Biosensors and Bioelectronics 24 (2008) 162–166

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

A potentiometric protein sensor built with surface molecular imprinting method

Yantian Wang[a], Yanxiu Zhou[b], Jonathon Sokolov[a], Basil Rigas[c], Kalle Levon[b], Miriam Rafailovich[a,*]

[a] Department of Materials Science and Engineering, Stony Brook University, Stony Brook, NY 11794-2275, USA
[b] Department of Biological and Chemical Sciences, Polytechnic University, Brooklyn, NY 11201, USA
[c] Department of Medicine, Stony Brook University, Stony Brook, NY 11794, USA

ARTICLE INFO

Article history:
Received 8 January 2008
Received in revised form 23 March 2008
Accepted 8 April 2008
Available online 20 April 2008

Keywords:
Sensor
Self-assembled monolayer
Surface molecular imprinting
Protein
Potentiometer

ABSTRACT

Surface molecular imprinting, as compared to molecular imprinted bulk polymers, has the advantages of higher re-occupation percentage of the reception sites, fast response, integration of sensing element and transducer, etc. In this study, a potentiometric protein sensor was developed based on the surface molecular imprinting technique. Using the self-assembled monolayers of alkanethiol with hydroxyl terminal groups as the matrix material, and target protein molecules as the template, the sensing layer was created on the surface of the gold-coated silicon chip-an electrochemical transducer. Potentiometric measurement demonstrated that the sensor could selectively detect myoglobin or hemoglobin molecules, either with or without the presence of other protein molecules in the same solution.

1. Introduction

Considerable attention has been paid to the research and investigation in the biosensors which has brought dramatic increase in the development level of new biosensors in the past two decades (Wilson and Gifford, 2005; Kissinger, 2005; Murphy, 2006). Molecular imprinting (MI), as a fast developing technology, has also been investigated for the sensing applications (Mosbach, 2006; Vlatakis et al., 1993; Shea, 1994; Wulff, 1995; Yano and Karube, 1999; Haupt, 2003; Sellergren, 2000; Bowma et al., 1998; Bradley and Kenneth, 2001; Rachkov and Minoura, 2001; Hayden et al., 2003; Guo et al., 2004). However, the 3D MI methods using the bulk polymer matrix, which have been studied extensively, still suffer from several limitations such as: the transduction mechanism is usually separated from the recognition element, the low re-occupation percentage of the cavities in the bulk polymers, long response time for the target to be captured by the reception site buried deeply inside the polymer, etc. All these would lead to a slow response of the sensor. To overcome these problems, MI on the surfaces (2D imprinting) could provide an efficient solution. Self-assembled monolayers (SAMs) have several attractive features which make them ideal candidates to be used as the imprinted matrix materials (Nirmalya and Vijayamohanan, 2002; Wink et al., 1997; Flink et al., 2000; Lotierzo et al., 2004). First, the easy procedure for the SAM formation and chemical binding with the metal substrates makes it possible for building an integrated sensor platform; second, the high degree of order and the dense packing of the long alkane chains of the SAM provide a stable matrix and hence makes a good insulation layer (Chen et al., 2005); third, the modification of the end functional groups of the SAM molecules provides a flexible way for non-covalent binding with the sensing molecules. Tabushi and coworkers first applied the imprinting technique with SAMs for molecular recognition purpose. They co-implanted inert guest (n-hexadecane) into the octadecylsiloxane (ODS) monolayer on the SnO_2 electrode, after extracting the guest molecules, the electrode showed significant electrochemical response to vitamin K-1, which had hydrophobic tails, but little response to vitamin K-3 which is more hydrophilic (Tabushi et al., 1987). Zhou et al. built the chemical sensor by imprinting the target molecules in the ODS SAM on an indium–tin oxide (ITO) electrode and demonstrated successful detection for chiral amino acid, methylphosphonic acid, and dipicolinic acid (Zhou et al., 2003, 2004, 2005). Imprinting with thiol SAMs has also been successful in recognizing small molecules such as cholesterol and barbituric acid (Piletsky et al., 1999; Mirsky et al., 1999). Although this technique has been applied in the small organic molecules, literature on the development of MI with SAMs for the recognition of proteins is rare, due to several technical difficulties: the relative complexities of the protein surfaces, which

* Corresponding author. Tel.: +1 631 632 8483.
E-mail address: mrafailovich@notes.cc.sunysb.edu (M. Rafailovich).

