Supplementary Information

Stimulated mass enhancement strategy-based highly sensitive detection of a protein in serum using quartz crystal microbalance technique†

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Experimental Details

1. Reagents and equipments

Monoclonal anti-IL-6 antibody (produced in mouse), polyclonal anti-IL-6 antibody (produced in goat), interleukin-6 (IL-6), glucose oxidase from *Aspergillus niger*, (E.C.1.1.3.4, Type X-S, 100-250 units/mg), peroxidase from horseradish (E.C. 232-668-6, Type VI, 250-330 units/mg) *N*-hydroxysuccinimide (NHS), 1-ethyl-3 (3-(dimethylamino)-propyl) carbodiimide (EDC), β-glucose, 4-chloro-1-naphthol, ferricyanide, sodium chloride, glycine, protein A (*Staphylococcus aureus*, PA), bovine serum albumin (produced in mouse), carcinoembryonic antigen (CEA), and prostate specific antigen (PSA) were obtained from Sigma Co. Human α-thrombin (specific activity: 3,725 Units/mg, concentration: 9.7 mg/ml) was obtained from Haematologic Technologies Inc. Carboxylic acid groups-functionalized magnetic beads (COOH-MB) (dynabeads®Myone™ Carboxylic acid, 1 μm diameter) was obtained from Invitrogen. The phosphate buffer saline solution (PBS) was prepared by mixing 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 with 0.9% sodium chloride (NaCl). All other chemicals were of extra pure analytical grade. All aqueous solutions were prepared with de-ionized distilled water obtained from a Milli-Q water purifying system (18 MΩcm).

Quartz crystal microbalance (QCM) experiments were performed with a model 430B time-resolved electrochemical quartz crystal microbalance (CH Instruments Inc. USA) utilizing the gold-coated (area, 0.205 cm^2) 8 MHz AT-cut quartz crystal (International Crystal Manufacturing Co. Inc.). Cyclic voltammograms (CV) were recorded using a model 430B potentiostat/galvanostat (CH Instruments Inc. USA). In CV experiment, Ag/AgCl (in saturated KCl), and a platinum (Pt) wire were used as reference and counter electrodes, respectively. Transmission electron microscope (TEM) and scanning electron microscopic (SEM) images were obtained using field-emission transmission electron microscope (JEM-2100F, JEOL) and scanning electron microscope (Model JSM-7000F, JEOL). Impedance spectra were recorded with a CHI 660D electrochemical workstation (CH Instruments Inc.

USA). The frequency was scanned from 0.1 to 100 kHz at an open circuit voltage with the AC voltage amplitude of 5 mV.

2. Design of the QCM immunosensor

At first, a self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA) formed on a gold-coated QCM electrode by dipping it for 18 h in a phosphate buffer saline solution (PBS) solution containing 5.0 mM of MPA. The uncovered sites of the QCM electrode were treated with 5.0 mM of 2-mercaptoethanol and washed three times with a PBS solution. The QCM/MPA modified electrode was then soaked in a PBS solution containing 10 mM 1-ethyl-3 (3-(dimethylamino)-propyl) carbodiimide (EDC) and 10 mM *N*-hydroxysuccinimide (NHS) for 6 h at room temperature to activate the –COOH groups in MPA. The modified electrode was washed repeatedly (3 times) with PBS buffer in order to remove excess EDC and NHS. The EDC/NHS treated-MPA modified QCM electrode was incubated for 8 h in a PBS solution containing 3.0 mg mL^{-1} of PA at 4^{o}C. By this step, PA was covalently attached onto the QCM /MPA surface through the interaction between the –COOH groups of MPA and the $–NH_2$ groups of PA. Then, monoclonal interleukin-6 (IL-6) antibody (Ab_1) was immobilized onto the QCM/MPA/PA electrode by incubating it in a 0.1 M phosphate PBS solution (pH 7.0) containing 0.1 mg mL^{-1} Ab_1 for 24 h at 4^{o}C. After washing with 0.1 M PBS, the QCM/MPA/PA/Ab_1 electrode (QCM immunosensor) was blocked by dipping it in 0.1% BSA solution for 2 h at 4^{o}C for minimizing the non-specific binding events. Various concentrations of IL-6 protein were introduced into the QCM cell and the QCM immunosensor was incubated for 1 h at 37^{o}C. After the immunointeraction between Ab_1 and IL-6 protein, the QCM/MPA/PA/Ab_1/IL-6 immunosensor was treated with Ab_2/MB/HRP/GOx bioconjugate for 1 h followed by washing three times with PBS to remove nonspecifically bound bioconjugates. The final QCM/MPA/PA/Ab_1/IL-6/Ab_2/MB/HRP/GOx probe was assembled into a QCM cell and connected to an oscillator for frequency measurements.

