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Carbon nanotube-assisted enhancement of surface plasmon resonance signal

Eun Gyo Lee, Kyung Mi Park, Jin Young Jeong, Seung Hui Lee, Jung Eun Baek, Hong Weon Lee, Joon Ki Jung, Bong Hyun Chung *

Korea Research Institute of Bioscience and Biotechnology, Yuseong, Daejeon 305-600, Republic of Korea

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ABSTRACT

We describe a method of amplifying the biosensing signal in surface plasmon resonance (SPR)-based immunoassays using an antibody–carbon nanotube (CNT) conjugate. As a model system, human erythropoietin (EPO) and human granulocyte macrophage colony-stimulating factor (GM–CSF) were detected by sandwich-type immunoassays using an SPR biosensor. For the amplification of the SPR signal, the CNT was conjugated with a polyclonal antibody, and then the conjugates were reacted with antibodies coupled with the target proteins. This amplification strategy increases the dynamic range of the immunoassays and enhances the detection sensitivity. The SPR immunoassays, combined with the CNT-assisted signal amplification method, provided a wide dynamic range over four orders of magnitude for both EPO and GM–CSF (0.1–1000 ng/ml). The CNT amplification method is expected to realize the detection of picogram levels and a wide dynamic detection range of multiple proteins, enabling it to offer a robust analysis tool for the development of biopharmaceutical production.

Surface plasmon resonance (SPR)[1] is an affinity optical sensor based on the detection of changes in mass concentration at a biospecific interface [1]. However, the lack of a highly sensitive analytical method for detecting an analyte at low concentrations is a major impediment to SPR biosensor technology. SPR offers the several advantages compared with various other sensing techniques; it does not require labeling reagents or hazardous labeling procedures, it is capable of rapid analysis, and it allows for on-site or in situ detection. The most important point for the application of SPR biosensors is to monitor the biomolecules sensitively and precisely. Because the detection limit of current SPR biosensors is approximately 1–100 ng/ml proteins [2,3], this sensitivity is sometimes insufficient for measuring biological samples [4].

Erythropoietin (EPO) is a 34-kDa glycoprotein hormone that promotes the production of red blood cells, and its abuse results in serious side effects such as hypertension and heart failure. The concentration range of EPO is approximately 6–100 pg/ml in urine [5], and it is produced approximately 150 μg/ml from Chinese hamster ovary (CHO) cell culture [6] and 50 μg/ml from transgenic pig milk [7]. Therefore, EPO biosensors require monitoring of the concentration range of 0.01–10,000 ng/ml to control the product quality accurately and rapidly. Granulocyte macrophage colony-stimulating factor (GM–CSF) is a pleiotropic cytokine for the survival, differentiation, and proliferation of granulocyte macrophage populations. It is expressed at approximately 130 μg/ml in yeast and animal cell cultures [8]. Currently, the commercially available enzyme-linked immunosorbent assay (ELISA) for EPO and GM–CSF are generally based on sandwich immunoassays and the horseradish peroxidase reaction for realizing higher sensitive detection. Their dynamic detection range is approximately 0.01–1.0 ng/ml, and the broad range is required for process design and quality assurance.

Many studies on the improvement of the detection limit or sensitivity of SPR in label-free ways have been reported [9–13]. Despite the successful use of various nanoparticles for signal amplification and sensitivity enhancement of sandwich immunoassays of biomolecules (peptides, proteins, and oligonucleotides), biomolecules of interest are detected with less sensitivity and less specificity and the steric hindrance of the nanoparticles may block the approach of the antigen to the conjugated antibody [14]. We also expect the use of an SPR biosensor system as a process development tool to evaluate downstream processing operations for biopharmaceuticals [15]. SPR systems have a number of advantages over traditional bioanalytical techniques (ELISA, flow injection analysis, and radioimmunoassay) that are currently in use to aid biopharmaceutical process development. SPR is especially capable of fast and on-site detection and does not require any labeling reagents [16].

* Corresponding author. Fax: +82 42 879 8594.
E-mail address: chungbh@kribb.re.kr (B.H. Chung).

