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A New Concept for Efficient Sensitivity Amplification of a QCM Based Immunosensor for TNF-α by Using Modified Magnetic Particles under Applied Magnetic Field

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This study introduces a new concept for a simple, efficient and cheap sensitivity amplification of a Quartz Crystal Microbalance (QCM) based immunosensor system for the detection of tumor necrosis factor-alpha (TNF-α, TNF) by using an in-built magnetic system. The frequency shift due to the applied magnetic field was successfully observed on magnetic particles labeled detection antibodies, *anti*-human TNF-α, which were bound to the immunologically captured TNF-α on the gold coated quartz crystals. In the present system, the magnitude of frequency shift depends on both the strength of magnetic field and the amount of target antigen applied. Significant signal amplification was observed when the additional built-in residual stress generated by the modified magnetic particles under the magnetic field applied. Used in conjunction with a sandwich type non-competitive immunoassay format, the lower detection limit was calculated to be 25 $ngmL^{-1}$ and showed good linearity up to TNF-α concentrations as high as 2.0 μgmL^{-1}. The sensitivity, most importantly, was improved up to 4.3 times compared with the same QCM system which was used only an antigen-antibody binding without additional magnetic amplification.

Key Words : Biosensor, Immunosensor, Quartz crystal microbalance, Magnetic particles, TNF-α

Introduction

Tumor necrosis factor-alpha (TNF, also known as TNF-α or cachectin) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction and plays important roles in the regulation of immune cells.[1,2] TNF-α is also identified as products of lymphocytes and macrophages that caused the lysis of cells, especially tumor cells.[3,4] The potent biological effects of TNF-α participate in human diseases and maybe harnessed to ameliorate certain illnesses.[5,6] During the last decades, pharmaceuticals to inhibit TNF-α have been developed which control previously recalcitrant inflammatory condition and TNF-α is being targeted for therapies against widespread human disease such as septic shock, cancer, AIDS, transplantation rejection, multiple sclerosis, diabetes, trauma, meningitis, ischemia-reperfusion injury, and adult respiratory distress syndrome.[7-9]

As regarding TNF-α very crucially, the importance of the quantitative and sensitive monitoring of TNF-α has been increased dramatically. Various detection methods have been employed for the monitoring of TNF-α such as ELISA, reverse transcription polymerase chain reaction (RT-PCR), flow cytometric assay, surface plasma resonance (SPR) and quartz crystal microbalance (QCM).[10-16] However, the high cost and slow turnaround times of conventional enzyme linked photometric or PCR based methods indicate a need for more sensitive but simpler analytical techniques. To meet this need, a sensor-based system which is simple to use, inexpensive, disposable and highly sensitive, is becoming increasingly important in TNF-α analysis.[17-19]

Among the sensor based systems reported previously, QCM based systems may have some technical advantages: the QCM measurement may detect the numbers, attachments and spreadings of the protein on the surface in real time and also has constant sensitivity to temperature fluctuation. In addition, it is possible to measure a protein concentration or mass change directly for signal transducing.[11] It is known that most desirable detection systems for protein analysis often require high sensitivity to distinguish nanogram change in mass. However, the conventional measurement systems including QCM, show insufficient sensitivity for the desired requirements.[17]

The specific binding of *anti*-human TNF-α antibody to TNF-α as primary or secondary antibody, is well established and well known and this specificity is widely used in commercial TNF-α immunoassay kits such as the AlphaLISA® assay Kit (PerkinElmer, Inc., Finland). Additionally, standard sandwich-type immunosensor systems for TNF-α were reported previously.[30,31] In the present system, we presents a new concept to improve the sensitivity to detect TNF-α by applying magnetic field to a QCM based sandwich type immunosensor system coupled with magnetic particle labelled antibody. In corporation of magmatic particles with the specific sandwich type TNF-α assay format allows higher QCM selectivity and additional signal amplification after binding with TNF-α-antibody immobilized on the sensor surface under the magnetic field. It is, therefore, suggested that the additional magnetic field system can improve the QCM sensor characteristics compared with QCM alone

system without the magnetic system.