3. Preparation of the Ab_2/HRP/GOx conjugate

The preparation Ab_2/HRP/GOx bioconjugate was performed as follows: 0.5 mg mL^{-1} of HRP in 0.1 M PBS solution (pH 7.0) and 0.5 mL of glutaraldehyde (25%) were mixed for 18 h at 4 °C. Excess glutaraldehyde was removed by using a sephadex G-25 column equilibrated with 0.9% NaCl. Then, 0.5 mg mL^{-1} of GOx and 5 μg mL^{-1} of secondary IL-6

antibody was added to the glutaraldehyde-treated HRP, and the mixture was incubated for 24 h at room temperature with stirring for cross-linking Ab_2, GOx, and HRP. After blocking the remaining glutaraldehyde-treated sites of HRP with 0.1% BSA solution, the Ab_2/HRP/GOx conjugate was dialyzed. Finally, the conjugate was filtered using a sterile Millipore membrane (0.20 μm), and the filtrate was stored at −20 °C.

4. Preparation of the Ab_2/MB/HRP/GOx conjugate

The secondary anti-IL-6 antibody (anti-IL-6 produced in rabbit, Ab_2)-based MB/HRP/GOx bioconjugate was prepared using carboxylic acid group functionalized magnetic beads (COOH-MB) as a bioconjugation platform. The COOH groups of MB were activated by treating with 10 mM NHS/EDC in 50 mM MES buffer at pH 5.8 for 6 h. The activated COOH-MB was separated from the free NHS/EDC by magnetic separation. 5 μg mL^{-1} of secondary IL-6 amtibody, 0.5 mg mL^{-1} of each HRP and GOx in 0.1 M PBS were added to the activated COOH-MB and stirred for 24 h at 4^{o}C. By this step, Ab_2, HRP, and GOx were covalently attached to the MB through the covalent bond formation between the carboxylic acid groups of MB and amine groups of Ab_2, HRP, and GOx. The Ab_2/MB/HRP/GOx bioconjugate was separated from the free Ab_2, HRP, and GOx by magnetic separation and was treated with 0.1% BSA solution followed by washing several times with a PBS solution. Finally, the Ab_2/MB/HRP/GOx bioconjugate was diluted with 1.0 mL of 0.1 M PBS (pH 7.4) and kept at 4^{o}C.

5. Dynamic light scattering characterizations of the bioconjugate.

Dynamic light scattering (DLS) measurements were performed with the ELS-Z instrumentation (Otsuka Electronics, Japan). The hydrodynamic size of the bare magnetic beads (MB) and the conjugate in aqueous media were determined and compared. Fig. S1 shows the size distributions of bare MBs and Ab_2/MB/HRP/GOx bioconjugates. The hydrodynamic diameter of the bare MBs was estimated to be ~1119 nm. After Ab_2, HRP, and GOx conjugation with MB, the hydrodynamic diameter of the Ab_2/MB/HRP/GOx conjugate increased to ~1250 nm. This data clearly shows that Ab_2, HRP, and GOx covalently attached on the surface of the MBs through the formation of the amide bond between the –COOH groups of MB and the $–NH_2$ groups of Ab_2, HRP, and GOx.

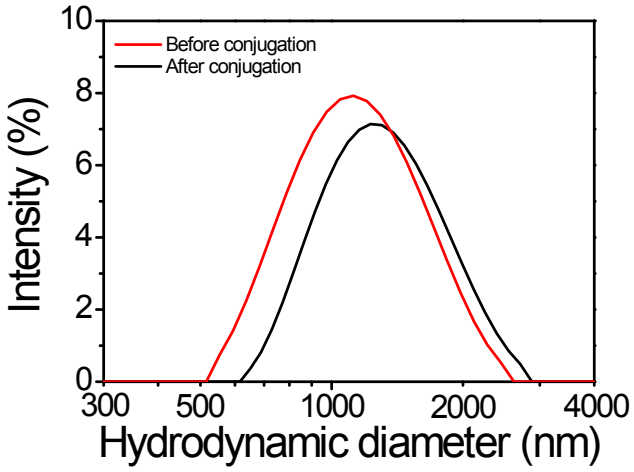

Fig. S1. The size distributions of the bare MB (red line) and the Ab_2/HRP/GOx-conjugated MB (black line) in aqueous media by DLS measurements.

6. Effect of blocking on the non-specific adsorption or non-specific binding

The effect of blocking the QCM immunosensor surface for minimizing the non-specific adsorption of the conjugates or non-specific binding of other molecules was studied. In the case of blocking, the QCM/MPA/PA/Ab_1 electrode was dipped into a 0.1% BSA solution for 2 h and then interacted with IL-6 protein. However, in the case of non-blocking, no BSA treatment was used. Figure S2 shows the frequency responses measured for 10 pg mL^{-1} IL-6 in serum (i) with or (ii) without blocking the immunosensor surface. In the case of without blocking, the frequency response was about 5 times higher than that of with blocking. The 5 times larger frequency response might be related to the non-specific binding of other molecules or the non-specific adsorption of the conjugates. These results clearly showed that the BSA treatment is necessary for minimizing the non-specific binding of other molecules or the non-specific adsorption of the conjugates.

