Development of chemiluminescent biosensing of nucleic acids based on oligonucleotide-immobilized gold surfaces

The Analyst

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A chemiluminescent biosensing method was developed based on the immobilization of DNA probes on a gold surface by the self-assembled monolayers technique. The complementary sequence was detected by coupling avidin–alkaline phosphatase to the biotinylated oligonucleotide and measuring the chemiluminescent signal obtained from the hydrolysis of the substrate 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane by this enzyme. The method has a wide calibration range of five orders of magnitude and the detection limit is 15 pM (signal-to-ratio = 3). The factors affecting the probe immobilization, target hybridization and sensitivity were investigated. The method was also applied to detect specific DNA fragments relative to the hepatitis B plasmid.

In recent times, the number of possible applications of nucleic acid hybridization techniques in molecular biology and medicine has grown immensely. In traditional hybridization methods, the denatured target DNA has to be bound to a solid support such as nitrocellulose or nylon filter membranes and subsequently hybridized, hence this process is laborious. In addition, this type of hybridization is difficult to automate and provide data for quantitative determinations. In contrast, a DNA based biosensor in which the single-stranded DNA (ssDNA) probe is attached to the surface of the sensing devices has the potential to allow rapid, quantitative monitoring of hybridization with the target DNA.[1,2] The basis of operation for this type of bioaffinity sensor is the complementary coupling between the specific DNA sequences within target analytes and the specific nucleotide sequence immobilized on the solid support.

Electrochemical sensors have widely been applied coupled with the highly specific biochemical reaction of hybridization,[2–13] in which the captured probes are immobilized on the electrodes by covalent reaction[3–5] or adsorption[6–13] and the amount of target DNA is detected by hybridization indicators. However, most of these methods have detection limits only of the order of nanograms level. This could be attributed to the immobilization procedure and detection method. In order to study the immobilization method further, quartz crystal microbalance (QCM)[14–18] and surface plasmon resonance[19,20] techniques have been employed to assess the degree of DNA binding and hybridization. As a result, molecular self-assembly, the Langmuir–Blodgett technique and the avidin–biotin system were used to design the biosensing devices for DNA. However, no detection limits for nucleic acids were reported. Recently fiber-optic biosensors based on fluorescence detection have been developed to assay oligomers,[21–24] in which oligonucleotide probes were attached to the core of the fibres *via* an avidin–biotin bridge[21] or covalent immobilization.[22–24] In addition, fluorescence detection has also been employed in DNA chip microfabrication technology[25–30] and such methods were reported to have potentially important appliations in high throughput sequencing and discrimination of point mutations.

Chemiluminescence as an alternative optical detection approach is sensitive enough to compete with chromogenic and radioisotopic tracers to detect viral genomes directly in clinical samples. Chemiluminescent assays have been developed to determine DNA fragments spotted on membranes,[31–33] but these methods involve complicated operations. This paper reports a new chemiluminescent biosensing method in which the captured probe is immobilized on a gold surface by the self-assembly technique and the chemiluminescent signals are detected after hybridization through conjugation of alkaline phosphatase. This can greatly simplify the assay operations and improve the concentration range of detection. In addition, this method was applied to determine specific DNA fragments relative to the hepatitis B plasmid by sandwich hybridization.

Experimental

Reagents and materials

Avidin–alkaline phosphatase and dithiothreitol (DTT) were supplied by Sigma (St. Louis, MO, USA). 3-(2′-Spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) was obtained from Tropix (Bedford, MA, USA). Calf thymus DNA, Ficoll 400, bovine serum albumin and sodium dextran sulfate were purchased from Sino-American Biotechnology (Beijing, China). Other chemicals were of analytical-reagent grade.

All oligonucleotides in this study were synthesized by standard phosphoramidite chemistry, responding to a portion of the hepatitis B core antigen DNA segment. Thiolated single-stranded DNA (HS-ssDNA) is a 20-base oligonucleotide with the sequence 5′-HS-$(CH_2)_6$-CTG TTC AAG CCT CCA AGC TG-3′ (immobilized probe). Biotinylated single-stranded DNA consists of two 20-mer ssDNA with biotin attached to the 5′-phosphate end and the sequence 5-CAG CTT GGA GGC TTG AAC AG-3′ (labeled probe A; complementary to the immobilized probe) and 5′-CCG CGT CGC AGA AGA TCT CAA-3′ (labeled probe B; complementary to the DNA fragment; see below). The non-complementary control has the same sequence as the immobilized probe but with biotin instead of HS-$(CH_2)_6$ attachment at the 5′-end.

A 566 base pair (bp) DNA fragment from the hepatitis B virus DNA plasmid was amplified by conventional polymerase chain reaction (PCR) with primers 5′-CTG TTC AAG CCT CCA AGC TG (forward) and 5′-TTG AGA TCT TCT GCG ACG CGG (reverse). PCR amplication was carried out on a PTC-51B

DNA thermocycler (Beijing Institute of Radiation Medicine, Beijing, China) according to the following protocol: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 100 s for 30 cycles. PCR products were purified using an Advantage PCR-Pure Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol.

Instrumentation

Light emission data were collected with an Ultra-Weak Luminescence Analyzer (Beijing Institute of Biophysics, Beijing, China) and data were processed *via* a computer connected to the analyzer. Hybridization experiments were performed with a Model 1339 Hybridization Incubator (Beijing Institute of New Technology Application, Beijing, China). Gold disc electrodes were prepared by potting 1 mm diameter gold wire (supplied by Peiking University, Beijing, China) in epoxy and used for DNA probe immobilization. Prior to the probe immobilization, the gold surfaces were polished with 0.05 μm alumina and cleaned ultrasonically followed by rinsing with distilled water.