Experimental

***Anti*-TNF-α Immobilization.** *Anti*-human TNF-α (Sigma Aldrich, USA) was used as capture and detection antibodies (Figure 2). For the capture, mercaptoundecanoic acid (MUA, Sigma Aldrich, USA) was used as a self-assembled monolayer (SAM) linker for the attachment of proteins onto the surface of gold coated quartz crystals. AT-cut 9 MHz quartz crystals with gold coating (5 mm in diameter, Princeton Applied Research, USA) formed uniformly on both sides were used as the QCM measurements. The quartz crystal was immersed into 10 mM MUA in 30% (v/v) ethanol for 16 hours in room temperature to form a self-assembled monolayer. After incubation, the quartz crystal was rinsed with ethanol followed by deionized water. For *anti*-human TNF-α antibody (Sigma Aldrich, USA) immobilization, the carboxylic acid-terminated SAMs were immersed into an aqueous solution of 0.4 $mgmL^{-1}$ *N*-hydroxysuccinimide (NHS) (Sigma Aldrich, USA) and 1.1 $mgmL^{-1}$ *N*-ethyl-*N*′-(dimethylaminopropyl) cabodiimide (EDC) (Sigma Aldrich, USA) for 30 min. After removal of the quartz crystal from the protein solution, the surface was exhaustively rinsed with deionized water. In order to immobilize antibody on the surface of the modified MUA layer, an aliquot of 2.0 $mgmL^{-1}$ TNF-α antibody solution prepared in 100 mM PBS (pH 7.4), was applied on the surface of the modified quartz crystals for 1 hour at room temperature. After antibody incubation, the quartz crystal was removed from the solution and was gently rinsed by PBS. Subsequently, the quartz crystal surface was blocked with 0.1% bovine serum albumin (BSA, Sigma Aldrich, USA) solution by immersing the quartz crystal for 30 minutes and was then rinsed thoroughly by PBS. All aqueous solutions in the present study were prepared in doubly distilled water, which was obtained from a Milli-Q water purifying system (USA). *Anti*-TNF-α was also used for the detection (Figure 2). The preparation of *anti*-TNF-α detection antibodies labeled with magnetic particles has been carried out by using commercial MPs (1.08 μm in diameter, Dynabeads® MyOne™ Tosylactivated, Invitrogen, Norway). The Dynabeads are uniform, superparamagnetic, polystyrene beads coated with a polyurethane layer. The hydroxy groups are activated by reaction with *p*-toluensulphonyl chloride. The resulting sulphonyl ester can subsequently react covalently with proteins or other ligands containing amino or sulfhydryl groups. 50 μg of *anti*-TNF-α and 1.0 mg Dynabeads were gently mixed in 5.0 mL of 0.5 M ammonium sulphate in 0.1 M sodium borate buffer (pH 9.5) for 24 hours at 25 °C with slow tilt rotation. After labeling, the MPs were washed and blocked by using a PBS (pH 7.4) containing 0.1% BSA, 0.05% Tween20 for 24 hours at 25 °C with slow tilt rotation. The labeled antibodies were then thoroughly washed with the PBS and kept in the PBS at 4 °C until use. All chemicals used were obtained from Sigma and used without further purification.

QCM Measurments. The resonant frequency of a quartz crystal was measured with a QCM analyzer (model 922, Princeton Applied Research, USA). An entire apparatus setup for QCM measurements were shown in Figure 1. The distance between a circular permanent magnet (Nd-Fe-B, 5000 G, 10 mm diameter) and the quartz crystal was manually controlled by using a homemade magnetic jig which is integrated with a conventional micrometer-head.

Figure 1. Apparatus setup for QCM measurements.

Applying Magnetic Field on QCM Measurement. The antibody modified quartz crystal was placed on a homemade QCM Teflon cell (Figure 1) to investigate the influence of the magnetic field to the antigen modified magnetic beads: tensile force or compressional force due to the position of magnet decrease (tensile force) or increase (compressional force). An aliquot of 30 μL of an operation buffer (100 mM PBS, pH 7.4) was placed into the cell. The magnetic field was applied when the frequency change converged and frequency change was measured under the magnetic field without protein reaction. To find out the influence of magnetic field on the antibody-antigen reaction, measurements were repeated with a series of the TNF-α with and without the detection antibodies. Both solutions were prepared in the operation buffer. A distance between the permanent magnet and the quartz crystals was changed by controlling the jig and frequency changes in QCM analyzer were measured under the various distance conditions.