0956-5663/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2008.04.010

carry large numbers of competing binding sites; their conformational sensitivity to temperature, pH, the nature of the solvent; poor solubility in apolar solvents; biocompatibility and relatively large molecular sizes (Sellergren, 2001; Turner et al., 2006). In most of the reports for the application of MI technique in protein sensors, elegant chemistry complementarities and polymerization procedures are essential for successful recognition (Lin et al., 2004; Turner et al., 2006).

In this study, we report the fabrication of a sensor built with surface molecular imprinting of thiol SAMs which can detect complex molecules with parts per million accuracy. We show that the technique is applicable to the detection for globular proteins such as myoglobin and hemoglobin with good sensitivity and selectivity.

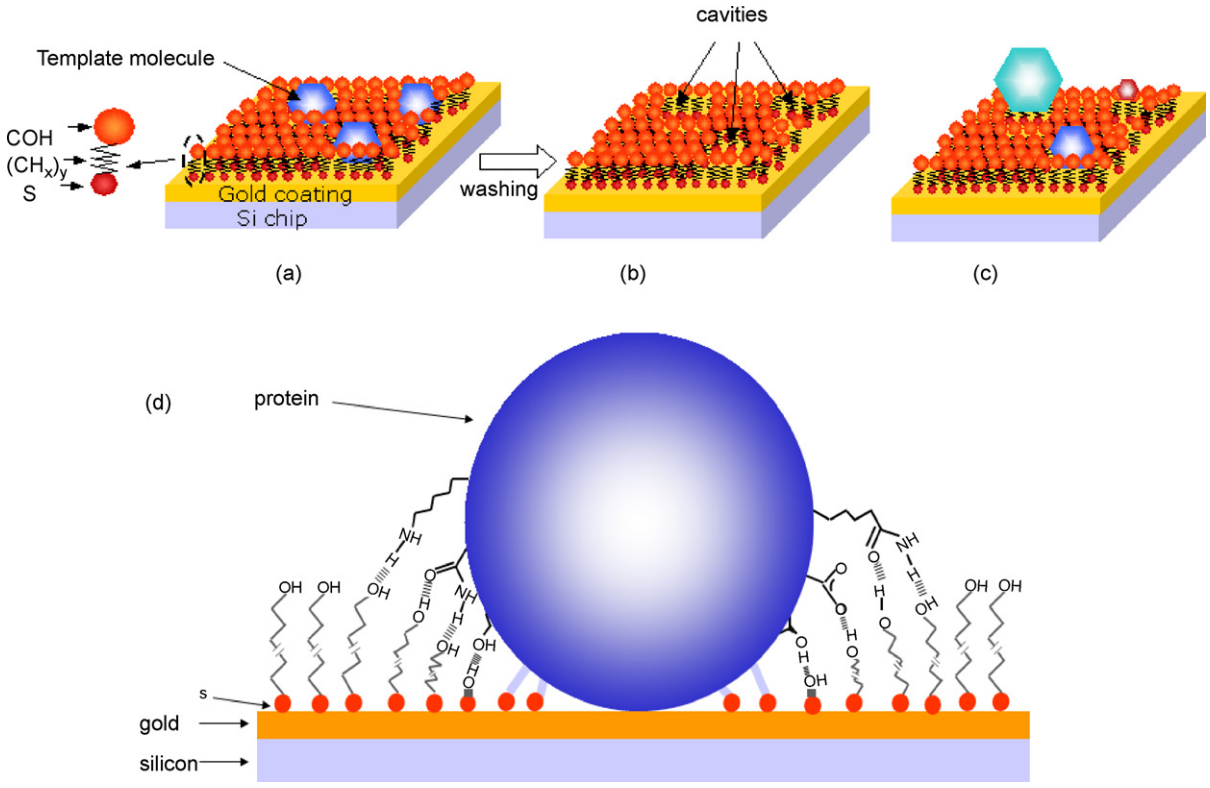
2. Experimental method

2.1. Materials and electrochemical measurement set-up

Myoglobin from equine skeletal muscle (M_w = 17.6K), hemoglobin from bovine blood (M_w = 64.5K), ovalbumin from chicken egg white (M_w = 44.29K), 11-mercapto-1-undecanol (97%) (thiol) were purchased from Sigma–Aldrich and stored in refrigerator. All the proteins and chemicals were used as received. Two kinds of electrodes were used as the sensing electrode: the gold (50 nm) coated silicon wafer (1 cm × 2 cm) (a) modified with imprinting the template molecules in the thiol SAM and (b) modified with pure thiol SAM. The potentiometric measurements were made in 8 mL of Dulbecco's phosphate buffered saline (DPBS: pH ~ 7.15) in a 10 mL beaker, equipped with a magnetic stirrer. The two-electrode system consisted of an Ag/AgCl as the reference electrode and the sensing plate as the working electrode. The potentials of the working electrode against the reference electrode were measured with an Orion 920A potentiometer. The electrochemical response was defined as the potential change after the testing molecules were added into the solution compared with that before addition of the test molecules (i.e., $\Delta E = E - E_0$, where E is the potential after adding the test molecules and E_0 is the potential before adding the test molecules). For selected experiments, a pH electrode (Orion Ross combination electrode) was connected to the other channel of the potentiometer to measure the pH value of the solution.