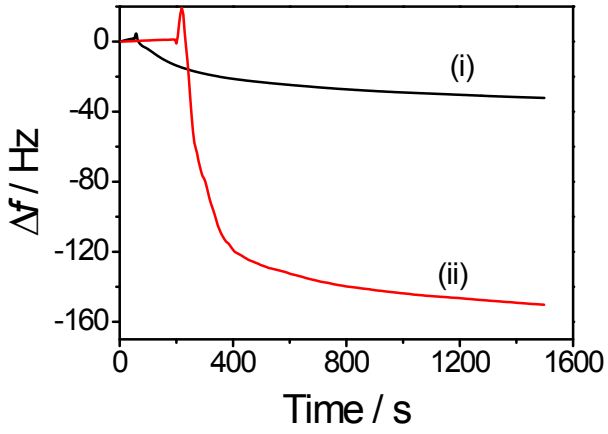

Fig. S2. The frequency responses obtained for 10 pg mL^{-1} IL-6 with (i) or without (ii) BSA blocking. .

7. Effect of magnetic beads (MB) in the signal amplification

The use of MB in the bioconjugate has a great effect on the signal amplification. Fig. S3 shows the frequency responses obtained by using the conjugate with or without MB. The frequency response for using MB was about 6 times higher than that of without using MB. This is due to the fact that MB has multifunctional sites for covalently attaching more amount of HRP and GOx, which enhanced the amount of precipitation formed. Thus, the frequency response was significantly higher for MB based bioconjugates.

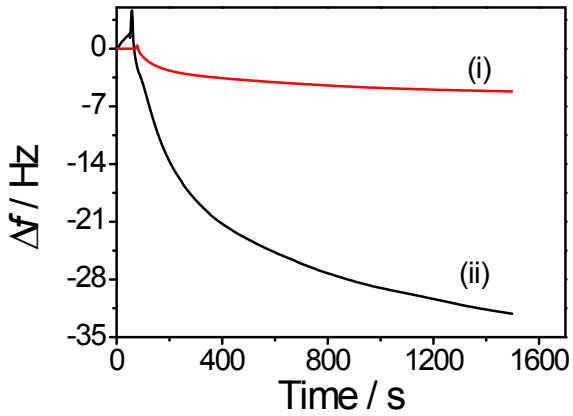

Fig. S3. The frequency responses obtained for 10 pg mL^{-1} IL-6 using the bioconjugate (i) without (Ab2/HRP/GOx) and (ii) with (Ab2/MB/HRP/GOx).

8. Selectivity

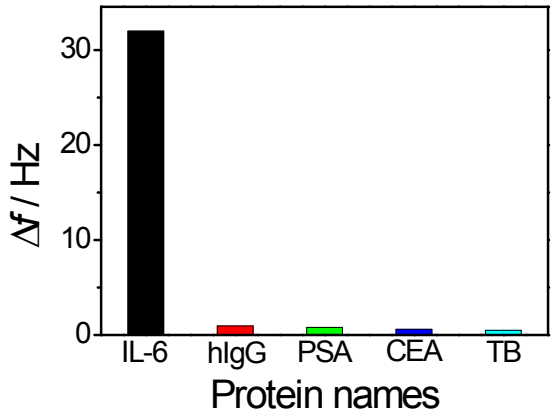

Fig. S4. The bar graph representation of the selectivity performance on QCM IL-6 immunosensor. The concentration of the IL-6 was 10 pg mL^{-1}.

9. Stability and regeneration

The stability of the proposed IL-6 immunosensor was checked by measuring the frequency responses of 10 pg/mL IL-6 for two months by regenerating the immunosensor probe. After each measurement, the immunosensor surface was regenerated by dipping it into a 0.2 M glycine-hydrochloric acid (Gly-HCl) solution (pH=2.8) for 5 min followed by washing with a PBS solution. When stored in dry condition at 4°C, the initial Δf responses did not change significantly for a period of six weeks. The Δf responses retained almost 90 % of its initial response indicating that the long time stability of the immunosensor was good.

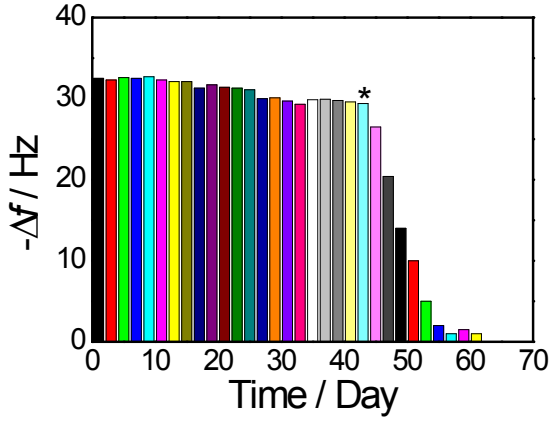

Fig. S5. The bar graph representation of the stability of the QCM IL-6 immunosensor. The concentration of the IL-6 was 10 pg mL^{-1}.