Results and Discussion

Sensing Principal. A quartz crystal vibrates with a natural frequency at resonance and the resonant frequency can be given when the stress is applied.[23] Apparently, magnetic beads which bound to the QCM quartz crystal attract a stress, the tensile residual stress, when the magnetic field is applied. Eq. (1) explains the effect of the built-in residual stress to the resonant frequency change in QCM.

$$f_r = f_n\sqrt{1+1.2\frac{FL^2}{Ebh^3}} = f_n\sqrt{1+1.2\left(\frac{F}{bh}\right)\left(\frac{L^2}{Eh^2}\right)} = f_n\sqrt{1+1.2\sigma\frac{L^2}{Eh^2}} \quad (1)$$

Where f_n is a natural frequency, b, h and L are the charac-

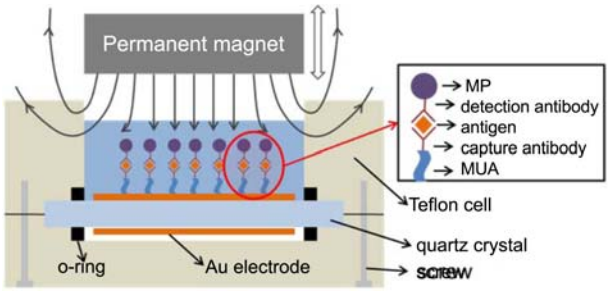

Figure 2. Schematic diagram of the sensing principle of QCM measurement for TNF-α under the magnetic field.

teristic width, height and length of system, respectively. F is an applied magnetic force and σ is the built-in residual stress, and E is the Young's modulus of system.[23] Previous mechanical studies[20-22] were demonstrated that the applied magnetic field could increase the resonant frequency of the quartz crystal. It is, however, note that there were no studies previously reported regarding the tensile residual stress as sensing chemistry for biosensor applications. Most combined studies of QCM coupled with magnetic beads were for selective collections or separations of biological materials such as proteins or cells.[24,25] A schematic diagram of the sensing principle of QCM measurement for TNF-α under the magnetic field is illustrated in Figure 2. As can be seen, significant signal amplification could be take place when the additional built-in residual stress incorporated with magnetic beads applied to the quartz crystal, compared with the results from conventional QCM based biosensors which uses only an antigen-antibody binding.[26,27]

Surface Morphology of QCM Electrode. The surface morphology changes of the quartz crystals have been investigated by using a scanning electron microscope (SEM, Model; HITACHI-S3500N, Hitachi corp., Japan) to evaluate the influences after the application of firstly, the capture antibodies, secondly, target antigen, and finally, the detection antibodies. For immune-binding, a quartz crystal immobilized with the capture antibody was incubated for 15 min with

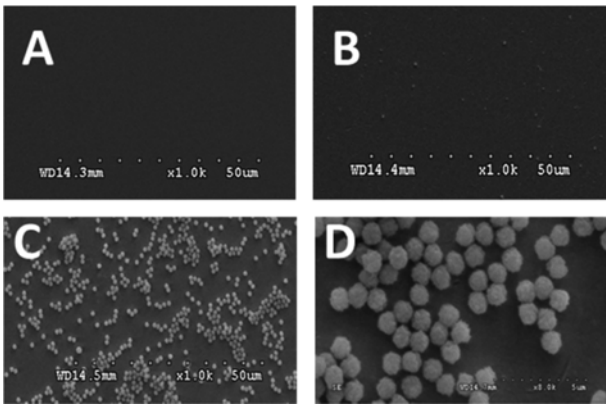

Figure 3. Scanning electron microscope images of only capture antibody modified (a), capture-antibody/TNF-α modified (b) and capture-antibody/TNF-α/detection-antibody modified (d and d) on gold coated quartz crystals.

40 μgmL^{-1} TNF-α. The crystal was then gently washed and incubated with 0.1% BSA in PBS for 15 min. Finally, the quartz crystal was removed from the solution and gently washed again with PBS and incubated with the capture antibody for 15 min. As can be seen, SEM images obtained from bare (image not shown) and the capture antibody modified quartz crystals without incubation with target antigen (Figure 3(a)) was similar to the image after the antigen incubation (Figure 3(b)). There were, however, significant differences in SEM images after the incubation of the capture antibodies (Figure 3(c) and 3(d)) compared with the images obtained from the results without the MPs. These clearly confirmed that the capture antibody immobilization, the immune binding with TNF-α and the MP labeled detection antibody application were successful.