2.2. Sensor fabrication

The mechanism of the surface molecular imprinting is illustrated in Scheme 1. The gold-coated silicon chip was used after cleaning with de-ionized water and dried with pure nitrogen gas. Due to the poor solubility of thiol in aqueous medium and the denaturation of proteins in non-aqueous medium or extremely low pH, a compromising dissolving process was used. Proteins were dissolved in de-ionized water and the thiol was dissolved in acetic acid. Then the mixture solution was made by blending them in the 19:1 (water:acetic acid) volume ratio. In this way, a good solubility was obtained for thiol and no precipitation of proteins occurred. Following the literature value for stable monolayer formation, the concentration of the thiol was chosen to be 0.1 mM (Porter et al., 1987). The concentration of the proteins was 30 μg/mL (i.e., 1.7 μM for myoglobin, 0.465 μM for hemoglobin), far less than 10 mole% ratio to the thiol to avoid aggregation of the proteins and formation of the supermolecular structure (Singh et al., 1999). Then the Au coated plate was immersed into the solution for at least 2 h to co-absorb the protein and thiol. While the thiol molecules can be tightly attached to the electrode surface through sulfur-metal bond, the proteins are adsorbed onto the gold surface mostly through hydrophobic interactions and electrostatic forces, in absence of



Scheme 1. Fabrication of protein imprinted SAM sensor and its proposed working mechanism. (a) Co-adsorption of template protein molecules and thiol SAMs onto the gold surface. (b) Cavities created after washing off the templates. (c) Selective adsorption of the template protein molecules against other molecules. (d) Hypothetical binding mechanism showing the hydrogen bonds between protein and –OH end groups of the thiol.

strong chemical bindings (Kaufman et al., 2007). Cavities that are complementary with the template proteins can be then created in the SAM matrix by removing the protein molecules through repetitive rinsing with DI water. Due to the unique complentarity, the so-prepared electrode is expected to have a higher affinity to adsorb the same kind of template protein molecules as compared to the other "guest" molecules. The electrode was then dried at room temperature overnight before electrochemical measurements. The pure thiol modified electrode was made in the same procedure in absence of the proteins.

