Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytica Chimica Acta 481 (2003) 191–198
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

# Piezoelectric immunosensor based on magnetic nanoparticles with simple immobilization procedures

Jishan Li, Xiaoxiao He, Zhaoyang Wu, Kemin Wang, Guoli Shen*, Ruqin Yu

*State key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China*

Received 25 July 2002; received in revised form 10 January 2003; accepted 21 January 2003

**Abstract**

A novel method for immobilizing antibodies (antigens) based on magnetic nanoparticles has been proposed for piezoelectric immunoassay. The goat-anti-IgG antibody (IgGAb) as the model analyte was first covalently immobilized to magnetic nanoparticles, which were surface modified with amino-groups. The magnetic bio-nanoparticles (MBN-s) formed were attached to the surfaces of quartz crystal with the help of a permanent magnet. The detection of immunoglobulin G (IgG) was performed with the sensor prepared. The process of immobilization and immunoreaction was monitored by frequency recording. From the SEM images of the sensor surface before and after immobilization of MBN, one can see that the MBN was homogeneously adsorbed on sensor surface. The piezoelectric immunosensor can determine IgG in the range of 0.6–34.9 $\mu g\,ml^{-1}$ with a detection limit of 0.36 $\mu g\,ml^{-1}$. The MBN and immunocomplex layer can easily be removed simply by taking away the magnetic field, making the piezoelectric sensor easy to be regenerated.
© 2003 Elsevier Science B.V. All rights reserved.

*Keywords:* Magnetic bio-nanoparticle; Piezoelectric immunosensor; Protein immobilization

## 1. Introduction

The immobilization of substrate materials for immunosensors has been the subject of recent investigations [1–5]. The immobilization procedures of the antibodies (antigens) on the surfaces of base transducers play an important role in the construction of the immunosensors. A general immobilization approach has been the precoating of the base transducers with polymer films capable of forming hydrophobic and/or covalent bonds with antibodies. Searching for simple immobilization methods on the surface of base transducers is of practical interest. Magnetic nanoparticles as special biomolecule immobilizing carriers offer a promising alternative to the conventional coating methodology for immunosensors. Due to its attractive properties, magnetic nanoparticles have been used in radio immunology [6,7] and cell separation processes [8,9]. Some recent reports [10–12] demonstrated the successful applications of magnetic nanoparticles in the immobilization of biomolecule. In this paper, an immobilization method for antibodies (antigens) based on magnetic nanoparticles for piezoelectric immunosensor will be reported.

For magnetic nanoparticles with the amine groups immobilized on the surface, the later can provide plenty anchor sites for covalently bonding to glutaraldehyde. Via glutaraldyhyde the antibodies/antigens were attached onto the surface of core/shell magnetic nanoparticles, forming magnetic bio-nanoparticles (MBN-s). Then the MBN-s was immobilized on the

* Corresponding author.
*E-mail address:* shenguo@cs.hn.cn (G. Shen).

0003-2670/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0003-2670(03)00089-8

electrode surfaces of QCM under the magnetic field, and the detection of antigen/antibody was reached. Finally, the MBN-s carrying immunocomplex layers can be rinsed out, via controlling the magnetic field. Therefore, the piezoelectric sensor can be regenerated repeatedly. This regeneration was of practical significance for immunoassay, especially for those with expensive base transducers.

To investigate its feasibility for immunosensing application, the IgGAb was used as the model system immobilized with the aforementioned immobilization procedure. The piezoelectric immnnosensor prepared has been successfully used for the detection of IgG. Comparing with the conventional methods of immobilization, the procedure with the magnetic nanoparticle adsorption immobilization shows advantages of simplicity, low cost and the possibility of repeated regeneration.

## 2. Experimental

### 2.1. Apparatus

Nanoparticle bioconjugate was prepared with a model LRH-250Z incubator purchased from Guangdong Medical Equipment (Guangzhou). Quartz crystal resonators purchased from Beijing Chenxing Radio Equipment (Beijing) consisted of 9 MHz, AT-cut quartz wafer with vacuum-deposited silver electrodes with a diameter of 6 mm on both sides. A home-made transistor–transistor logic integrated circuit (TTL-IC) was used to drive the quartz crystal at its resonant frequency. The resonant frequency was monitored with a frequency counter (Model CN3165 Wellstar).