[1] *Abbreviations used:* SPR, surface plasmon resonance; EPO, erythropoietin; CHO, Chinese hamster ovary; GM–CSF, granulocyte macrophage colony-stimulating factor; ELISA, enzyme-linked immunosorbent assay; CNT, carbon nanotube; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; BSA, bovine serum albumin; MUA, 11-mercaptoundecanoic acid; MHA, 16-mercapto-1-hexadecanoic acid; MUOH, 11-mercapto-1-undecanol; SAM, self-assembled monolayer; DW, distilled water; PBS, phosphate-buffered saline; SEM, scanning electron microscopy; RU, resonance units.

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Recently, carbon nanotubes (CNTs) have evolved as a new class of nanomaterials that possess high surface area as well as unique electronic, thermal, and mechanical properties [17,18]. Owing to their excellent biocompatibility and ability to improve the electron transfer rate, they are extremely useful for applications in electrochemical biosensors [19]. In addition, large molecular mass is expected to be sensitive detection for an SPR system. Major barriers for the preparation of a CNT-based biosensor are the poor dispersibility of CNTs in water solvents and hydrophobicity [20]. If these barriers could be eliminated, the CNT-assisted biosensor might be able to detect biomolecules sensitively and precisely.

Here we have developed a signal enhancement procedure of SPR to overcome the technical difficulties related to the very low concentration of biomolecules such as EPO and GM–CSF. We did so by using the CNT–antibody complex for the first time. For stable and specific interaction between antibody and antigen, the optimal conditions of the surface of the chip and CNT were investigated. The logarithmic correlation between the SPR angle shift and antigen concentration exhibited a good linear relationship for both EPO and GM–CSF. The proposed method can offer a wide dynamic detection range as well as improve the detection limit compared with conventional label-free SPR analysis.

Materials and methods

Materials

Recombinant EPO was purchased from the Korea Food and Drug Association, and GM–CSF was purchased from R&D Systems (Minneapolis, MN, USA). Primary monoclonal capture antibodies (MAB2871 and MAB215) and secondary polyclonal antibodies (AB-286-NA and AF-215-NA) for ELISA analysis were obtained from R&D Systems. *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and 1.0 M ethanolamine–HCl (pH 8.5) were obtained from Biacore (Uppsala, Sweden), and bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). 11-Mercaptoundecanoic acid (MUA), 16-mercapto-1-hexadecanoic acid (MHA), and 11-mercapto-1-undecanol (MUOH) were purchased from Aldrich (Milwaukee, WI, USA). Multiwalled CNTs were purchased from Iljin Nanotech (Korea).

Preparation of gold-coated glass plate

The gold-coated glass plate was immersed in 10 mM MUA at room temperature for 20–24 h to form MUA self-assembled monolayer (SAM) immediately after cleaning with a 3:1 (v/v) solution of H_2SO_4/H_2O_2 at 50 °C for 30 min. Mixed SAM solutions were obtained by mixing 1 mM MHA and 1 mM MUOH, both in ethanol, at a 1:9 (v/v) ratio. The plate was washed with ethanol and distilled water (DW), sonicated for 5 min in ethanol, and dried under a nitrogen stream. This protocol produces stable and fully covered SAMs on gold. After the plate was immersed in a solution of 0.2 M EDC and 0.05 M NHS in DW for 10 min, monoclonal antibody for EPO or GM–CSF capturing was immobilized on the activated surface by immersing 0.1 mg/ml solution of antibody in phosphate-buffered saline (PBS, pH 7.4) for 45 min. The unreacted esters on the chip surface were inactivated by immersing the chip in a solution of 1.0 M ethanolamine (pH 8.5) for 10 min and then in a solution of 1% BSA for 45 min.