Influence of Magnetic Field on QCM System. To evaluate the effect of the magnetic effect on a conventional QCM system, a series of measurements were carried out. An unmodified bare and a capture-antibody modified quartz crystal were prepared and were set up into the QCM cell and then 30 μL of PBS was applied in to the cell. When the measured frequency has been converged, a magnetic field was applied to the QCM system by using the in-built permanent magnet. As can be seen in Figure 4(a), the resonance frequency of the bare quartz crystals (Figure (b)) were not altered under the magnetic field which was repeatedly applied and removed with 50 seconds time intervals. Similar results were observed with the quartz crystals after the MUA modification. During the immune-binding between the immobilized capture antibody and 10 μgmL^{-1} TNF-α, as shown in upper curve in Figure 4(b), an apparent QCM frequency shift was observed. After TNF-α binding the solution was removed and the crystal was gently washed and incubated with 0.1% BSA in PBS for 15 min. After blocking by BSA, the quartz crystal was gently rinsed with PBS and incubated with 2 μgmL^{-1} non-labeled (i.e. without MPs) detection antibodies for 15 min. As it can be

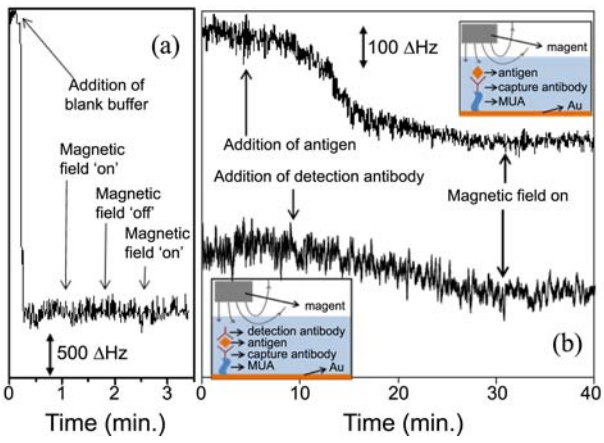

Figure 4. Influence of magnetic field on a QCM measurement without employing MPs. A: Influence of magnetic field on a bare QCM quartz crystal. B: Influence of magnetic field on the capture antibody modified QCM quartz crystal after the immune reaction with TNF-α.

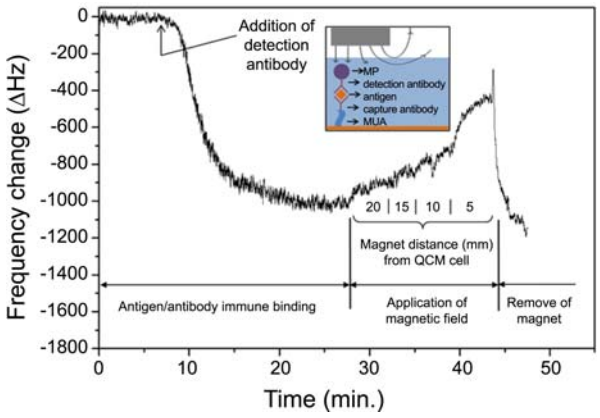

Figure 5. Influence of the strength of magnetic field on a QCM measurement coupled with a magnetic system.

expected, similar frequency shift was observed after the addition of the non-labeled detection antibodies (lower curve in Figure 4(b)). Consequently, these results indicated that the magnetic field applied did not show any detectable influence on the conventional QCM measurement.

Further experiments were carried out to investigate the influence of magnetic field on a QCM measurement after the application of the detection antibodies: quartz crystals after the application of the capture antibody and 10 μgmL^{-1} TNF-α were incubated in 30 μL of the prepared detection *anti*-body solution for 15 min. As shown in Figure 5, immediate resonance frequency decay was observed just after the addition of the detection antibody solution. The frequency decay was similar to the shift (Figure 4(b)) observed when the non-labeled detection antibody was added but the decay was more stiff and rapid. When the resonance frequency reached a steady state the permanent magnet applied to generate a magnetic field onto the QCM cell. The MPs bound to the detection antibodies were attracted by applied magnetic force generated from the in-built permanent magnet with 20 mm distance and bringing about a resonant frequency increase of about 140 Hz. The frequency increase was elevated while the magnet was closer to the quartz crystal from 20 mm to 5 mm. From these results, it could assume that the tensile force on the link between the MPs bound to detection antibodies and the surface of the quartz crystal reduced the applied mass onto the quartz crystals. Consequently, the removal of the magnetic field applied produced an immediate decrease in resonance frequency. Therefore, the QCM coupled with modified MPs can be applied as a simple and efficient QCM based biosensor system for the detection of proteins. It is also noted that, in the present system, the unbound detection antibodies were effectively removed from the surface of the quartz crystals by the applied magnetic field.