### 2.2. Materials

Goat-anti-human IgG antibody (IgGAb, affinity purification) and normal human reference serum (NHRS, containing 10 mg $ml^{-1}$ of immunoglobulin G (IgG)) were obtained from Sino-Americal Biotechnology Company (Shanghai). Phosphate buffer solution (PBS, pH = 7.05 ) was prepared using 1/15 mol $l^{-1}$ of $KH_2PO_4$ and 1/15 mol $l^{-1}$ of $Na_2HPO_4$. Other reagents were of analytical purity, and doubly distilled water was used throughout all experiments.

### 2.3. Fabrication of the piezoelectric sensor

The configuration of the sensor is illustrated in Fig. 1. Immersing only one side of piezoelectric sensor into solution is essential for stable oscillation in liquid. At the same time, inner removable ferrite permanent magnet (diameter 8 mm, height 12 mm, producing an inhomogeneous magnetic field, 0.5 T at the surface) provides controllable magnetic field for the immobilization of MBN on the surface of the transducer.

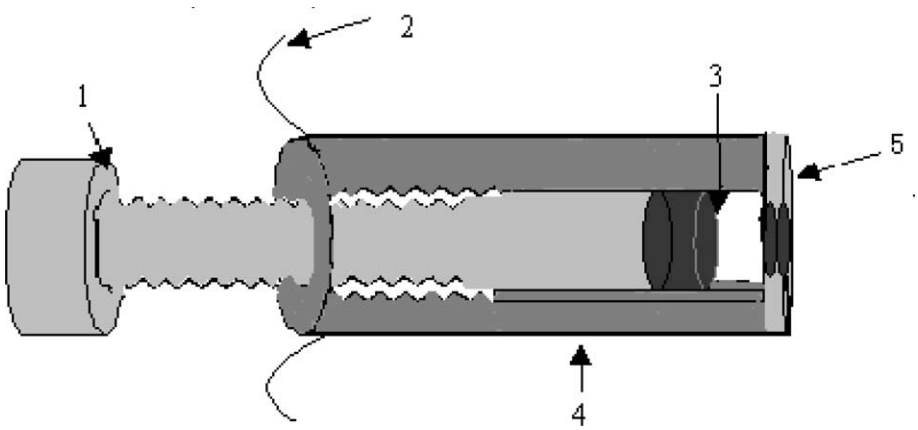


Fig. 1. Schematic of piezoelectric quartz crystal biosensors: (1) PVC nut; (2) electric wire; (3) magnet; (4) PVC tube; (5) piezoelectric quartz crystal.

### 2.4. Preparation of core/shell magnetic nanoparticles surface modified with amine groups

The magnetic nanoparticles coated with silica (40 nm) were prepared by the hydrolysis of tetraethoxysilane (TEOS) in water-in-oil microemulsion with the initiation of ammonia as previously reported [13]. Briefly, the procedure includes the synthesis of suspension of ferrofluid by precipitation from the chloride mixture (ferrous sulphate and ferric chloride) with the ammonia and the core/shell silica coating of the particles formed using water-in-oil microemulsion technique.

#### 2.4.1. Synthesis of the magnetite core in water

A 10 ml portion of 0.1 mol $l^{-1}$ ferric chloride solution in water was mixed with 2.5 ml of solution of 2 mol $l^{-1}$ ferrous sulphate in 2 mol $l^{-1}$ HCl. The chloride mixture was added quickly to 125 ml of 0.7 mol $l^{-1}$ ammonia in a vessel under vigorous stirring (non-magnetic). After 30 min of stirring, the precipitate was isolated by a columnar ferrite magnet (diameter 22.5 mm, height 12 mm), which produces an inhomogeneous magnetic field (0.5 T at the surface). Then the precipitate was diluted with 20 ml 1.0 mol $l^{-1}$ tetramethylammonium hydroxide and double distilled water to a total volume of 100 ml. The dispersion was filtered through a glass filter with 40–80 μm pore to remove magnetite clusters. Then the ferrofluid was titrated with 0.5 mol $l^{-1}$ HCl up to pH = 10.