Preparation of CNT–antibody complex

CNTs were functionalized by heating in 3:1 H_2SO_4/HNO_3 at 120 °C for 1 h and shortened by sonication for 3 h. The resulting dispersion was washed with DW and filtered until its pH was neutral. This acidic treatment generates carboxylic groups on the surface of CNTs to be conjugated with antibodies. The resulting CNTs (1 mg) were dispersed in 1 ml of PBS buffer (pH 7.4), mixed with 0.02 M EDC, and then incubated with EPO or GM–CSF polyclonal antibody (0.5 mg/ml) overnight at 4 °C. The reaction mixture was then repeatedly centrifuged at 12,000 rpm for 20 min to remove the unreacted EDC and free antibody. The CNT–antibody complex was dispersed in PBS buffer, sonicated for homogeneous dispersion, and stored at 4 °C.

Scanning electron microscopy

The shortened and functionalized CNTs with antibody were characterized by scanning electron microscopy (SEM, FEI, Netherlands) at an acceleration voltage of 10 kV after platinum coating using a platinum coater (Bal-Tec, Switzerland).

SPR immunoassay in BIAcore system

All SPR measurements were performed on a BIAcore X SPR biosensor system (GE Healthcare, Sweden). The instrument was operated using the BIAcore X control software (BIAevaluation 4.1). PBS buffer was used as a running buffer for SPR assays. The chip was mounted onto the chip carrier and inserted into a Biacore X SPR biosensor system, which operated at a constant flow rate of 5 μl/min and 25 °C. The analyte solution of EPO or GM–CSF (0.1–1000 ng/ml) in PBS buffer was applied to the chip for 25 min. Then the CNT–antibody complex was injected for 15 min at a flow rate of 5 μl/min, as shown in Fig. 1 (strategy I). The unbound molecules were washed away using PBS for 5 min. The sensor chip was regenerated by injecting 0.1 M glycine–HCl buffer (pH 2.0).

For strategy II in the schematic diagram of Fig. 1, the CNT–monoclonal antibody complex was reacted with the analyte and the unreacted analyte was washed away with PBS. A chip immobilized with the polyclonal antibody (anti-EPO or anti-GM–CSF) was prepared, and then the signal was amplified by injecting the solution containing the CNT–antibody and analyte for 15 min.

Results and discussion

Surface preparation and blocking of the chip and CNT

The schematic diagram (Fig. 1) describes our procedure for the sandwich-type immunoassay via primary antibody (e.g., monoclonal antibody) immobilization on a chip surface for binding with an analyte and a secondary antibody (e.g., polyclonal antibody) for capturing the analyte. The immunoassay of EPO and GM–CSF consisted of its monoclonal antibody immobilization, the capture of each antigen, and CNT-assisted signaling. To specifically capture the analyte using the antibody combined with CNT, the blocking of both the gold chip surface and CNT is one of the critical factors in minimizing nonspecific adsorption on the chip surface. MUA and a mixture of MHA and MUOH were examined as an SAM on the chip surface. For this experiment, the CNT–antibody complex, as a binding material, was injected into the flow cell. The formation of a monolayer with 10 mM MUA resulted in relatively minor adsorption compared with the mixture of MHA and MUOH (Fig. 2A). In addition, BSA showed a slightly lower background signal than amino dextran treatment when the chip surface was prepared with both MUA and MHA/MUOH SAMs (Fig. 2A). BSA was a superior blocking agent for the chip surface following the immobilization of the monoclonal antibody of EPO, whereas amino dextran was a better blocking agent against the CNT–antibody complex in capturing the antigen specifically (Fig. 2B). The intention of using a blocking agent in the SPR immunoassay was

