



Label-free detection of C-reactive protein using reflectometric interference spectroscopy-based sensing system

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ABSTRACT

Reflectometric interference spectroscopy (RIFS) is a label-free, time-resolved technique, and suitable for detecting antibody–antigen interaction. This work describes a continuous flow biosensor for C-reactive protein (CRP), involving an effective immobilization method of a monoclonal antibody against CRP (anti-CRP) to achieve highly sensitive RIFS-based detection of CRP. The silicon nitride-coated silicon chip (SiN chip) for the RIFS sensing was first treated with trimethylsilylchloride (TMS), followed by UV-light irradiation to in situ generation of homogeneous silanols on the surface. Following amination by 3-aminopropyltriethoxysilane, carboxymethyl dextran (CMD) was grafted, and subsequently, protein A and anti-CRP was monitored with the RIFS system by consecutive injections of an amine coupling reagent, protein A and anti-CRP, respectively, to confirm the progress of each step in real time. The sensitivity was enhanced when all of the processes were adopted, suggesting that the oriented immobilization of anti-CRP via protein A that was coupled with the grafted CMD on the aminated surface of TMS-treated SiN chip. The feasibility of the present sensing system was demonstrated on the detection of CRP, where the silicon-based inexpensive chips and the simple optical setup were employed. It can be applied to other target molecules in various fields of life science as a substitute of surface plasmon resonance-based expensive sensors.

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1. Introduction

Diverse biosensors, based on electrochemical, optical and micromechanical detection principles, have been developed as simple and convenient tools for determining target molecules in complicated matrices [1–5]. Among them, optical biosensing techniques using surface plasmon resonance (SPR) phenomena, have been increasingly attractive for biomolecular interaction analyses, due to allowing label-free, time-resolved diagnosis [6–8]. Recently, reflectometric interference spectroscopy (RIFS)-based sensing system has been alternatively used for label-free detection of biomolecules. RIFS is based on interference of reflection wave by a multiple thin layer bearing a different refractive index each other. When molecular recognition elements are immobilized on the surface of the multilayer and analytes are adsorbed on the surface, the optical thickness of the surface undergoes change, leading to the modulation of reflectometric interference patterns. As a result, detection of analytes can be inferred from changes in reflectometric interference spectrum. For example, Mehne et al. investigated

the detection of three kind ligands on mixed poly(ethylene glycols) immobilized silicon surfaces, which had properties of high loading capacity and minimal non-specific adsorption of biomolecules [9]. Huang et al. have reported a competitive protein adsorption of albumin, fibrinogen and IgG on the surface of fluorocarbon end-capped poly(carbonate) urethane (PCUF) and polystyrene [10]. The group of Albrecht et al. have developed a new sandwich assay for C-reactive protein based on a high affinity polypeptide scaffold immobilized on the sensing surface [11]. We also reported the detection of glycoproteins by using immobilized lectin on the silicon nitride-coated sensor chips [12].

C-reactive protein (CRP) is known as a marker for infections and inflammatory processes in human blood serum. The level of CRP may rise as high as 1000-folds with the presence of inflammation. CRP was found to be the only marker of inflammation that independently predicts the risk of a heart attack [13–15]. When the CRP level is elevated to be over $3 \mu\text{g mL}^{-1}$, a risk for cardiovascular diseases is considered to be high [16]. Therefore, for the determination of CRP, varied analytical methods, e.g., magnetic permeability assay, surface plasmon resonance sensing, and so on are often performed for the concentrations below $0.12 \mu\text{g mL}^{-1}$ [17–20].

In this study, we developed a RIFS-based label-free CRP detection system, where an incident light is perpendicularly irradiated

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from above the sensor chip [12], unlike the previously reported RfS systems having the principle irradiation point located to the back side of the sensor chip [11,21]. A 66.5-nm thickness silicon nitride coated silicon substrate (SiN chip) was employed as a base sensing chip, which enabled the detection of an interference spectrum at visible light region with a single rereflectance minimum [22]. When an analyte is bound to the surface, this bottom of the spectrum is red-shifted due to the increase of optical thickness, and the difference in wavelength of the bottom before and after the adsorption is an index of analyte binding ($\Delta\lambda$). A transparent micro-flow-cell prepared from polydimethylsiloxane (PDMS) was placed on the sensor chip to construct the microfluidics continuous flow system.