#### 2.4.2. Silica coating on the magnetite cores

The silica coating on the magnetite core was prepared with the water-in-oil microemulsion technique. The (W/O) microemulsion was first prepared by mixing TritonX-100, *n*-hexanol and cyclohexane (1:1:4 (v/v)). The ferrofluid suspension was then added into the above solution as the aqueous phase and the mixture was stirred for an hour. On the presence of tetraethoxysilane, a polymerization reaction was proceeded by adding ammonia (28–30% W). The stirring was continued for 24 h. After the reaction was completed, the particles were isolated by the columnar ferrite magnet. TEM images of: (a) magnetic

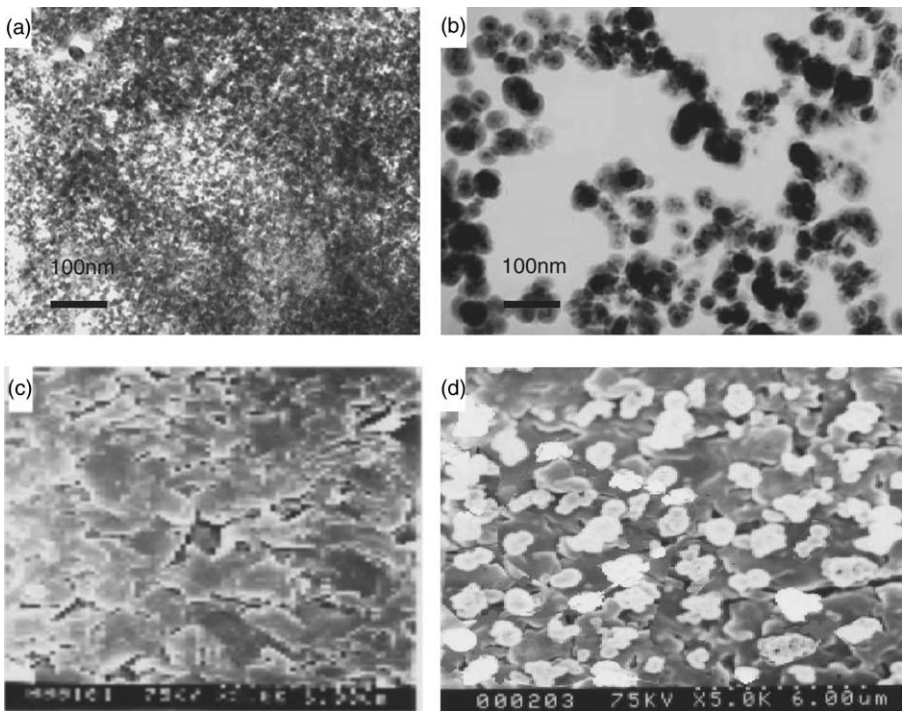


Fig. 2. TEM images of: (a) magnetic core (80000×) and (b) silica coated magnetic nanoparticles (80000×) were acquired from a Hitachi TEM 800; SEM images of sensor surface before (c) and after (d) immobilization of MBN were acquired from a Hitachi SEM 8010.

core and (b) silica coated magnetic nanoparticles are shown in Fig. 2. The core/shell silica magnetic nanoparticles was modified with *N*-(β-amimoethyl)-γ-aminopropyltyiethoxysilane according to [14].

### 2.5. Formation of the bioconjugate and immobilization of MBN

The conjugation procedure of MBN is shown in Fig. 3a. Magnetic nanoparticle bioconjugate was formed by using glutaraldehyde as the cross-linking reagent. Under the agitation, 100 mg of amine-modified magnetic nanoparricles was added to 6 ml of glutaraldehyde (2.5 wt.%). After reaction at room temperature for 11 h, the mixture was washed with PBS three times. The separation of the treated magnetic nanoparticles from the washing solutions was accomplished with the help of a columnar ferrite magnet (diameter 22.5 mm, height 12 mm), producing an inhomogeneous magnetic field (0.5 T at the surface). After adding 6 ml of PBS, 2 mg of IgGAb was added, and the mixture was agitated for 8 h [15] at 4 °C. Excess protein (not conjugated to particles) was removed by washing with PBS and stored in refrigerator at 4 °C.

