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Molecularly imprinted hydroxyapatite thin film for bilirubin recognition

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ABSTRACT

A novel piezoelectric sensor has been developed for bilirubin (BR) detection, based on the modification of molecularly imprinted hydroxyapatite (HAP) film onto a quartz crystal by molecular imprinting and surface sol–gel technique. The performance of the developed BR biosensor was evaluated and the results indicated that a sensitive BR biosensor could be fabricated. The obtained BR biosensor presents high-selectivity monitoring of BR, better reproducibility, shorter response time (37 min), wider linear range (0.05–80 μM) and lower detection limit (0.01 μM). The analytical application of the BR biosensor confirms the feasibility of BR detection in serum sample.

1. Introduction

Bilirubin, BR, a common metabolite of hemoglobin, is normally conjugated with albumin to form a water-soluble complex and excreted from hepatocytes into bile mainly as BR glucuronides (Lee et al., 2002; Ahmad et al., 2006). Serum BR can be regarded as an important index for judging liver function and identifying a variety of liver diseases. In the past years, some analytical methods have been developed for BR detection, including enzymatic assay (Kurosaka et al., 1998), fluorimetric method (Andreu et al., 2000), capillary electrophoresis (Klemm et al., 2000), high-performance liquid chromatographic (HPLC) (Zelenka et al., 2008), electrochemical method (Klemm et al., 2000) and chemiluminescence (Lu et al., 2004). However, these methods have been found to have practical limitations with respect to cost, efficiency, instrumental complexity and manipulation. Therefore, the innovation of a new method for BR detection is necessary.

Molecular imprinting has been recognized as a representative technique to prepare specific binding sites for given molecules in appropriate matrices. In this approach, the shape and functionality of a template can be transcribed onto microporous materials. Various sensors based on molecularly imprinted polymer (MIP) have been developed for BR recognition (Syu et al., 2005, 2006; Baydemir et al., 2009; Wu and Syu, 2006; Huang et al., 2007; Yang et al., 2008a). Recently, inorganic matrices have been employed for molecular imprinting. Compared with MIP, molecularly imprinted inorganic matrices not only possessed a better stability and structural rigidity, but relieved some drawbacks of organic material such as low binding efficiency, slow binding process, and difficult fabrication (Yang et al., 2008b; Makote and Collinson, 1998; Pinel et al., 1997). Silica and titania sol–gel materials have been used to prepare molecularly imprinted thin film, which was used for the detection and separation of organism (Lee et al., 2005; Liu et al., 2010). HAP ($Ca_{10}(PO_4)_6(OH)_2$), a main constituent of bones and teeth, has been used extensively in such fields as bone repairs, bone implant, bioactive materials and purification and separation of biological molecules due to its excellent biocompatibility, slow biodegradation, good mechanical stability and great absorption property (Wang et al., 2003; Kokubo et al., 2003; Ozeki et al., 2007). To the best of our knowledge, the study on molecularly imprinted HAP film has not been reported up to now.

The QCM is a simple, portable, cost effective, high resolution mass sensing instrument, which has been favorably adopted for analytical application due to its extreme sensitivity to the nanogram level of mass change loaded onto surface of the QCM resonator. As a mass sensor, QCM has been widely used in biochemistry, environment, food, life science, and clinical analysis because the instrument provides a label-less method for the direct study of biospecific interaction process (Nunalee et al., 2006; Park et al., 2004; Shen et al., 2007; Min et al., 2008).

In the present work, combining the advantages of high selectivity from the piezoelectric microgravimetry using molecular imprinting technique and high sensitivity from QCM detection, a novel biosensor has been developed for BR detection based on the modification of BR-imprinted HAP film onto a quartz crystal by surface sol–gel technique.