Signal Amplification by Magnetic System. As shown in Figure 5 and as stated in previous section, there was rapid and stiff frequency decay when the MPs labeled detection antibody applied. This could be caused by a simple physical mass addition of MPs from the detection antibodies. It is, therefore, apparent that the simple mass addition can be easily removed by the applied magnetic field and cannot generate any tensile force without proper immune bindings between the detection antibodies and TNF-α. As a result, the decreased frequency was rapidly shifted back almost its initial level. There were, however, interesting results were observed: the magnitude of frequency decay caused by the addition of the non-labeled detection antibody (lower curve in Figure 4(b)) is much lower than the magnitude of frequency increase observed in Figure 5 which was based on the tensile force generated by the applied magnetic system. In the present system, the resonance frequency of the crystal shows an effective improvement in the sensitivity when the MPs were applied. In order to investigate the amplification of QCM sensitivity by the magnetic system, a series of QCM measurements were repeated with TNF-α samples prepared in PBS over the concentration range from 0.1 to 2.0 μgmL^{-1}. In these experiments, the distance between the magnet and the modified quartz crystals was set as 5 mm and 10 mm for the signal amplification by the magnetic system. As can be seen in Figure 6, the sensitivity of 302.6 Hz μg^{-1}mL was obtained when the distance between the magnet and each quartz crystal was set at 5 mm (▲). This value was approximately 4.3 times greater than the sensitivity, 70.3 Hz μg^{-1}mL, obtained employing the non-labeled MPs (■). However, the raw values of frequency change recorded, as shown in Figure 4, 5 and 6, show relatively higher noise compare with other QCM based biosensor

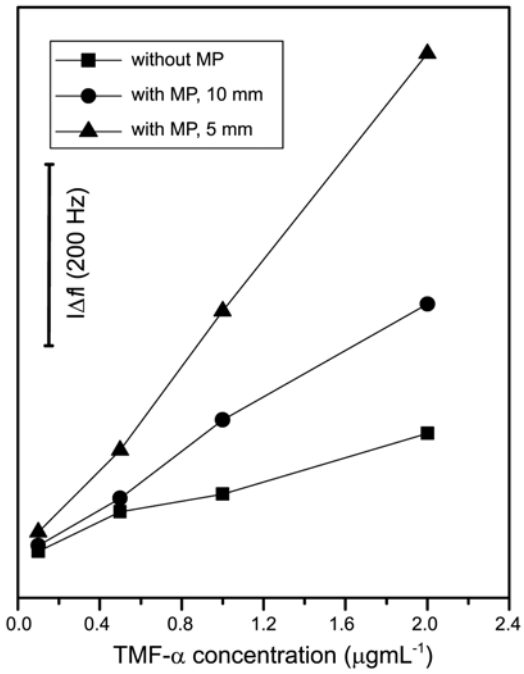

Figure 6. QCM signal amplification by magnetic system. resonance frequency change obtained from TNF-α bound to immobilized capture and non-labeled detection antibodies without magnetic system (■), from TNF-α bound to capture and MPs labeled detection antibodies under the magnetic field applied with the distance between the magnet and the quartz crystals were set as 5 mm (▲) and 10 mm (●).