The permanent magnet was moved inside the PVC tube by turning the PVC nut to put the magnet close to but not in touch with the inside surface of the quartz chip. Under a stable magnetic field formed and an upside down position of the sensor or with outer side of the quartz chip turned to the top, 15 μl of MBN in PBS was added onto the crystal surface. After 60 min, the surface was washed with PBS. The MBN was firmly immobilized onto the quartz crystal electrode surface and ready for bioassay.

### 2.6. Measurement procedure

The MBN-coated quartz crystal was inserted in 5 ml of PBS containing 60 μg ml$^{-1}$ BSA. After equilibrium for about 30 min, an appropriate amount of NHRS was

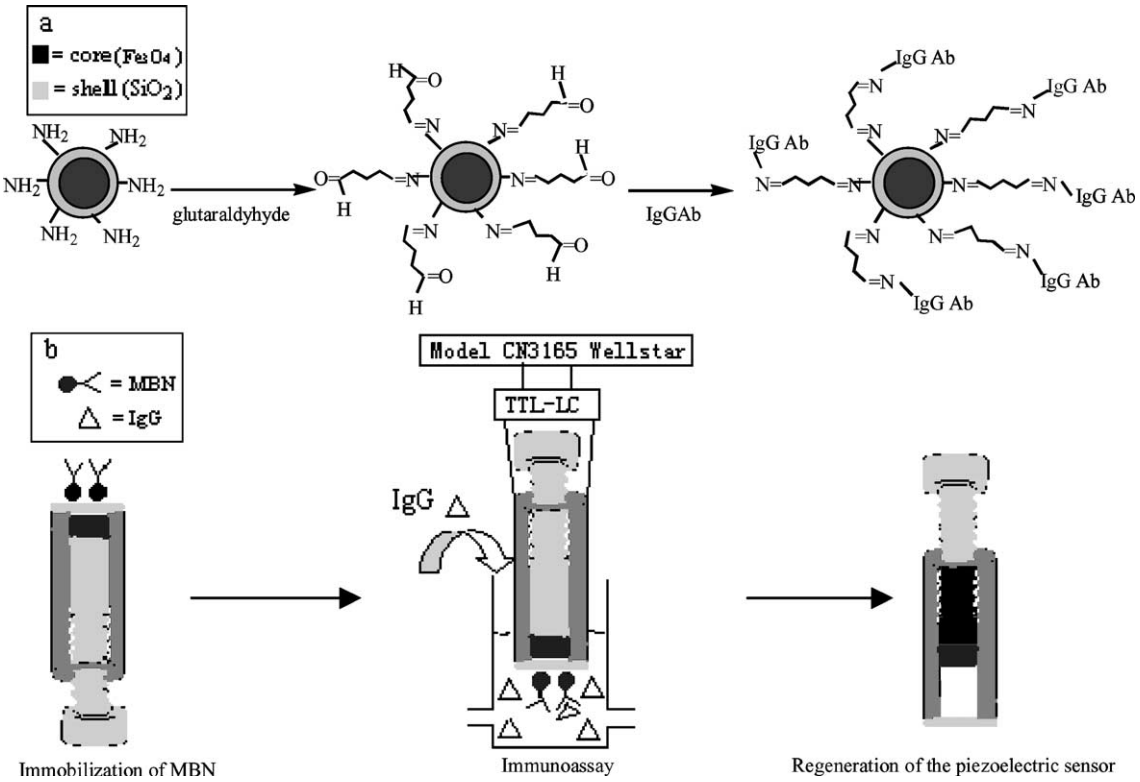


Fig. 3. Schematic diagram for preparation of magnetic bio-nanoparticle (a) and procedure for the immunoassay (b).

rapidly introduced into the flow-type detector cell. The resonance frequency of crystal was recorded as time went on and 20 min was selected as the immunoreaction time. The procedure of immunoassay is shown in Fig. 3b.

### 2.7. Estimation of the MBN loading

When necessary, the MBN loading is estimated by recording the resonance frequency of the crystal before and after coating. The mass of loaded MBN is calculated from the air frequency shift ($\Delta F_{air}$).