2.3. Principles of potentiometric detection

Proteins in aqueous solutions are polyelectrolytes and have a net electrical charge the magnitude of which depends on the isoelectric point of the protein and on the ionic composition of the solution. It has been demonstrated that when the charged proteins are trapped into the thin insulating layer which is deposited onto a metallic conductor, the change of the surface potential will occur, and this change can be measured potentiometrically using a reference electrode immersed in the same solution (Janata, 1975).

3. Results and discussion

3.1. Hemoglobin sensor

Hemoglobin imprinted thiol modified electrode was fabricated using the above described method (Scheme 1). The sensitivity and selectivity of sensor were tested and shown in Fig. 1(a).

It can be seen that the potential of the hemoglobin sensor changed drastically with the addition of hemoglobin before reaching a stable value at the concentration of around 40 μg/mL, after which E did not change remarkably. For the non-imprinted thiol SAM, only very slight potential response was observed. This result indicated that the imprinted electrode had a much higher adsorption ability to the template molecules than the non-imprinted ones. From Fig. 1(a), we can also see that the hemoglobin sensor showed no obvious response to the myoglobin or ovalbumin, demonstrating that the hemoglobin imprinted sensor had little or no affinity to the other protein molecules. This is consistent with the sensing mechanism that the hemoglobin was initially incorporated into the adsorbed film and extracted away to create the molecular recognition cavity.

Since the potential of the sensing electrode is closely related to the charge of the protein, and the net charge of the proteins is highly dependent on the pH of the solution, the pH of the testing medium is important to the response of the electrode. The isoelectric point of the protein is defined as the pH at which the net charge on the protein surface is zero. Usually, in the solution where pH is higher than the isoelectric point of the protein, it's negatively charged, while in the media with pH lower than its isoelectric point, the protein is positively charged. The pH of the DPBS used as the medium is 7.15 ± 0.1. The isoelectric point of myoglobin, hemoglobin and ovalbumin is 7.0, 6.8 and 4.6, respectively. Hence, in the above experimental condition, the myoglobin might be charged or neutral due to the extremely close value of its isoelectric point to the pH of the test medium. To see if the pH is the reason that contributed to the insensitivity of the hemoglobin sensor to myoglobin, the pH of PBS was adjusted to 6.0, and the potentiometric measurement of the hemoglobin sensor was carried out in this buffer solution. As shown in Fig. 1(b), the hemoglobin sensor showed an obvious potential response to the hemoglobin while it was still insensitive to the presence of the myoglobin, even at a pH well below the isoelectric point of myoglobin. This result clearly excluded the possibility that the insensitivity of the imprinted sensor to other proteins was due to the zero net charge of the target molecules.