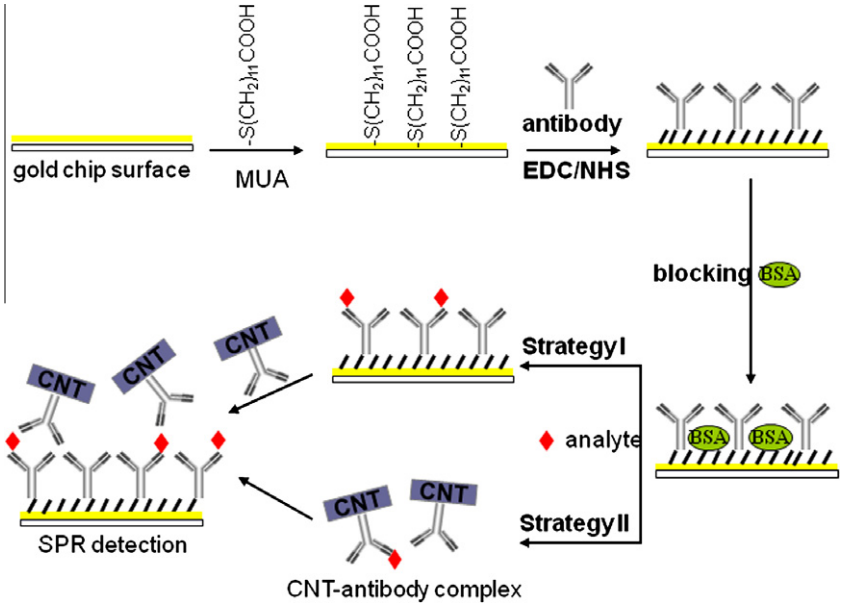

Fig.1. Schematic diagram of SPR immunoassay to enhance sensitivity with the CNT–antibody complex.

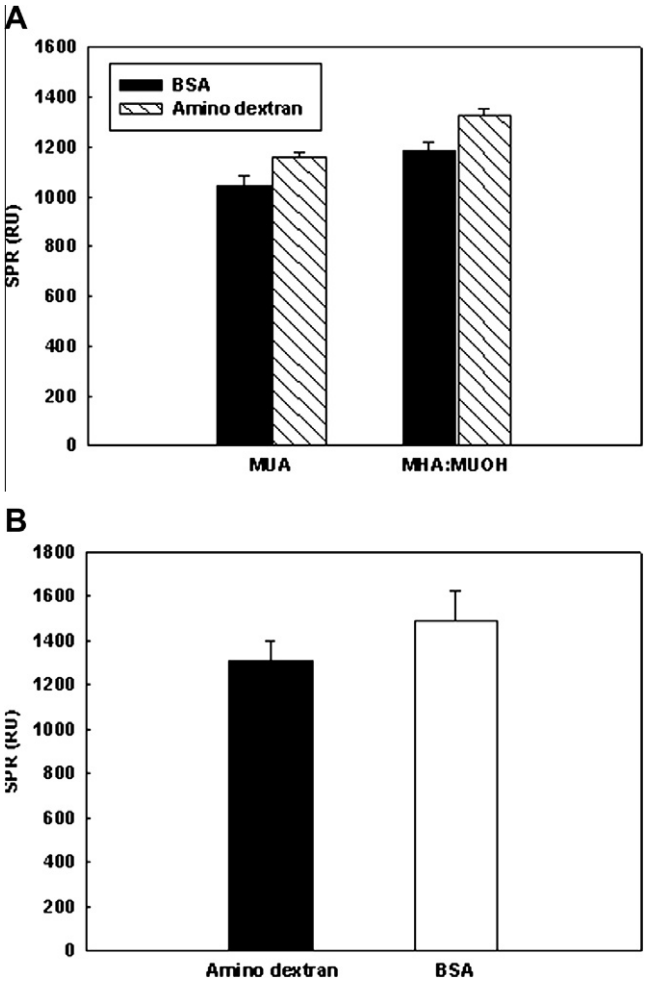

Fig.2. Effects of the blocking agent on the exposed surface of the CNT–antibody complex (A) and both SAMs and blocking agents on the chip surface (B) by using a nonspecific binding test of the CNT–antibody complex.

to avoid the nonspecific binding of impurities with the exception of the specific antigen on the chip surface.