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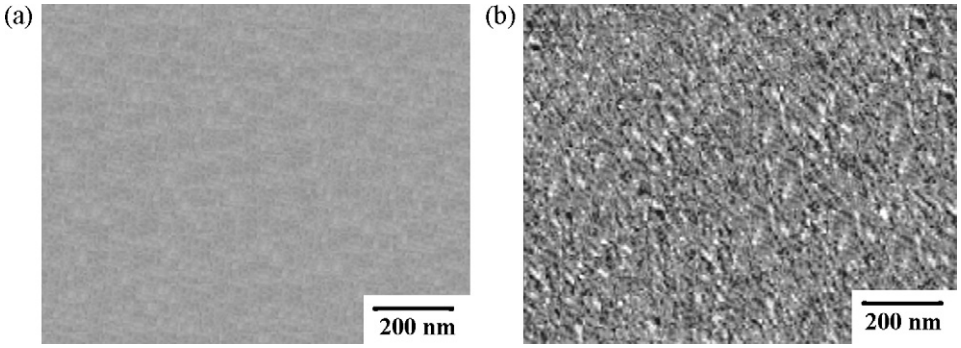

Fig. 1. SEM micrographs of quartz crystals before (a) and after (b) molecularly imprinted HAP film modification.

2. Experimental

2.1. Materials

BR, biliverdin, cholesterol, testosterone and benzoquinone (BQ) were purchased from Sigma Chemical Co., and used without further purification. Triethyl phosphite ($P(C_2H_5O)_3$) and calcium nitrate ($Ca(NO_3)\cdot 4H_2O$) were obtained from Shanghai Aladdin Chemical Co., China. All other chemicals were of analytical grade purchased from Shanghai Chemical Co., China. Deionized (DI) water (resistivity of 18 MΩ cm) was obtained from a Milli-Q system (Millipore Inc.), and was used for rinsing and for makeup of all aqueous solutions.

BR stock solution (1 mM) was prepared daily by dissolving 5.8 mg of BR in 10 ml of 50 mM NaOH solution. Standard solutions of lower concentrations were prepared by further dilution of the BR stock solution. All solutions were stored in amber glass vials wrapped with aluminum foil and placed in the dark to prevent light-initiated BR oxidation. The pH of the solutions was adjusted by adding an adequate amount of diluted HCl and buffered at the desired pH by the phosphate buffer solution. BR solution (1.0 μM, pH = 7.4) was used in our experiments, unless otherwise stated.

2.2. Apparatus

QCM measurements were performed using a Q-sense instrument (Gothenburg, Sweden). Mechanically polished AT-cut 9-MHz quartz crystals (diameter of 12 mm, Beijing Chenjing Co., Beijing, China) were vacuum deposited with gold electrodes (6-mm diameter) on both sides of the crystal. To reduce background frequency drift, one electrode was completely insulated from the test liquid. This was accomplished by the method reported in our previous study (Yang et al., 2008c). The working crystal was connected to a transistor–transistor logic integrated circuit (TTL-IC) oscillator (IC 74LS04, Kuan Hsi Co., Taiwan). Both the detector cell and the oscillator were put in a copper Faradaic cage to remove the surrounding electromagnetic noise. The frequency was monitored using a universal frequency counter (Iwatsu SC-7201, Tokyo, Japan) attached to personal computer, and the data was acquired and stored using a computer-based data acquisition system.

The cyclic voltammetry (CV) experiments were conducted by the CHI660 electrochemical station (CH Corporation, USA). The curves of current versus potential were recorded between −500 and +200 mV, at 100 mV s^{-1} scan rate. The surface morphology of molecularly imprinted HAP film was characterized by scanning electron microscopy (SEM, JEOL JSM-5400, Japan).

2.3. Fabrication of molecularly imprinted HAP film at the surface of quartz crystal

The procedure for preparation of molecularly imprinted HAP film at the surface of quartz crystal was described as follows. Triethyl phosphite and BR were first dissolved in toluene–ethanol (v/v, 1:1) mixture, and then vigorously stirred at room temperature until BR was dissolved completely. Subsequently, a fixed amount of DI water with a molar ratio of water/phosphite equal to 4 was added for hydrolysis under vigorous stirring for 24 h, and then 2 M solution of calcium nitrate in ethanol with Ca/P = 1.67 was added dropwise into the hydrolyzed phosphite sol. The resulting mixture was then stirred for 24 h and aged for 3 days. The obtained sol–gel was spin-coated onto the surface of quartz crystal at a speed of 3000 rpm for 30 s in ambient atmosphere. Thereafter, the films were dried in N_2 gas. The spin-coating-drying step caused the incorporation of BR into HAP thin film, and the thickness of imprinted film could be controlled by the number of "spin-coating-drying" cycles. Before and after HAP films modification, the frequency of the quartz crystal was measured, and the thickness of HAP films was estimated according to the amount of HAP at the surface of quartz crystal. The film with around 1.2 μm thickness was used in our experiments, unless otherwise stated. In order to form imprinted sites for template molecule, the BR-incorporated film was treated with ammonia aqueous solution (1 wt%) and rinsed with DI water. The procedures were repeated until a constant frequency was obtained. As a result, the template molecules were eliminated, and a HAP thin film with cavities imprinted with BR molecule was formed. The non-imprinted HAP film was prepared using the same procedure in the absence of BR.