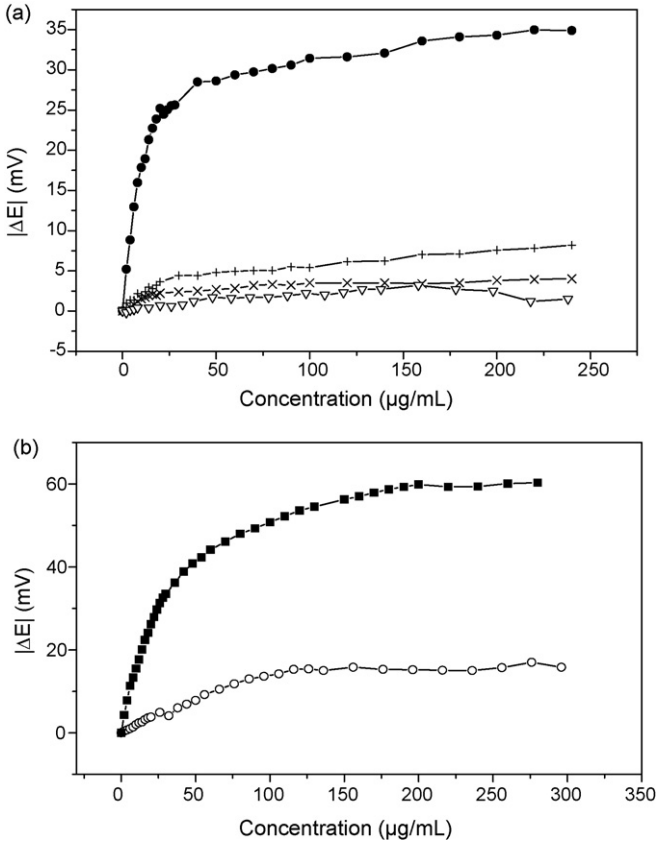


Fig. 1. (a) Potentiometric response of hemoglobin sensor to hemoglobin, ovalbumin, myoglobin, respectively; non-imprinted electrode response to hemoglobin ((•) hemoglobin sensor tested with hemoglobin; (+) non-imprinted thiol tested with hemoglobin; (×) hemoglobin sensor tested with ovalbumin; (▽) hemoglobin sensor tested with myoglobin). (b) Hemoglobin sensor response to hemoglobin, myoglobin, at pH 6 ((■) hemoglobin; (◯) myoglobin).

3.2. Myoglobin sensor

Similarly, the myoglobin sensing electrode was prepared by imprinting the myoglobin molecules with the thiol SAM.

Fig. 2(a) shows that the imprinted sensor had a big potential response to the myoglobin, while very slight or no response to hemoglobin or ovalbumin. Also, when the sensing electrode was not imprinted, reduced sensitivity to the myoglobin was observed.

Since in the practical application, target analyte usually coexists with a large number of interfering species, the sensor is expected to be able to identify a target molecule in complex mixtures without complicated separation step. For this purpose, its ability to recognize a specific target in the multi-component system was further tested, the results are shown in Fig. 2(b). Myoglobin sensor was tested with two kinds of blend solutions: (A) myoglobin/hemoglobin/ovalbumin (1:1:1 in wt) and (B) hemoglobin/ovalbumin (1:1 in wt). The potential of the sensor was plotted as a function of the concentration of a single component in the mixture solution. The big potential change of the sensor with the addition of protein solution (A) compared to the relative slight change with that of (B) suggested that the sensor could selec-

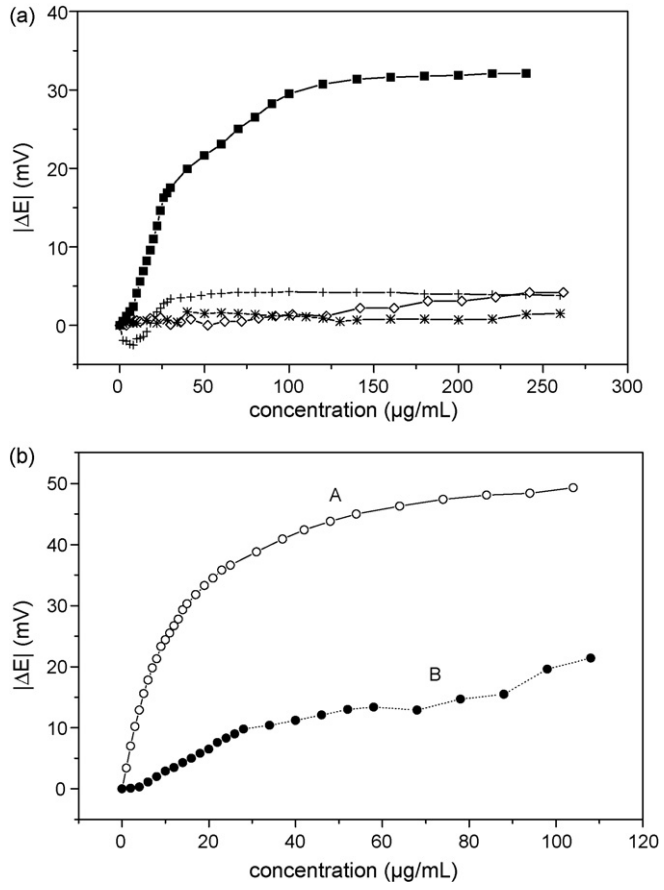


Fig. 2. (a) Potentiometric response of myoglobin sensor to myoglobin, hemoglobin, ovalbumin, respectively; non-imprinted electrode response to myoglobin ((+) myoglobin sensor tested with hemoglobin; (◊) non-imprinted thiol tested with myoglobin; (*) myoglobin sensor tested with ovalbumin; (■) myoglobin sensor tested with myoglobin). (b) Potential response of myoglobin sensor to the mixture solution of (A) myoglobin, hemoglobin and ovalbumin (1:1:1 in wt), and (B) hemoglobin and ovalbumin (1:1 in wt), as a function of the concentration of a single component in the solution.