Signal-amplified SPR immunoassay

The SPR immunoassay of EPO was investigated by direct assay and two different types of CNT–antibody complex. For the preparation of the chip surface, the antibody of EPO (100 μg/ml) was immobilized on the activated monolayer and blocked with 1 M ethanolamine and, subsequently, 1% BSA. For direct assay, EPO (1 μg/ml) was injected into the flow cell and the response due to monoclonal antibody–EPO reaction was monitored (Fig. 3). The SPR signal of EPO bound on the immobilized monoclonal antibody was approximately 33 resonance units (RU), whereas the signal shift by binding between the antigen and the polyclonal antibody was approximately 29 RU (Fig. 3A). Instead of the polyclonal antibody itself, the CNT–polyclonal antibody complex extensively amplified the SPR signal by 1925 (Fig. 3B), which contained the background signal. This means that there is an increase of signals by immunoreaction between the antigen and the CNT–antibody complex, as well as CNT itself, due to nonspecific hydrophobic interaction between the chip surface and CNT. This nonspecific binding may be minimized by selecting a suitable blocking agent for both CNT and the chip surface, but it cannot be eliminated. The CNT as an SPR signal-enhancing material had approximately 800–1250 signal responses for a loading time of 300 s on the chip surface under blocking treatments. Therefore, to evaluate a relationship between the antigen and CNT–antibody by excluding the CNT effect, the SPR signals in all experiments were calculated by subtracting the positive response from the control experiment, which was performed by injecting the CNT–antibody complex without the antigen on the surface of the chip in the same conditions.

In spite of the signal enhancement by CNT, the CNT-assisted SPR detection has weak points for precise quantification in terms of the inherent hydrophobicity, the steric hindrance of binding due to rod type, and the wide range of sizes, resulting in false sensor signals in SPR measurement. To lessen the problematic properties of CNT, the nonspecific adsorption of the CNT–antibody complex should be minimized. The hydrophobicity of the exposed CNT surface was reduced with amino dextran as a blocking agent, as mentioned above. In addition, because the CNT–antibody complex itself was supposed to have a higher nonspecific adsorption on the chip surface, the CNT–monoclonal antibody complex previously bound with its antigen (EPO or GM–CSF) injected into the flow cell, where

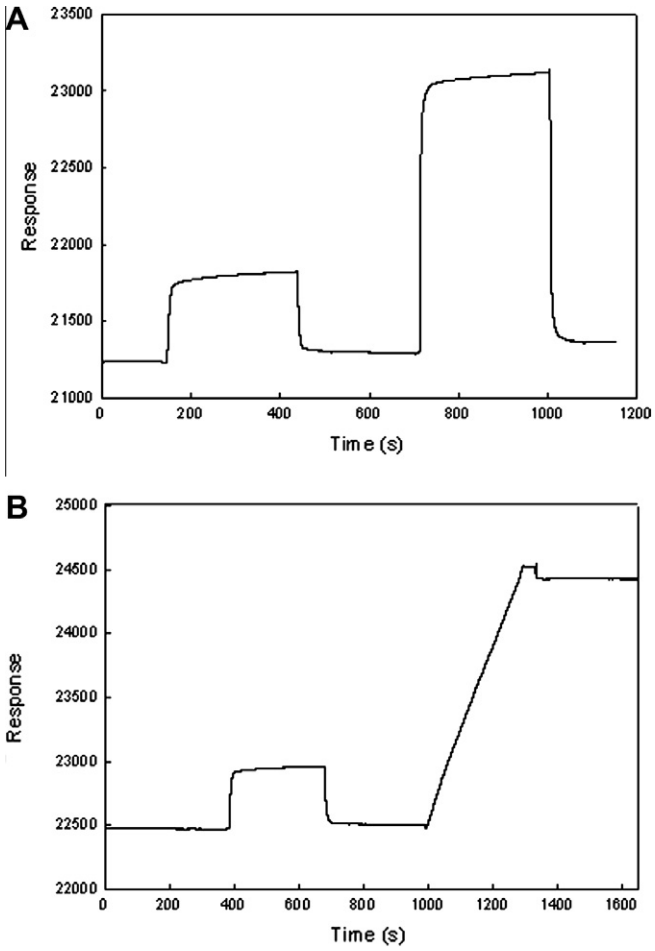

Fig.3. SPR sensorgrams of the EPO (1 μg/ml) immunoassay with the antibody (A) and CNT–antibody complex (B).