2.4. QCM measurements

To monitor the in situ response of the BR biosensor to the BR molecule, the following procedure was adopted. After mounting the crystal in the cell, a small amount of aqueous solution was introduced into the cell by a peristaltic pump Ismatec (IPC-8 model) with a constant flow-rate. When the frequency became stable, the solution containing BR was injected into the cell within 5 s. Time-dependent change in the frequency was recorded continuously by a frequency counter and stored in a microcomputer during the reaction process of BR.

3. Results and discussion

3.1. Characterization of molecularly imprinted HAP film

The surface morphology of the quartz crystal before and after modification of molecularly imprinted HAP film is observed by an

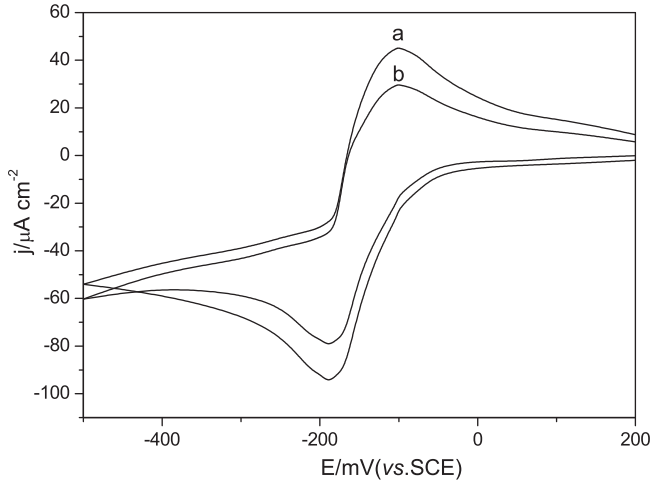

Fig. 2. Cyclic voltammograms of 0.5 mM BQ in 0.1 M KCl aqueous solution on molecularly imprinted HAP film/Au electrode (a) and non-imprinted HAP film/Au electrode (b). $v = 100\,mVs^{-1}$.

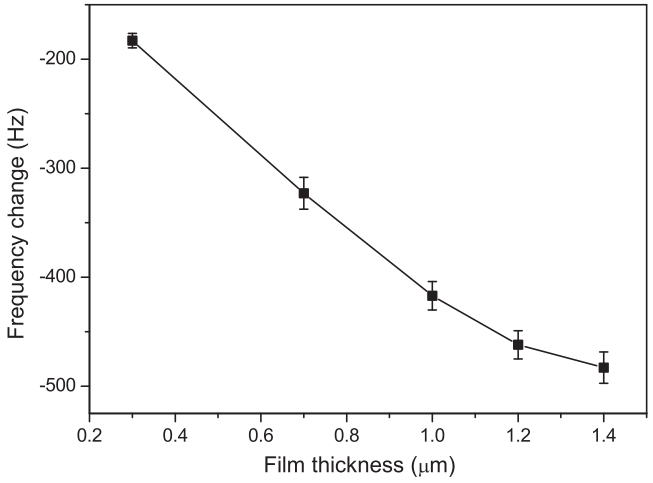

Fig. 3. Effect of the imprinted film thickness on response of the BR biosensor. Experimental conditions: 1.0 μM BR (pH = 7.4) and 37 °C. Average of three measurements (mean ± S.D.).

SEM as shown in Fig. 1. Obviously, the surface conditions in Fig. 1(a) and (b) were different. Compared with bare surface of a QCM electrode, the quartz crystal modified by molecularly imprinted HAP film was actually rather rough, and plenty of cavities appeared among the HAP matrix. These relative uniform cavities can be attributed to the extraction of imbedded templates in the process of molecularly imprinted HAP film formation. SEM observation strongly indicated the presence of molecularly imprinted HAP film at the surface of quartz crystal.