tively recognize the myoglobin molecules in the mixture, inducing a larger response signal.

3.3. Response time of the sensor

The response time of the sensor, which was the time needed for reaching a stable reading of the potentiometer after a stepwise increase of the target molecule concentration, was 2–10 min.

3.4. Discussion about the binding mechanism

Unlike the traditional MI recognition mechanism which mainly depends on the precise spatial arrangement of functional groups in the matrix to ensure selective recognition of the target molecules, our surface imprinting method does not involve any covalent bindings between the matrix and the template molecule. The combination effect of the attraction forces between the protein molecule and gold surface, the hydrogen bonds between the hydrophilic groups of the protein surfaces and the –OH groups at the thiol end, as well as the specific arrangement of these interactions in shape and orientation, determines the recognition and selectivity of the sensor (Shi et al., 1999; Kaufman et al., 2007). Although the sensor has been demonstrated to work well with the proteins tested so far, a question remains to answer: how important is the geometric match between the sensing molecules and the imprinting molecules for the sensor to function properly? The myoglobin and hemoglobin are very similar in structure and chemistry, still the larger size hemoglobin imprinted sensor is insensitive to the smaller size myoglobin, indicating the geometrical complementarity is crucial for the sensor to function. While the molecules with size and shape complementary to the cavities can be captured on the electrode surface, for the molecules with a smaller size than the imprinted cavities, binding with the surrounding thiols is far more difficult (Scheme 1c). Even though the molecules can enter the cavities, two possible processes may prevent them from inducing an electrochemical signal: first, the protein molecule tightly adsorbed onto the pure gold surface, without the surrounding hydrophilic thiol molecules to retain its shape, may be denatured quickly (Höök et al., 1998), and hence loses its electrochemical activity; second, the loosely attached protein molecules that do not have hydrogen bonds with the thiol molecules have a bigger chance to escape into the solutions instead of staying in the monolayer matrix, so that the adsorption is unstable for kinetic reasons. Since the potential of the sensing electrode is closely related to the amount of charges on the electrode surface, the less accumulated charges due to the escaping of the approached molecules into the solution will lead to an unchanged potential of the sensing electrode.

4. Conclusion

In this study, a protein sensor was built by the surface molecular imprinting method and demonstrated to be efficient in recognizing myoglobin and hemoglobin. The selective adsorption of the target protein molecules onto the sensing electrode induced a significant potential change of the electrode and this change became more gradual above a certain concentration due to saturation of the accepting sites. The sensor also had the ability to recognize the specific protein in a mixture solution of multiple proteins. The size and shape match was demonstrated to be crucial for the precise recognition.

