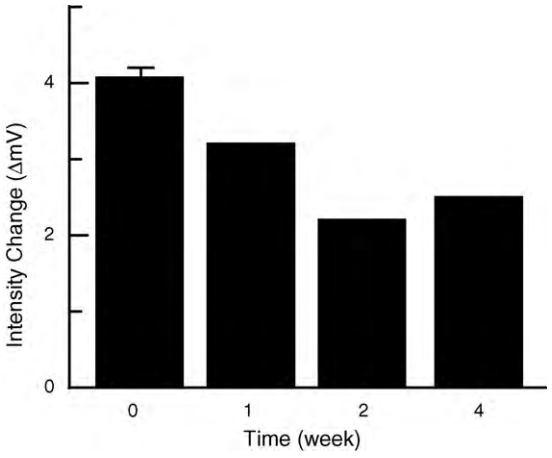

Fig. 6. Biosensor response to 50 pmol/ml cGMP after storage at 4 °C for up to 4 weeks.

apply a photomultiplier tube in our setup to further enhance our resolution.

4. Conclusion

The LSPR biosensor with the immobilized anti-cGMP antiserum at the surface of the gold nanoparticles has been demonstrated for determining intracellular cGMP concentration. The optimal antiserum dilution and incubation time for preparing the probe are 1:250 and 2 h, respectively. The sensor shows a wider detection range than EIA with a cGMP detection range of 0.1–100 pmol/ml. The sensitivity of the biosensor is three orders of magnitude higher in measuring acetylated cGMP vs. cGMP. The biosensor produces a stable response after stored at 4 °C for up to 4 weeks. In addition, the biosensor provides a label-free and low background detection for determining the dissociation constant between antibody and antigen. Furthermore, not like EIA or RIA, the procedures of the biosensor measurement involve no washing steps.

Acknowledgements

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