## 3. Result and discussions

### 3.1. Immobilization of MBN

$SiO_2$-coated $Fe_3O_4$ magnetic nanoparticles have high stability and super-paramagnetism, which can be immobilized onto solid surface under magnetic field. The immobilization of protein on the surface of piezoelectric quartz crystal can be realized via the core/shell structural magnetic nanoparticles. Fig. 4 shows the frequency shift of sensors with different immobilization time of MBN in 19.96 μg ml$^{-1}$ of IgG. The immobilization time of MBN on the sensor surface under the magnetic field was varied from 10 to 70 min with an interval of 10 min. Fig. 4, when the immobilization time of MBN was over 30 min, the frequency shift of quartz crystal tended to stabilize. It indicates that the adsorption immobilization process has essentially reached equilibrium. In order to obtain a stable and renewable surface of sensor, 60 min was used as the immobilization time of the MBN on the sensor surface.

### 3.2. The role of magnetic field existing

Fig. 5 shows the time response of two MBN sensors with (a) and without (b) permanent magnet inserted. The experiment was carried out as decried in Sections 2.5 and 2.6 and the frequency recording started just after 20 μl of NHRS was introduced into the detector cell. Without the permanent magnet inserted, a frequency shift of ca 45 Hz was recorded as compared to a 200 Hz shift for the case of magnetic field existing. These results clearly show the essential role of presence of the magnet in the immobilization of MBN. The aforementioned immobilizing procedure of MBN could form a usable immunosensing surface. Without the magnetic field, the MBN could be rinsed away

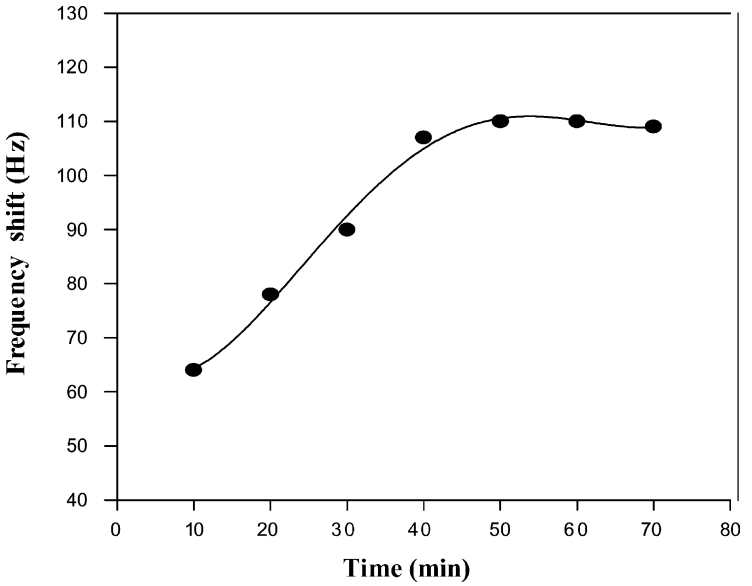


Fig. 4. The frequency shifts of sensors with different immobilization time of MBN on the sensor surface under the magnetic field in 19.96 μg ml$^{-1}$ of IgG.

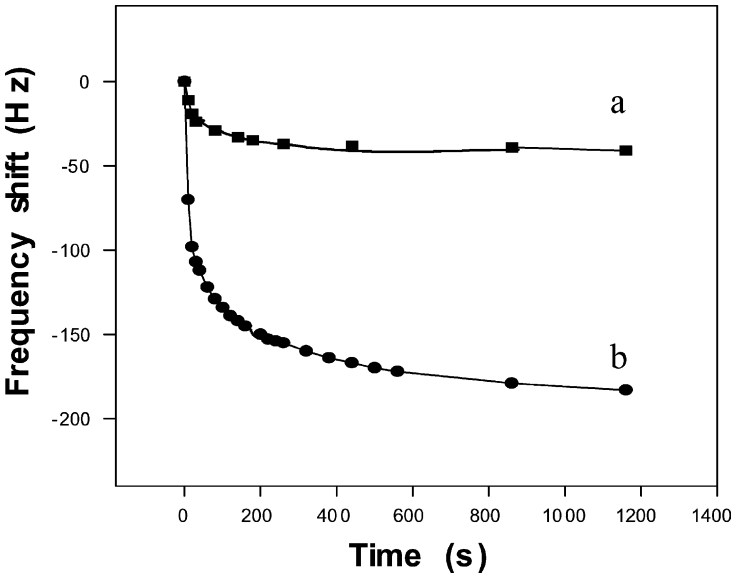


Fig. 5. Dependence of the frequency shift ($\Delta F$) on time: (a) with the magnetic field; (b) without. The sensors were prepared by adding 15 μl of MBN in PBS to the surface for 60 min. The frequency was recorded started after introduction of 20 μl of NHRS into the cell.

during the step of washing with PBS. Moreover, one can see that the resonance frequency of the quartz crystal tended to stable after 20 min, so 20 min was selected as the immunoreaction time in this experiment.