References

Bradley, R.H., Kenneth, J.S., 2001. J. Am. Chem. Soc. 123, 2072–2073.
Bowma, M.A.E., Allender, C.J., Brain, K.R., Heard, C.M., 1998. Methodol. Surv. Bioanal. Drugs 25, 37–43.
Chen, H., Heng, C.K., Puiu, P.D., Zhou, X.D., Lee, A.C., Lim, T.M., Tan, S.N., 2005. Anal. Chem. Acta 554, 52–59.
Flink, S., Veggel, F.C.J.M., Reinhoudt, D.N., 2000. Adv. Mater. 12, 1315–1328.
Guo, T.Y., Xia, Y.Q., Hao, G.J., Song, M.D., Zhang, B.H., 2004. Biomaterials 25, 5905–5912.
Haupt, K., 2003. Chem. Commun., 171–178.
Hayden, O., Bindeus, R., Haderspöck, C., Mann, K.J., Wirl, B., Dickert, F.L., 2003. Sens. Actuators B 91, 316–319.
Höök, F., Rodahl, M., Kasemo, B., Brzezinski, P., 1998. Proc. Natl. Acad. Sci. U.S.A. 95, 12271–12276.
Janata, J., 1975. J. Am. Chem. Soc. 97, 2914–2916.
Kissinger, P.T., 2005. Biosens. Bioelectron. 20, 2512–2516.
Kaufman, E.D., Belyea, J., Johnson, M.C., Nicholson, Z.M., Ricks, J.L., Shah, P.K., Bayless, M., Pettersson, T., Feldotö, Z., Blomberg, E., Claesson, P., Franzen, S., 2007. Langmuir 23, 6053–6062.
Lin, T.Y., Hub, C.H., Choub, T.C., 2004. Biosens. Bioelectron. 20, 75–81.
Lotierzo, M., Henry, O.Y.F., Piletsky, S., Tothill, I., Cullen, D., Kania, M., Hock, B., Turner, A.P.F., 2004. Biosens. Bioelectron. 20, 145–152.
Mirsky, V.M., Hirsch, T., Piletsky, S.A., Wolfbeis, O.S., 1999. Angew. Chem. Int. Ed. 38, 1108–1110.
Mosbach, K., 2006. Sci. Am. 295, 86–91.
Murphy, L., 2006. Curr. Opin. Chem. Biol. 10, 177–184.
Nirmalya, K.C., Vijayamohanan, K., 2002. Biosens. Bioelectron. 17, 1–12.
Piletsky, S.A., Piletskaya, E.V., Sergeyeva, T.A., Panasyuk, T.L., El'skaya, A.V., 1999. Sens. Actuators B 60, 216–220.
Porter, M.D., Bright, T.B., Allara, D.L., Chidseyi, C.E.D., 1987. J. Am. Chem. Soc. 109, 3559–3568.
Rachkov, A., Minoura, N., 2001. Biochim. Biophys. Acta 1544, 255–266.
Sellergren, B., 2000. Angew. Chem. Int. Ed. 39, 1031–1037.
Sellergren, B., 2001. Molecular Imprinted Polymers, Man-made Mimics of Antibodies and Their Applications in Analytical Chemistry. Elsevier, New York.

Shea, K.J., 1994. Trends Polym. Sci. 2, 166–173.
Shi, H., Tsai, W.B., Garrison, M.D., Ferrari, S., Ratner, B.D., 1999. Nature 398, 593–597.
Singh, S., Sasaki, D.Y., Cesarano III, J., Hurd, A.J., 1999. Thin Solid Films 339, 209–215.
Tabushi, I., Kurihara, K., Naka, K., Yamamura, K., Hatakeyama, H., 1987. Tetrahedron Lett. 28, 4299–4302.
Turner, N.W., Jeans, C.W., Brain, K.R., Allender, C.J., Hlady, V., Britt, D., 2006. Biotechnol. Prog. 22, 1474–1489 (and references therein).
Vlatakis, G., Andersson, L.I., Muller, R., Mosbach, K., 1993. Nature 361, 645–647.
Wilson, G.S., Gifford, R., 2005. Biosens. Bioelectron. 20, 2388–2403.
Wink, Th., Zuilen, S.J., Bult, A., Bennekon, W.P., 1997. Analyst 122, 43R–50R.
Wulff, G., 1995. Angew. Chem. Int. Ed. Engl. 34, 1812–1832.
Yano, K., Karube, I., 1999. Trends Anal. Chem. 18, 199–204.
Zhou, Y., Bin, Y., Levon, K., 2003. Chem. Mater. 15, 2774–2779.
Zhou, Y., Yu, B., Shiu, E., Levon, K., 2004. Anal. Chem. 76, 2689–2693.
Zhou, Y., Yu, B., Levon, K., 2005. Biosens. Bioelectron. 20, 1851–1855.