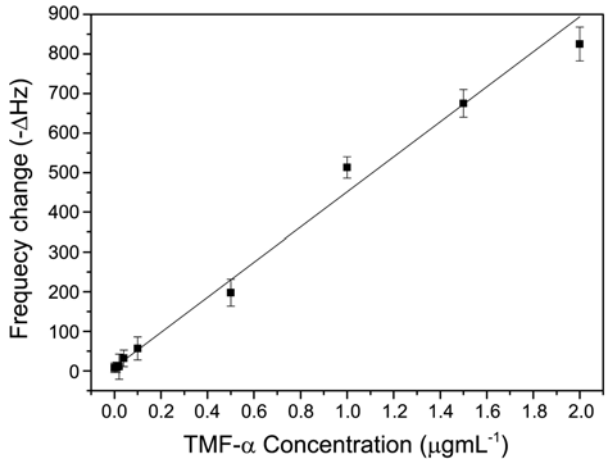

Figure 7. Calibration curve for TNF-α obtained by using QCM based biosensor with in-built magnetic system. All experimental condition was as same as Figure 6(b) (▲).

systems.[26,28] To enhance the sensor performance, all raw values from QCM measurements in this study were statistically processed and a mean value from each measurement were calculated by using a commercial PC software based on the Savitzky-Golay smoothing method.[29]

Sensor Calibration. A calibration curve for TNF-α was constructed using the QCM with in-built magnetic system under the condition described for Figure 6 (5 mm distance). Aliquots of TNF-α samples ranged from 10 ngmL^{-1} to 2.0 μgmL^{-1} were prepared in PBS. The initial frequency decay caused by immune-binding between the capture antibodies were observed and reached a steady-state in about 10-15 min. The sensors were then rinsed and blocked by BSA as described in the previous sections. 30 μL aliquots of the detection antibodies applied and allowed for 15 min for another immune binding. Finally, the sensors were exposed to the magnetic field and subsequent frequency change was monitored for 10 min.

The calibration curve constructed was shown in Figure 7 and each point represents the mean value of four measurements and error bars represent one standard deviation. In the curve obtained using the magnetic system in the present study, the equation of the regression line was calculated to be y (−ΔHz) = 0.44 x (ngmL^{-1}) + 8.9 (n = 4, r^2 = 0.99). Responses to TNF-α could be detected at TNF-α concentration as low as 25 ngmL^{-1} and showed good linearity ranged from 40 ngmL^{-1} to TNF-α concentration as high as 2.0 μgmL^{-1}. The lower detection limit of the system was taken as three times of the standard deviation of the frequency change due to the addition of a blank solution. The reproducibility of the system was investigated using TNF-α concentrations between 0.5 μgmL^{-1} and 1.5 μM. The QCM sensor performance demonstrated a maximum CV (coefficient variation) of 9.1% over the whole concentrations range.

Conclusion

This study has demonstrated that the magnetic particles coupled with QCM can be used for the enhancement of QCM sensitivity in concert with protein separations as the common uses of magnetic particles in QCM studies.[24,25] In the present study it was found that the magnitude of frequency shift due to the additional magnetic system was directly dependent on both the strength of magnetic field and the concentration of the target antigen, TNF-α, which was immunologically bound to the immobilized antibody. The detection limit of the system was found to be 25 ngmL^{-1} and the sensitivity of this QCM based biosensor system with MPs was improved 4.3 times compared with the results without the magnetic system. Although the detection limit obtained rather higher than other results from QCM based immunosensors (sub-ngmL^{-1}) without magnetic system,[26,27] in practice, the magnetic system in this study could be employed as a simple, effective and cheap 'add-in' for lower grade commercial QCM based immunosensor systems to enhance the sensitivity. The selectivity of the present system can be easily controlled strictly by the additive well-known selectivity of the *anti*-TNF-α antibodies as described in the Introduction Section. Additionally, ionic interferences can be avoided by using the QCM based biosensor system with in-built magnetic amplification as the underlying transducer during the fabrication of such an immunosensor instead of amperometric or potentiometric biosensors. Consequently, the QCM based biosensor system could be used for the monitoring of TNF-α in biological or clinical analysis.