A commonly used immobilization procedure of antibodies is to form covalent bond between amino groups on antibodies and surface carboxyl groups on sensing chips. Carboxymethyl dextran (CMD)-modified sensor chips are often used for this purpose, as they have a high density of carboxyl groups on the aminated surface and exhibit a low non-specific binding property, with an easy modification procedure using a crosslinking reagent such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) with the combined use of N-hydroxysuccinimide (NHS) [23,24].

Major problems related to sensitivity and specificity are sometimes caused by the orientation of antibodies on sensor chips. In order to achieve a comparable detection range with the current RfS system to the previously reported methods for CRP, an oriented immobilization procedure of anti-CRP on the sensing chip of the RfS system should be carefully considered. A promising approach to oriented immobilization of antibodies would be to utilize protein A. When protein A is immobilized on the surface, antibodies can be bound in a controlled directional state on the protein A-immobilized surface [25–27], since it can bind specifically to the Fc region of immunoglobulin G (IgG) from various animals [28–30].

In the present study, a pre-treatment of the sensor chip was carried out with trimethylsilylchloride (TMS) before the preparation of an aminated surface on the sensor chip to immobilize CMD, since TMS can be transformed into siloxane/silanol by UV-light irradiation, resulting in a homogeneous silane coupling agent-reactive surface [31]. Amino groups were grafted by further silanization with 3-aminopropyltriethoxysilane (APTES), followed by attachment of CMD covalently by amine coupling reaction batchwise. The protein A was then immobilized by the same coupling reaction. A monoclonal antibody against CRP (anti-CRP) was finally bound on the surface via protein A. The process for the protein A immobilization onto the CMD-immobilized surface was carried out in situ by injecting each reagent successively into the RfS system, and monitored in real time to confirm the completion of each process (Fig. 1). Here, we investigated the feasibility of the proposed immobilization method via protein A, involving the use of TMS, APTES and CMD as a surface modification, and evaluated the binding behavior and the enhancement of sensitivity toward CRP on the prepared immuno-sensing chips using the RfS system.

2. Materials and methods

2.1. Experimental and apparatuses

Trimethylsilylchloride (TMS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and protein A were purchased from Nacalai Tesque Co. Ltd (Kyoto, Japan). Carboxymethyl dextranomer (C-50-120, CMD) and N-hydroxysuccinimide (NHS) were obtained from Fluka (Sigma, St. Louis, MO). 3-Aminopropyltriethoxysilane (APTES) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan).

2-Aminoethanol and glycine were purchased from Wako Pure Chemical Co. (Osaka, Japan). C-reactive protein (CRP) was purchased from Merck KGaA (Darmstadt, Germany). A monoclonal antibody against C-reactive protein (anti-CRP, mouse, clone C2) was purchased from HyTest Ltd. (Turku, Finland). Other reagents and solvents were used without further purification.

A RfS-based molecular interaction analyzer, MI-Affinity LCR-01 was purchased from Konica Minolta Opto, Inc. (Tokyo, Japan). A pump (PU-980, Jasco, Tokyo, Japan), degasser (DG-980-50, Jasco, Tokyo, Japan) and an auto-sampler (AS-950-10, Jasco, Tokyo, Japan) were used to construct an automated flow system for the RfS device. Silicon nitride (SiN) chips (L 9 mm × W 9 mm × H 0.725 mm) and PDMS-based microfluidic cells (L 5 mm × W 1 mm × H 0.2 mm, cell volume: 1 μ L) were purchased from Konica Minolta Opto, Inc. (Tokyo, Japan).

2.2. Preparation of TMS-treated SiN chip

TMS was dissolved in toluene (1% (v/v), 5 mL) under nitrogen atmosphere and stirred for 15 min. A silicon nitride (SiN) chip was cleaned by a UV-O₃ cleaner (PC440, mercury vapor lamp, BioForce Nanosciences, Inc., Ames, USA) for 15 min. The SiN chip was then soaked in the TMS solution and incubated for 10 min at room temperature. The SiN chip was washed with toluene and distilled water, then dried under flow of N₂ blowing. The TMS-coated SiN chip was again placed in the UV-O₃ cleaner for 15 min to transform TMS residues into silanol/siloxane.