### 3.3. Estimation of the MBN loading

From Fig. 4 one notices that 1 h time is sufficient for the process of immobilization of MBN onto sensor surface under the magnetic field. In air the frequency change before and after the MBN immobilization step is $2427 \pm 200$ Hz as recorded by five parallel experiments with 15 μl of MBN in PBS. According to the Sauerbrey's equation [16], the mass change $\Delta m$ ($\mathrm{g\,cm^{-2}}$) on the surface of crystal can be estimated from the frequency shift ($\Delta F$)

$$\Delta m = \frac{-\Delta F}{2.26 \times 10^{-6} F_{\mathrm{q}}^2} \quad (1)$$

where $F_{\mathrm{q}} = 8996612$ Hz is the fundamental resonance frequency, $\Delta F$ is the measured frequency shift in Hz. So the mass change on the surface of crystal is estimated to be ca. $13.267\ \mu\mathrm{g\,cm^{-2}}$. SEM images of the sensor surface before (2c) and after (2d) immobilization of MBN are shown in Fig. 2. Although one can see from Fig. 2d that MBN was lightly aggregated on the sensor surface, the MBN is homogeneously adsorbed on sensor surface.

### 3.4. Determination of IgG by the immunosensor

To decrease the effect of non-specific adsorption of protein, BSA ($10\ \mathrm{mg\,ml^{-1}}$) was added into the detector cell. Table 1 shows the frequency shift of the piezoelectric immunosensor when the concentration

Table 1
The frequency shift of the piezoelectric immunosensor in the different concentration of BSA solution

| BSA (μg/ml) | $\Delta F$ (Hz) |
|---|---|
| 9.99 | 10 |
| 19.96 | 11 |
| 29.91 | 13 |
| 39.84 | 15 |
| 49.75 | 18 |
| 59.64 | 18 ± 2 |
| 69.51 | 18 |
| 79.27 | 18 |
| 89.20 | 17 |
| 99.00 | 18 |

The sensor was prepared as described in Section 2.5.

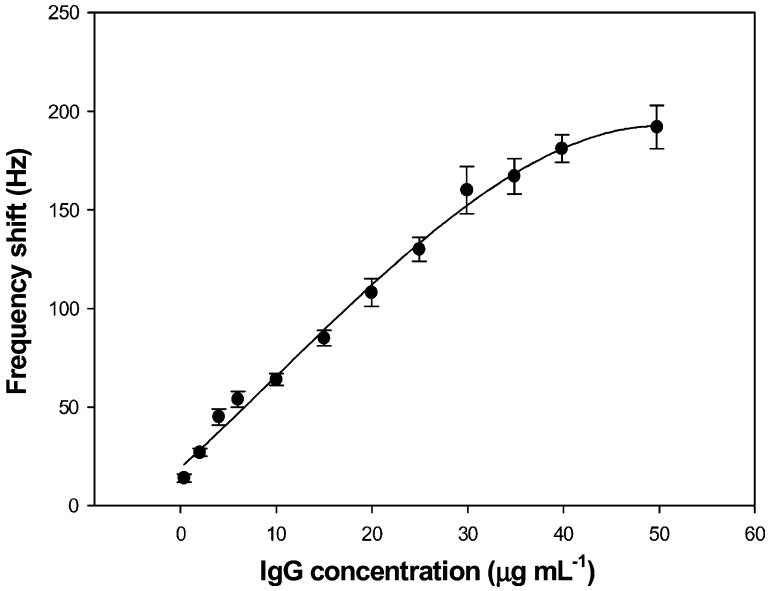


Fig. 6. The curve of frequency response of the sensor versus the concentration of IgG. The sensor was prepared as described in Section 2.5 and the frequency measurement was carried out as described in Section 2.6.

of BSA changed. The frequency response tended to stabilize when the concentration of BSA exceeded 59.64 μg ml$^{-1}$. It seems that the adsorbed layer of BSA has covered the non-specific adsorption sites of immunosensor surface. It is recommended to add 30 μl of BSA to suppress the non-specific adsorption of possibly co-existing protein species in analytical samples.