In order to further confirm the formation of molecularly imprinted HAP film, Fig. 2 shows cyclic voltammograms of 0.5 mM BQ in 0.1 M KCl aqueous solution on molecularly imprinted HAP film/Au electrode and non-imprinted HAP film/Au electrode. It can be seen from figure that the profiles were almost similar except for the difference of peak current, indicating two kinds of different films. Compared with non-imprinted HAP film/Au electrode, a clear increase in the peak current was observed on molecularly imprinted HAP film/Au electrode. Such phenomena further indicate the formation of molecularly imprinted HAP film. The enhanced peak current on imprinted film may be attributed to the change of surface/inner structure of HAP film caused by molecular imprinting, enhancing porosity of HAP film and resulting in faster BR diffusion through the film to the electrode surface.

3.2. Response characteristics of the BR biosensor

3.2.1. Effect of imprinted film thickness on response of the BR biosensor

The response of BR biosensor was closely related to the imprinted film thickness. As seen in Fig. 3, the value in frequency change increased with the increasing thickness of imprinted films, indicating that BR molecules can enter into the inner layer of BR-imprinted HAP film due to the high porosity of the imprinted HAP film with channels and holes. However, when the imprinted film thickness was above 1.2 μm, a further increase in the imprinted film thickness caused a slight enhancement of frequency response, such result may be attributed to the hindrance of HAP matrix in imprinted film, causing that it is difficult for BR to enter into the inner layer of more thick HAP film. Therefore, the BR-imprinted HAP film with 1.2 μm thickness was used in our experiments.

3.2.2. Response time, linear range, detection limit and selectivity

Dynamic response is a factor that measures the sensing ability of a sensor. The response time of the BR biosensor was measured by changing the BR concentration from 0.01 to 100 μM. The steady-state response was reached at about 37 min within the whole concentration range. The response time was shorter than 1 h (Wu and Syu, 2006) and 2 h (Yang et al., 2008a; Syu et al., 2005). It is believed that mass transfer resistance by the binding cavity, the HAP matrix and strength of the interaction could be major factors affecting the response tine of the BR biosensor.

The linear dynamic concentration range of the BR biosensor was investigated by monitoring the BR solution in the concentration range from 0.01 to 110 μM. Fig. 4 shows the plot of frequency response versus BR concentration. Over the low concentration range (0.05–80 μM), good linearity was obtained. The linear range was wider than 0.1–7.0 mg/dl (Huang et al., 2007), 0.45–11.0 mg/dl (Wu and Syu, 2006) and 1.0–5.0 mg/dl (Syu et al., 2006). The linear regression equation was y (Hz) = −377.5–103.9[BR] (μM), $R = 0.9990$, $n = 7$. When the BR concentration was higher than 80 μM, the frequency response started to level off with the increasing of BR concentration. The sluggish frequency change for the higher BR concentration can be attributed to the restriction of the imprinted cavities. When the concentration of substrate was overloaded, the imprinted cavities become saturated with BR molecules under these BR solution conditions and no more BR can be absorbed by the imprinted HAP film. The detection limit of the BR biosensor

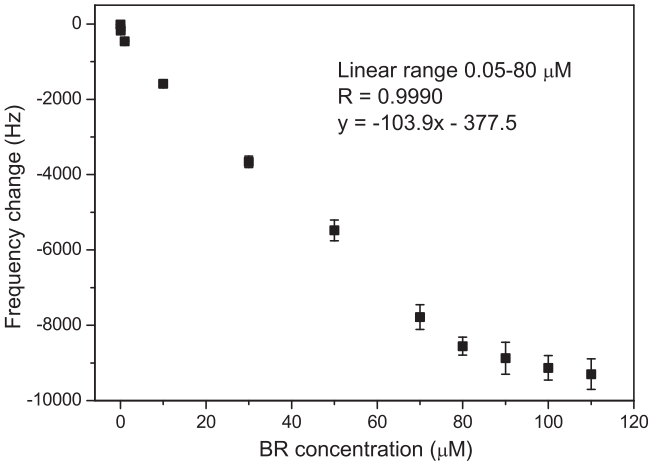

Fig. 4. Calibration graph obtained for the BR biosensor in a BR concentration range from 0.01 to 110 μM. Experimental conditions: pH = 7.4 and 37 °C. Average of three measurements (mean ± S.D.).