2.3. Amination of the SiN chip

APTES (0.1 mL) was added to 10 mL of 95% ethanol, and the solution was stirred for 60 min at room temperature. The TMS-treated SiN chip was immersed in 1% APTES solution for 60 min. Subsequently, the sensor chip was washed with distilled water, and nitrogen-dried. Finally, the chip was placed on a hot plate for 60 min at 80 °C to obtain an aminated SiN chip. The SiN chip without the TMS treatment was also aminated by the same manner.

2.4. Immobilization of CMD on the SiN chip

For the immobilization of CMD via covalent bonding on the aminated SiN surface, CMD (0.1 mg mL⁻¹ or 0.01 mg mL⁻¹) was dissolved in distilled water. Carboxyl groups in CMD were converted to activated esters by using EDC and NHS (0.2 M and 0.05 M, respectively) for 15 min and then the aminated SiN chip was immersed in CMD solution for 15 min [25,26]. The CMD-immobilized SiN chip was washed thoroughly with distilled water to remove any excess reagents and dried by N₂ blowing.

2.5. Immobilization of anti-CRP on the SiN chip

The CMD-immobilized SiN chips (with and without the TMS treatment) and the PDMS-based micro-flow-cell were equipped to the RfS apparatus, and 10 mM HEPES buffer (pH 7.4) containing 10 mM calcium chloride and 0.001% Tween 20, was flowed as a running buffer. Carboxyl groups on the surface of the CMD-immobilized SiN chip were again converted to activated esters by injecting a mixture of EDC (0.2 M) and NHS (0.05 M) in water (100 μ L) at a flow rate of 10 μ L min⁻¹. Protein A (50 μ g mL⁻¹, 100 μ L) and anti-CRP (50 μ g mL⁻¹, 50 μ L) dissolved in 10 mM acetate buffer (pH 5), were injected on the sensor surface at a flow rate of 10 μ L min⁻¹ in consecutive order. Then, 50 μ L of 2-aminoethanol (10 mM, pH 8.5) was injected for blocking the excess NHS-ester groups on a surface. An anti-CRP immobilized TMS-treated CMD-SiN chip was prepared without protein A, where

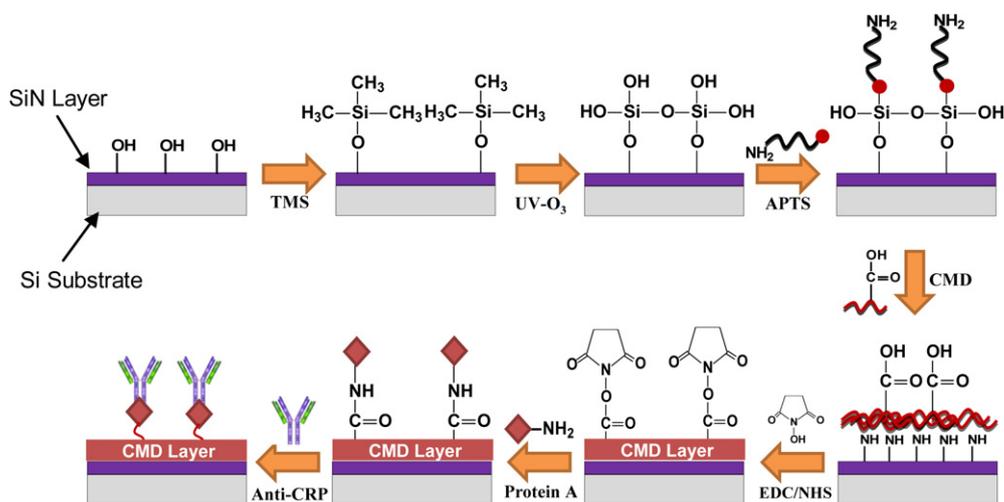


Fig. 1. Schematic illustration of the modification procedure on the SiN chip.

anti-CRP was directly immobilized on the CMD immobilized on the TMS-treated SiN chip.