To obtain the calibration curve, the frequency response of the immunosensors immobilized with MBN under magnetic field to IgG solutions of different concentrations are measured (Fig. 6). The results show that the frequency change ($Y$) and the IgG concentration ($X$) possess a linear relationship in the concentration range of 0.6–34.9 μg ml$^{-1}$. The regression equation is $Y = 4.35X + 22.44$ with a correlation coefficient of 0.9978. According to the above equation, the limit of detection can be estimated as 0.36 μg ml$^{-1}$ from the three times of standard deviation ($\sigma$) corresponding to the blank sample measurement. It indicates that the immunosensor can be used for the determination of IgG in a relatively wide range of concentration. The relative standard deviations of the determination estimated from five repeated measurements for 3.99, 19.96 and 29.91 μg ml$^{-1}$ of IgG were 10.2, 7.0 and 7.3%, respectively.

### 3.5. Regeneration of the sensor

After each immunoassay, a regenerating procedure was performed by turning away the magnet and rinsing away the MBN and immunocomplex layer with water for 10 min under the absence of magnetic field. The sensor was regenerated and can be repeatedly used.

## 4. Conclusion

A novel method for the immobilization of antibodies on the surface of piezoelectric sensor based on the core/shell magnetic nanoparticles surface modified with amine groups is described in this paper. Taking the advantage of the modification of amine groups on the surface of nanoparticles and the super-paramagnetism of these particles, the covalent immobilization of the antibodies on magnetic nanoparticles and the attachment of the magnetic nanoparticles with the help of a permanent magnet on the sensor surface have been realized. The proposed immunosensor shows improved performance in terms of the simplicity of preparation and possibility of regeneration.

## Acknowledgements

This work was supported by the NNSF of China (Nos. 29975006, 20075006, 20205004), The Foundation of Ministry of Education for Doctor (No. 20010532008).

## References

[1] D.S. Hage, Anal. Chem. 67 (1995) 455R.
[2] Z.H. Lin, G.L. Shen, Q. Miao, R.Q. Yu, Anal. Chim. Acta 325 (1996) 87.
[3] R. Nakamura, H. Muguruma, K. Ikebukuro, S. Sasaki, R. Nagata, I. Karube, H. Pedersen, Anal. Chem. 69 (1997) 4649.
[4] F. Caruso, K. Niikura, D.N. Furlong, Y. Okahata, Langmuir 13 (1997) 3427.
[5] Z.Y. Wu, Y.H. Yan, G.L. Shen, R.Q. Yu, Anal. Chim. Acta 412 (2000) 29.
[6] R.F. Borch, M.D. Bernstein, H.D. Durst, J. Am. Chem. Soc. 93 (1971) 28970.
[7] P.M. Dey, Eur. J. Biochem. 385 (1984) 140.
[8] B.A. Schuarty, G.R. Gray, Arch. Biochem. Biophys. 181 (1977) 542.
[9] S.V. Sonti, A. Bose, J. Colloid. Interface. Sci. 170 (1995) 575.
[10] T. Tanaka, T. Matsunaga, Anal. Chem. 72 (2000) 3518.
[11] K.M. Wang, X.X. He, Res. Commun. Med. 31 (4) (2002) 33.
[12] C.R. Martin, D.T. Mitchell, Anal. Chem. News Features 70 (1998) 322A.
[13] X.X. He, K.M. Wang, W.H. Tan, D. Xiao, X.H. Yang, J. Li, Proc. SPIE 4414 (2001) 394.
[14] G. Zhang, Y. Zhou, J. Yuan, S. Ren, Anal. Lett. 32 (1999) 2725.
[15] Z.H. Jiang, J.H. Zhang, in: Immobilizing Techniques and Application of Biomoleculer, Chemistry Industry Press, Beijing, 1998, p. 93.
[16] G. Sauerbrey, Z. Phys. 155 (1959) 206.