a sensor chip was immobilized with the polyclonal antibody. The CNT–monoclonal antibody complex combined with its antigen further increased the specificity, achieving a signal-enhancing effect (18-fold higher than direct assay) by CNT (Fig. 4). The appropriate binding formation between the antigen and the CNT–antibody complex was further considered by determining the suitable ratio

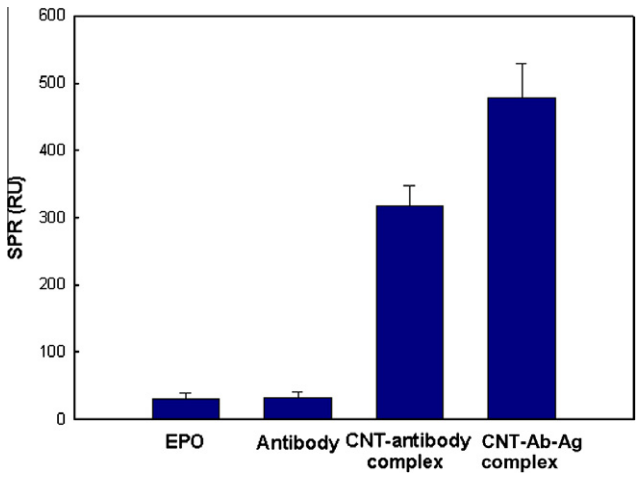

Fig.4. SPR signal shift by direct and CNT-mediated detections. CNT–Ab–Ag refers to the complex formation of CNT–antibody–antigen after the CNT–antibody and the sample are reacted.

of the antibody to CNT to detect the antigen. To investigate the effect of the ratio of CNT to antibody, we carried out the experiments in various ranges of CNT to antibody from 250 to 5. Although the preferable ratio of CNT to antibody depended on each antibody and on antibody concentration, the CNT concentration was chosen as 50:1 (mass:mass) for EPO- or GM–CSF-specific antibodies (each 10 μg/ml), reducing the background signals slightly. It is possible that excess CNT or antibody may increase the background signal or induce multiple bindings with a couple of antigens.

In the SPR sensing system, the antigen (EPO) binds with the immobilized monoclonal antibody on the SAM chip surface and the CNT–antibody complex is assembled onto the antigen captured on the immobilized monoclonal antibody. Because the CNTs are large, the relationship between the antigen and the CNT–antibody complex could be evaluated simply by SEM. The SEM pictures under relatively low (6.25 μg/ml) and high (62.5 μg/ml) concentrations of EPO are shown in Fig. 5. The size of the CNT showed broad ranges of 0.3–1.3 μm, and the CNT–antibody complex was observed to combine uniformly with its antigen, indicating that the CNT complex has good dispersibility in buffer solution and high specificity against the antigen.

Quantification of EPO and GM–CSF by SPR sensing using CNT–antibody complex system

The CNT-assisted SPR immunoassays were performed using varying concentrations of EPO and GM–CSF. The logarithmic correlation between the SPR angle shift and antigen concentration