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References

1. Beutler, B.; Cerami, A. *Biochemistry-Us* **1988**, *27*, 7575.
2. Vilcek, J.; Lee, T. H. *J. Biol. Chem.* **1991**, *266*, 7313.
3. Kriegler, M.; Perez, C.; Defay, K.; Albert, I.; Lu, S. D. *Cell* **1988**, *53*, 45.
4. Granger, G. A.; Shacks, S. J.; Williams, T. W.; Kolb, W. P. *Nature* **1969**, *221*, 1155.
5. Mohan, N.; Edwards, E. T.; Cupps, T. R.; Oliverio, P. J.; Siegel, J. N. *Arthritis Rheum* **2000**, *43*, S228.
6. Chan, F. K. M.; Siegel, R. M.; Lenardo, M. J. *Immunity* **2000**, *13*, 419.
7. Siegel, R. M.; Chan, F. K. M.; Chun, H. J.; Lenardo, M. J. *Nat. Immunol.* **2000**, *1*, 469.
8. Maini, R. N.; Taylor, P. C. *Annu. Rev. Med.* **2000**, *51*, 207.
9. Pasparakis, M.; Alexopoulou, L.; Episkopou, V.; Kollias, G. *J. Exp. Med.* **1996**, *184*, 1397.
10. Michelson, A. D. *Blood* **1996**, *87*, 4925.
11. Spangler, B. D.; Wilkinson, E. A.; Murphy, J. T.; Tyler, B. J. *Anal. Chim. Acta* **2001**, *444*, 149.
12. Baker, K. N.; Rendall, M. H.; Patel, A.; Boyd, P.; Hoare, M.;

Freedman, R. B.; James, D. C. *Trends Biotechnol.* **2002**, *20*, 149.
13. Bleve, G.; Rizzotti, L.; Dellaglio, F.; Torriani, S. *Appl. Environ. Microb.* **2003**, *69*, 4116.
14. Ioachim, H. L.; Medeiros, L. J., 4th ed.; Lippincott Williams and Wilkins: Philadelphia, 2008.
15. Battaglia, T. M.; Masson, J. F.; Sierks, M. R.; Beaudoin, S. P.; Rogers, J.; Foster, K. N.; Holloway, G. A.; Booksh, K. S. *Anal. Chem.* **2005**, *77*, 7016.
16. Kurita, R.; Arai, K.; Nakamoto, K.; Kato, D.; Niwa, O. *Anal. Chem.* **2010**, *82*, 1692.
17. Choi, C. J.; Belobraydich, A. R.; Chan, L. L.; Mathias, P. C.; Cunningham, B. T. *Anal. Biochem.* **2010**, *405*, 1.
18. Kramer, P. M.; Kess, M.; Kremmer, E.; Schulte-Hostede, S. *Analyst* **2011**, *136*, 692.
19. La Belle, J. T.; Demirok, U. K.; Patel, D. R.; Cook, C. B. *Analyst* **2011**, *136*, 1496.
20. Nunalee, F. N.; Shull, K. R. *Langmuir* **2004**, *20*, 7083.
21. Tilmans, H. A. C.; Legtenberg, R. *Sensor. Actuat. a-Phys.* **1994**, *45*, 67.
22. Pandey, A. K.; Venkatesh, K. P.; Pratap, R. *Sadhana-Acad. P. Eng. S.* **2009**, *34*, 651.
23. Pilkey, W. D. *Formulas for Stress, Strain, and Structural Matrices*, 2nd ed.; John Wiley & Sons: New York, 2005.
24. Pan, Y. L.; Guo, M. L.; Nie, Z.; Huang, Y.; Pan, C. F.; Zeng, K.; Zhang, Y.; Yao, S. Z. *Biosens. Bioelectron.* **2010**, *25*, 1609.
25. Laurent, S.; Forge, D.; Port, M.; Roch, A.; Robic, C.; Elst, L. V.; Muller, R. N. *Chem. Rev.* **2008**, *108*, 2064.
26. Vaughan, R. D.; O'Sullivan, C. K.; Guilbault, G. G. *Enzyme Microb. Tech.* **2001**, *29*, 635.
27. Michalzik, M.; Wendler, J.; Rabe, J.; Buttgenbach, S.; Bilitewski, U. B. *Sensor Actuat. B-Chem.* **2005**, *105*, 508.
28. Liu, Y.; Zhang, W.; Yu, X.; Zhang, H. W.; Zhao, R.; Shangguan, D.; Li, Y.; Shen, B. F.; Liu, G. Q. *Sensor Actuat. B-Chem.* **2004**, *99*, 416.
29. Savitzky, A.; Golay, M.J.E. *Anal. Chem.* **1964**, *36*, 1627.
30. Delamarche, E.; Cesaro-Tadic, S.; Dernick, G.; Juncker, D.; Buurman, G.; Kropshofer, H.; Michel, B.; Fattinger, C. *Lab. Chip.* **2004**, *4*, 563-569.
31. Wild, D. *The Immunoassay Handbook*, 2nd ed.; Nature Publishing Group: New York, 2001.