2.6. Label-free detection of C-reactive protein by the RfFS-based biosensor

Various concentrations (0.01, 0.1, 1.0, and $10 \mu\text{g mL}^{-1}$) of CRP dissolved in 10 mM HEPES (pH 7.4) buffer containing 10 mM CaCl_2 and 0.001% Tween 20 were prepared. The concentration-dependent change against the CRP injection ($50 \mu\text{L}$) was monitored by the RfFS system using HEPES buffer at a flow rate of $20 \mu\text{L min}^{-1}$. Removal of the bound CRP on the immobilized anti-CRP to regenerate the sensor chip was carried out by washing the surface with a glycine–HCl solution (10 mM, pH 1.5). The CMD immobilized SiN chip, prepared with 0.01 mg mL^{-1} CMD, was used for the repetitive injection of $10 \mu\text{g mL}^{-1}$ CRP to examine the reproducibility of the RfFS response.

3. Results and discussion

3.1. Effect of the TMS treatment on amination of the SiN chip surface

Since the refractive index of the silicon nitride thin film deposited on the SiN chip was around 2.2 due to the use of silicon-rich silicon nitride deposition, silanol/siloxane groups may mingle randomly with silicon nitride on the surface, which can react with a silane coupling reagent such as 3-aminopropyltriethoxysilane (APTES) to introduce amino groups on the surface [32]. It is reported that trimethylchlorosilane (TMS) monolayer on a silica surface can be converted into silanol groups by UV-light irradiation [31]. These experiments were conducted acting under the assumption that this freshly generated outermost silanol may facilitate a homogeneous silane coupling reaction by 3-aminopropyltriethoxysilane, resulting in more homogeneous amination on the surface of the SiN chip than that without the TMS treatment. To examine the effect of the pre-treatment of TMS, amination on the SiN chip surface by APTES was performed with and without the TMS treatment followed by UV-light irradiation for 15 min. The degree of amination was evaluated by the binding ability of an acidic protein, bovine serum albumin (BSA).

Just after the TMS treatment (without UV-light irradiation), the surface showed a high contact angle, and when UV-light was irradiated for 15 min, the hydrophobicity of the surface decreased (Fig. S1 in supporting information). After the APTES treatment, BSA was injected to examine the binding behavior. The BSA binding

ability appeared to be enhanced on the TMS treated chip, compared with that of the TMS-untreated chip (Fig. 2), suggesting that the TMS monolayer was decomposed and the density of silanol on the surface was enhanced by the UV-light irradiation. From these results, the TMS-treated SiN chip was adopted for subsequent experiments.

3.2. In situ monitoring of the modification procedures on anti-CRP immobilized sensor chip via protein A

To generate the carboxyl-functionalized surface on the SiN chip, CMD was immobilized on the TMS-treated SiN chip by using the EDC–NHS coupling reagent. After the CMD treatment, protein A was immobilized on the CMD-modified SiN chip with in situ monitoring of the immobilizing process. When the 0.2 M EDC/0.05 M NHS aqueous solution was injected (Fig. 3a), the $\Delta\lambda$ value increased up to around 1 nm. Following injection of $50 \mu\text{g mL}^{-1}$ protein A solution (Fig. 3b), the $\Delta\lambda$ value was increased to 2.4 nm. Subsequently, $50 \mu\text{g mL}^{-1}$ anti-CRP solution was injected (Fig. 3c), then the $\Delta\lambda$ increased to around 3.6 nm. The baseline came to be stable within 5 min of injection. As a control, anti-CRP was directly immobilized on CMD-modified SiN chip without protein A, where the $\Delta\lambda$ value was elevated by around 0.5 nm when the anti-CRP solution was injected, which was less than that of anti-CRP immobilization

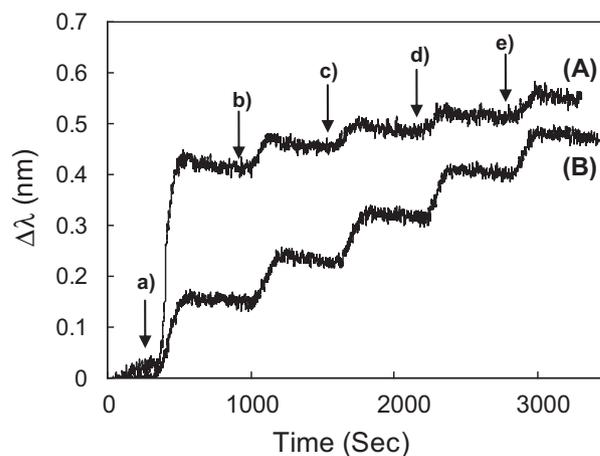


Fig. 2. Binding activity toward BSA on the aminated silicon nitride chips prepared (A) with and (B) without the TMS treatment, followed by 15 min UV/O₃ treatment. BSA conc.: (a) 3.125, (b) 6.25, (c) 12.5, (d) 25, and (e) $50 \mu\text{g mL}^{-1}$.