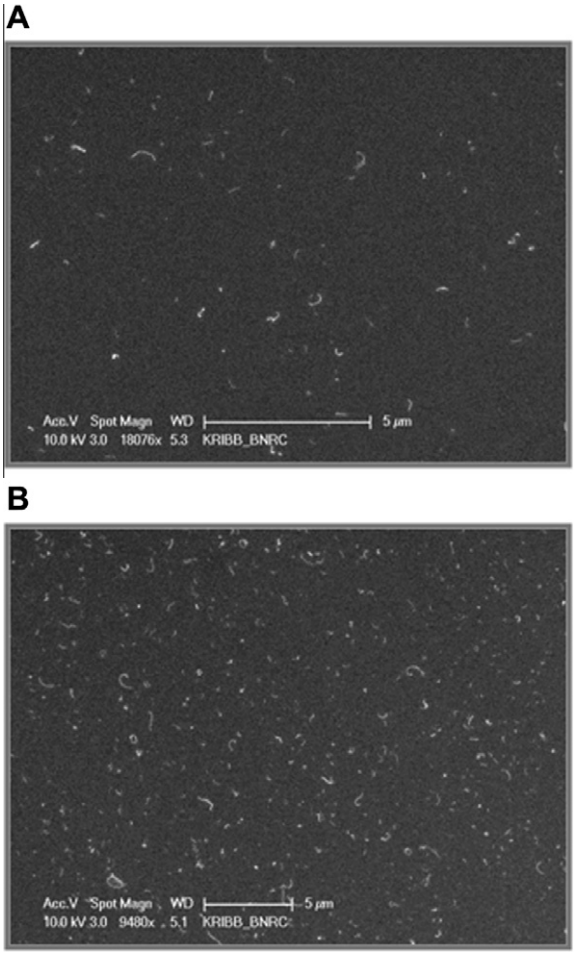

Fig.5. SEM images of chip surface bound with the CNT–antibody complex at low (6.25 μg/ml) (A) and high (62.5 μg/ml) (B) concentrations. The scale bar is 5 μm.

exhibits a good linear relationship of 0.98 and 0.96 for both EPO and GM–CSF in the range of 0.1–1000 ng/ml (Fig. 6), whereas that from a normal SPR immunoassay without signal amplification shows poor linearity over the analytical range. Here the slope refers to the sensitivity of capturing material against the antigen and reveals the extent of the nonspecific binding. The regression refers to the precision of the SPR sensor over the dynamic detection range. As a result, the CNT-assisted SPR sensor could be applied for the quantification of therapeutic proteins on the process qualification of culture and downstream processing when considering the correlating equation. In particular, the correlation slope (>0.30) (Fig. 7) of the CNT–monoclonal antibody complex, measured after reaction with the antigen, had higher sensitivity than that of the CNT–polyclonal antibody complex conducted in the general SPR sensing system (Fig. 6). The CNT-assisted assay resulted in a 31-fold higher signal amplification and higher correlation than the direct label-free immunoassay. In addition, it may extend five orders of magnitude and detect lower concentration (<0.1 ng/ml) of EPO and GM–CSF, as shown in Fig. 7, whereas the normal SPR immunoassay with CNT amplification had meaningless SPR signals with EPO and GM–CSF concentrations of less than 1 ng/ml.

The quantification of various proteins is required to profile multiple proteins present in biological samples over a broad range of concentrations, and cytokines in serum are present in concentrations ranging from 0.001 to 5×10^5 ng/ml [4]. Thus, the CNT-assisted SPR immunoassays developed in this study offer potential application to a protein profiling analysis in terms of a wider dynamic range and limit of detection than other detection methods.