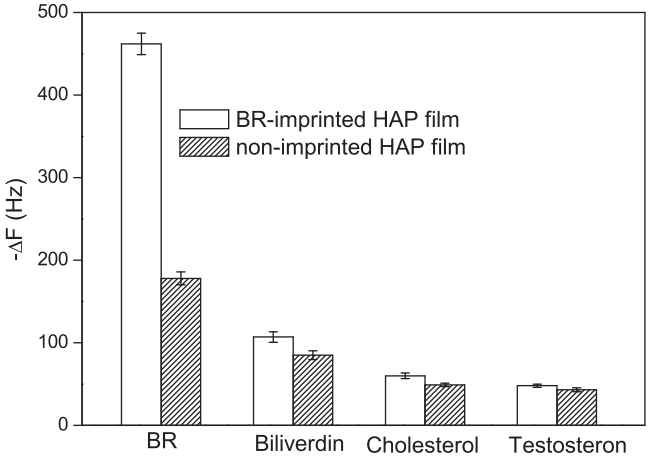

Fig. 5. Comparison of absorption selectivity of the QCM sensors. Concentration 1.0 μM, pH = 7.4 and temperature 37 °C. Average of three measurements (mean ± S.D.).

was found to be about 0.01 μM ($S/N = 3$), which was lower than 8×10^{-5} mol/L (Yang et al., 2008b). The result can be attributed to the high selectivity of BR-imprinted HAP film and extreme sensitivity of QCM.

Molecular recognition selectivity is an important parameter in evaluating sensor. In order to better study the selectivity of the BR biosensor, biliverdin, cholesterol and testosterone were chosen as competitive absorption molecules for comparison due to their similar size to BR and coexistence in the serum. Therefore, to know how they interfere binding of BR by imprinted HAP film is essential for the in vitro measurement of serum samples. A comparison of the frequency response caused by absorption of BR, biliverdin, cholesterol and testosterone was preformed on the imprinted and non-imprinted HAP film, and the obtained result was shown in Fig. 5. According to the frequency change, the relative selectivity coefficients of BR-imprinted HAP film for BR/biliverdin, BR/cholesterol and BR/testosterone pairs were 2.06, 2.14 and 2.33 times greater than that for non-imprinted HAP film, respectively, indicating that BR-imprinted HAP film favored BR much more than other molecules and therefore the absorption of BR by the imprinted film was much higher than that of other molecules. Obviously, the results revealed that BR-imprinted HAP film was highly selective to BR. This means that BR can be determined by the sensor modified with BR-imprinted HAP film even in the presence of other interferents.

3.3. Reusability and analytical application

Reusability of the same BR biosensor was evaluated by measuring the response signal eight times in a continuous manner. After each injection of BR solution and the attainment of equilibrium, the BR biosensor was recovered by sequential washes with ammonia aqueous solution (1%) and DI water until the frequency of QCM reached a steady value. The sensor could be stored in DI water at room temperature and reused for next experiments. The obtained results indicate that the BR biosensor exhibited better reproducibility, and the response signal of the sensor was about 2.6% loss at the 8th cycle. In addition, our experiments indicated that the BR biosensor was quite stable not only when operated for a long period under a continuous flow detection system but also when reused from time to time. The sensor has proved its stability for more than 8 months with a slight loss of sensitivity, which is a significant advantage of the molecular imprinting technique employed here.

To demonstrate the feasibility of the BR biosensor for analytical application, the quartz crystal modified by BR-imprinted HAP film was applied to the detection of BR in serum sample. No signal could be detected in blank serum sample, while the detection signal was obvious after BR was added to serum sample. As showed in Table 1, with five different additions of BR to the serum sample, the obtained recoveries ranged from 96.83 to 101.50%. The high recovery indicated that the BR-imprinted HAP film showed satisfactory absorption ability towards BR even in the presence of other compounds coexisting in serum sample. The results suggested that the BR biosensor was reliable for BR detection in serum sample.

Table 1
The recovery determination of BR in serum samples.

BR concentration (μM)			Recovery (%)
Added	Found		
	Mean[a]	R.S.D.[b] (%)	
0.10	0.10	3.4	100.00
10.00	9.84	2.9	98.40
30.00	29.42	4.8	98.07
50.00	50.75	4.1	101.50
70.00	67.78	5.6	96.83

[a] Average of three determinations.
[b] Relative standard deviation.