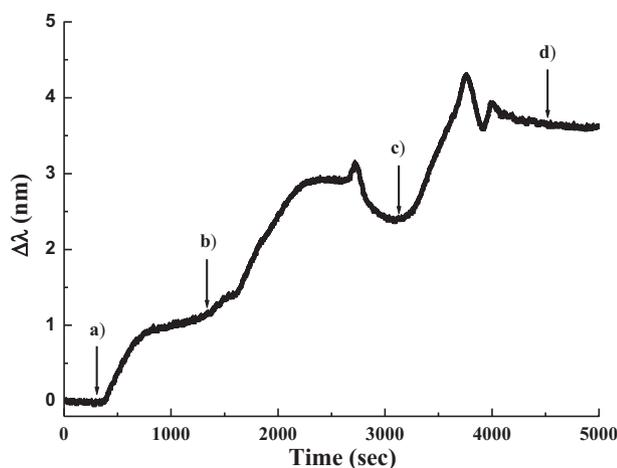


Fig. 3. In situ monitoring of the anti-CRP immobilization by the change of $\Delta\lambda$ values after injections of (a) EDC–NHS mixture (100 μL), (b) protein A (100 μL), (c) anti-CRP (50 μL) and (d) 2-aminoethanol (50 μL).

via protein A. As can be seen, each immobilization process was checked by monitoring the elevated baseline in real time, suggesting that anti-CRP was successfully immobilized on CMD-modified SiN surface under the coupling conditions employed.

3.3. Detection of CRP on the anti-CRP immobilized sensor chip

In blood serum, the normal concentration of CRP related to diagnosis for cardiovascular disease, has been reported to be 1–3 $\mu\text{g mL}^{-1}$ [15]. When the CRP level rises over 3 $\mu\text{g mL}^{-1}$ or goes down below 1 $\mu\text{g mL}^{-1}$, various disease symptoms appear in the body. Therefore, the detection of CRP was designed to obtain the detection range of below 1 $\mu\text{g mL}^{-1}$ and over 3 $\mu\text{g mL}^{-1}$. In order to confirm the feasibility of the proposed anti-CRP immobilized RfS-based biosensor, CRP was injected at a range from 0.01 to 10 $\mu\text{g mL}^{-1}$ (Fig. 4).

The cumulative increase in $\Delta\lambda$ values was observed after injection of each CRP solution. When 0.01, 0.1, 1.0, and 10 $\mu\text{g mL}^{-1}$ of CRP were injected into the anti-CRP immobilized TMS-treated CMD-SiN chip functionalized via protein A, $\Delta\lambda$ increased with the concentration of CRP injected, and a change of $\Delta\lambda$ was clearly observed

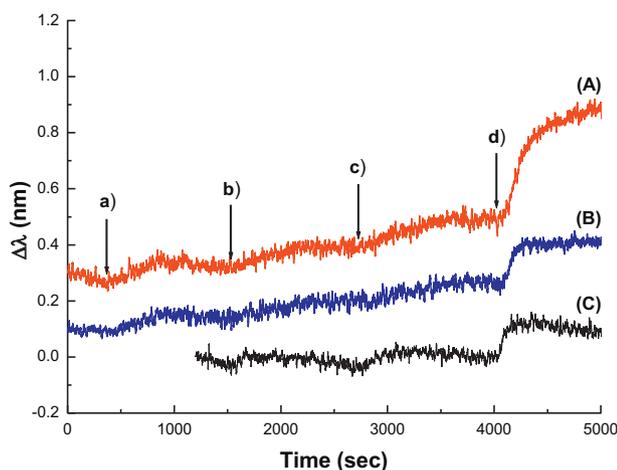


Fig. 4. Cumulative change of $\Delta\lambda_{\text{peak}}$ values after the consecutive CRP injections. (A) Anti-CRP immobilization via protein A on the TMS-treated CMD-SiN chip; (B) anti-CRP immobilization via protein A on the CMD-SiN chip (no TMS treatment); (C) anti-CRP immobilization on the TMS-treated CMD-SiN chip (without protein A); CRP conc.: (a) 10 ng mL^{-1} ; (b) 100 ng mL^{-1} ; (c) 1 $\mu\text{g mL}^{-1}$; and (d) 10 $\mu\text{g mL}^{-1}$.