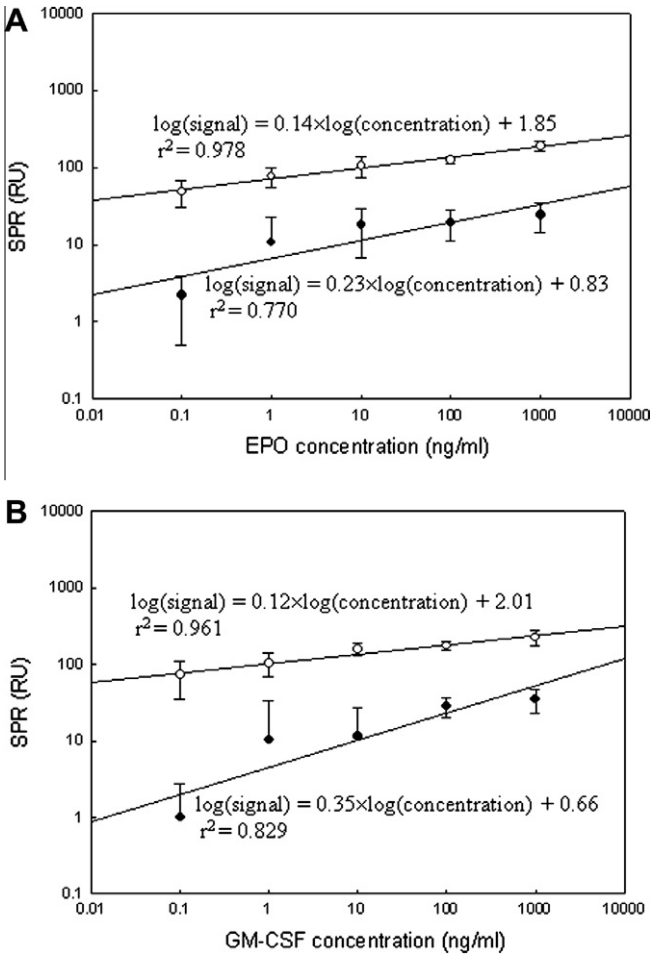

Fig.6. SPR angle shifts at various EPO (A) and GM–CSF (B) concentrations using the CNT–antibody complex. The error bars represent standard deviations in triplicate runs: ●, direct immunoassay; ○, signal-amplified immunoassay with CNT–antibody complex.

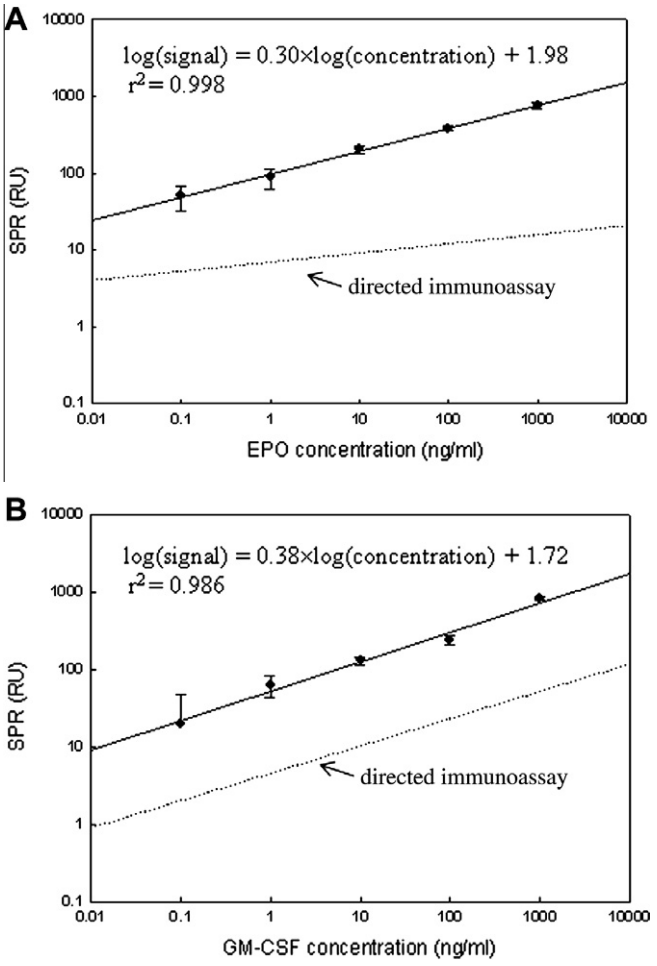

Fig.7. SPR angle shifts at various EPO (A) and GM–CSF (B) concentrations. The samples were previously reacted with the CNT–antibody complex.

Conclusions

We have demonstrated the use of a signal amplification method by CNT for SPR immunoassays. This amplification strategy enhances the detection sensitivity and enlarges its dynamic range. The SPR immunoassay combined with the signal amplification method provided a wide dynamic range over four orders of magnitude for EPO and GM–CSF (0.1–1000 ng/ml). In addition, it may extend to five orders of magnitude and detect lower concentrations (<0.1 ng/ml) of EPO and GM–CSF. The CNT amplification method is expected to realize the detection of picogram levels of both EPO and GM–CSF and a wide dynamic range analysis of multiple proteins, enabling its application for analysis of biomolecular interaction and to aid the development of pharmaceutical processes as a monitoring tool.

Acknowledgments

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