4. Conclusions

SEM and CV analysis indicated that the molecularly imprinted HAP film at the surface of quartz crystal could be successfully prepared by molecular imprinting and surface sol–gel technique. By using the BR-imprinted HAP film as the recognition material, a novel QCM sensor with good reproducibility, short response time, wide linear range, low detection limit and high selectivity has been developed for the determination of BR. The detection of BR by the BR biosensor is rapid and sensitive in the detection system. The analytical application of the BR biosensor demonstrates the feasibility of BR detection in serum sample.

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References

Ahmad, N., Arif, K., Faisal, S.M., Neyaz, M.K., Tayyab, S., Owais, M., 2006. Biochim. Biophys. Acta 1760, 227–232.
Andreu, Y., Galban, J., De Marcos, S., Castillo, J.R., 2000. Fresenius J. Anal. Chem. 368, 516–521.
Baydemir, G., Bereli, N., Andaç, M., Say, R., Galaev, I.Y., Denizli, A., 2009. Colloids Surf. B: Biointerfaces 68, 33–38.
Huang, C.Y., Syu, M.J., Chang, Y.S., Chang, C.H., Chou, T.C., Liu, B.D., 2007. Biosens. Bioelectron. 22, 1694–1699.
Klemm, J., Prodromidis, M.I., Karayannis, M.I., 2000. Electroanalysis 12, 292–295.
Kokubo, T., Kim, H.M., Kawashita, M., 2003. Biomaterials 24, 2161–2175.
Kurosaka, K., Senba, S., Tsubota, H., Kondo, H., 1998. Clin. Chim. Acta 269, 125–136.
Lee, K.H., Wendon, J., Lee, M., Costa, M.D., Lim, S.G., Tan, K.C., 2002. Liver Transpl. 8, 591–593.
Lee, S.W., Yang, D.H., Kunitake, T., 2005. Sens. Actuators B 104, 35–42.
Liu, Y.T., Liu, R.H., Liu, C.B., Luo, S.L., Yang, L.X., Sui, F., Teng, Y.R., Yang, R.B., Cai, Q.Y., 2010. J. Hazard. Mater. 182, 912–918.
Lu, C., Lin, J.M., Huie, C.W., 2004. Talanta 63, 333–337.
Makote, R., Collinson, M.M., 1998. Chem. Mater. 10, 2440–2445.
Min, K., Cho, M., Han, S.Y., Shim, Y.B., Ku, J., Ban, C., 2008. Biosens. Bioelectron. 23, 1819–1824.
Nunalee, F.N., Shull, K.R., Lee, B.P., Messersmith, P.B., 2006. Anal. Chem. 78, 1158–1166.
Ozeki, K., Janurudin, J.M., Aoki, H., Fukui, Y., 2007. Appl. Surf. Sci. 253, 3397–3401.
Park, I.S., Kim, D.K., Adanyi, N., Varadi, M., Kim, N., 2004. Biosens. Bioelectron. 19, 667–674.
Pinel, C., Loisil, P., Gallezot, P., 1997. Adv. Mater. 9, 582–585.

Shen, Z.H., Huang, M.C., Xiao, C.D., Zhang, Y., Zeng, X.Q., Wang, P.G., 2007. Anal. Chem. 79, 2312–2319.
Syu, M.J., Chiu, T.C., Lai, C.Y., Chang, Y.S., 2006. Biosens. Bioelectron. 22, 550–557.
Syu, M.J., Deng, J.H., Nian, Y.M., Chiu, T.C., Wu, A.H., 2005. Biomaterials 26, 4684–4692.
Wang, C.X., Wang, M., Zhou, X., 2003. Biomaterials 24, 3069–3077.
Wu, A.H., Syu, M.J., 2006. Biosens. Bioelectron. 21, 2345–2353.
Yang, D.H., Takahara, N., Lee, S.W., Kunitake, T., 2008a. Sens. Actuators B 130, 379–385.
Yang, Y., Long, Y.Y., Cao, Q., Li, K., Liu, F., 2008b. Anal. Chim. Acta 606, 92–97.
Yang, Z.P., Si, S.H., Zeng, X.M., Zhang, C.J., Dai, H.J., 2008c. Acta Biomater. 4, 560–568.
Zelenka, J., Leníček, M., Muchová, L., Jirsa, M., Kudla, M., Balaž, P., Zadinová, M., Ostrow, J.D., Wong, R.J., Vítek, L., 2008. J. Chromatogr. B 867, 37–42.