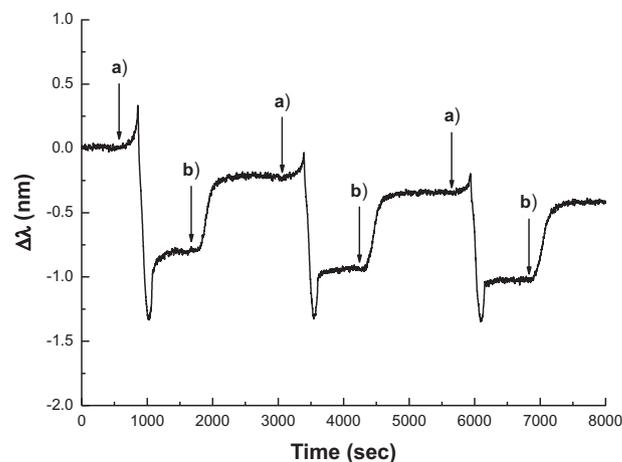


Fig. 5. Monitoring of the regeneration process on the anti-CRP immobilized TMS-treated CMD-SiN chip via protein A. (a) 10 mM glycine–HCl (pH 1.5) and (b) 10 $\mu\text{g mL}^{-1}$ CRP solution.

at 100 ng mL^{-1} of CRP, where the net RfS response, given by the difference between the average $\Delta\lambda$ value of 30 s before the injection and that before the end of the 20 min measurement interval, was 0.048 nm, which was more than three times greater than the standard deviation (0.013 nm) of the baseline drift for 30 s (Fig. 4A). When the CMD coupling was carried out with a lower concentrations of CMD (0.01 mg mL^{-1}), the response for 10 $\mu\text{g mL}^{-1}$ CRP was given to be the similar value as that prepared with 0.1 mg mL^{-1} CMD (data not shown). The sensor chip prepared without the TMS treatment showed less sensitivity (Fig. 4B). These results reveal that the TMS coated surface was effective for the preparation of the aminated surface and the successive immobilization of CMD and protein A, enhancing the sensitivity for CRP. Furthermore, the directly immobilized anti-CRP on the TMS-treated CMD-SiN chip (no protein A) showed a lower sensitivity than that done with protein A (Fig. 4C). This supports that the orientation of the immobilized anti-CRP is significantly important, and directly affects the sensitivity in the RfS system, as is in the case of other immunosensors.

Reproducibility of the RfS response for the repetitive injection of 10 $\mu\text{g mL}^{-1}$ CRP was examined, where 10 mM glycine–HCl (pH 1.5) was used as a regeneration reagent. As shown in Fig. 5, the injection of glycine–HCl allowed the sensor chip to be regenerated at an interval of 20 min, and the net RfS response for the binding of CRP to the proposed sensor chip was confirmed to be reproducible, where an average of the net RfS response was 0.59 nm, and a coefficient of variation was 0.87% ($n = 3$).

4. Conclusion

Immuno-sensing for CRP was demonstrated by using the RfS-based biosensor, in which anti-CRP was immobilized via protein A on the SiN chip. The pre-treatment on the SiN chip by TMS, followed by UV-light irradiation, resulted in a homogeneous silane coupling agent-reactive surface. After that, amino groups were grafted on the chip, to which CMD was covalently attached. After the addition of protein A, anti-CRP was immobilized on the surface through protein A, which could align the orientation of bound anti-CRP upward. Owing to the effects of both the TMS treatment and the oriented anti-CRP, the sensitivity of the RfS sensor toward CRP was enhanced, compared with the direct immobilization without the use of protein A. The reusability of the sensor chips was also confirmed by the alternative injection of CRP and the regeneration reagent. From these results, it is concluded that the

newly developed sensing system will provide a feasible way for the measurement of molecular interaction in life science, biotechnology, medical science, pharmaceutical sciences and related fields without using surface plasmon resonance-based expensive sensor chips and/or devices that have been used to date.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aca.2012.03.030>.

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