Immobilization procedure

Prior to use, the HS-ssDNA was reduced with DTT, as thiols are known to be oxidized to disulfides on prolonged standing.[34] The reduction was carried by reaction of 600 μl of HS-ssDNA (95 μg ml^{-1}) in TE buffer with 20 μl of 0.15 M DTT at room temperature for 60 min. In order to extract excess DTT from aqueous solution, ethyl acetate (3 × 0.6 ml) was added to the mixture. The mixture was vortex mixed for 30 s, centrifuged for 5 min, then the supernatant was removed. Finally, the aqueous solution was used for the immobilization procedure.

The polished gold surfaces were immersed in a solution containing the DNA immobilized probe for the required time at 4 °C and then washed with distilled water to remove the probes that were not adsorbed. The modified gold sensing devices were stored in TE buffer (10 mmol l^{-1} TRIS–HCl, 1 mmol l^{-1} EDTA, pH 8.0).

Hybridization

Hybridization experiments were performed by exposing the gold sensing devices with the immobilized DNA probes to 150 μl of the hybridization buffer [5 × SSC (1 × SSC: 150 mmol l^{-1} NaCl, 15 $mmol^{-1}$ sodium citrate; pH 7) 45% v/v formamide, 20 mM phosphate-buffered saline and 5% w/w sodium dextran sulfate] containing the complementary single-stranded biotinylated DNA. The reaction mixture was shaken for 1 h at 25 °C. After the reaction, the electrodes were sequentially washed with 2 × SSC–0.1% sodium dodecyl sulfate (SDS) and 0.2 × SSC–0.1% SDS for 10 min.

Sandwich hybridization

In the sandwich hybridization procedure, the target DNA was denatured by heating at 95 °C for 5 min followed by rapid cooling on ice for 5 min. A 5 μl sample and 5 μl of labeled probe DNA (probe B) were added to 150 μl of the hybridization buffer. The subsequent hybridization procedure was as described above.

Detection

The gold sensing devices were first incubated for 15 min with 150 μl (0.5 U) of avidin–alkaline phosphatase. After conjugation, they were washed twice for 5 min at room temperature with TRIS buffer (pH 7.5) containing 0.5% w/w Tween 20, followed by immersion in the glass detection cell containing 0.5 ml of 150 μg ml^{-1} AMPPD solution and incubation for 30 min except for the kinetic study. The chemiluminescent intensity was then constantly measured for the required time with the luminescence analyzer.

Results and discussion

Chemiluminescent detection

AMPPD is a direct chemiluminescent substrate for alkaline phosphatase. Enzymatic cleavage of the phosphate group destabilizes the molecule, which decomposes to produce light emission.[31] The rate of light emission is dependent on the enzyme concentration. On the other hand, avidin–alkaline phosphatase could be coupled to DNA probes *via* the avidin–biotin complex. As a result, this chemiluminescent system could be employed to assay oligonucleotides and DNA fragments through the probe hybridization.

A schematic diagram of the detection system for specific nucleic acid sequences using the chemiluminescent biosensor is shown in Fig. 1. Fig. 1(a) illustrates the measuring principle of the biosensor and it was employed to study the characterization of the biosensor and optimize the conditions of probe immobilization on gold surfaces. In addition, this detection approach was also used to study the hybridization and assay procedures. In order to demonstrate further the application possibilities, sandwich hybridization [Fig.1(b)] was developed to assay the specific nucleic acid sequences related to the hepatitis B virus.

$AMP^{-}D$ anion, produced upon dephosphorylation of AMPPD, is moderately stable, and this produces a kinetic delay which precedes the steady-state rate of light emission. It has been reported[31] that a constant rate of chemiluminescence is

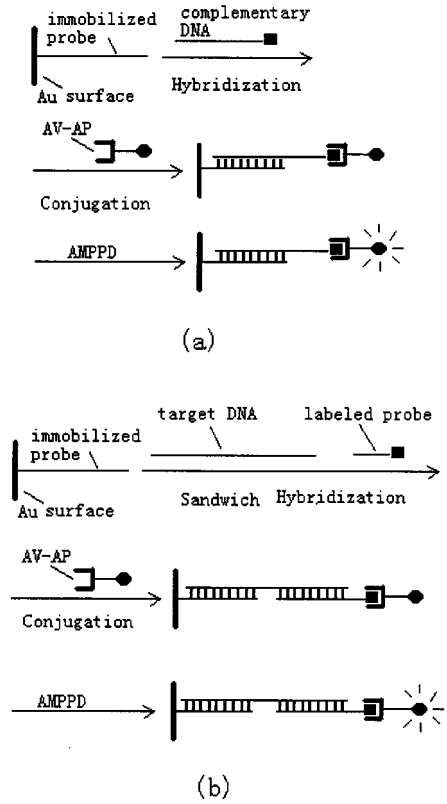

Fig. 1 Schematic representation of the chemiluminescent biosensing assay. (a) Conventional hybridization; (b) sandwich hybridization.

attained in approximately 20 min at pH 9.5. Fig. 2 illustrates the kinetics of the chemiluminescent detection signals. It was found that the time to reach the steady-state rate was related to the nucleic acid concentration. For lower concentration of DNA (*e.g.* <10 ng ml^{-1}), the light emission intensity could reach 90% of the maximum within 10 min. This could be attributed to the small amount of enzyme. For higher concentrations, on the other hand, the light emission intensity gradually increased with time up to 30 min. As a result, the chemiluminescent detection sensitivity became greater with the assay time. Fig. 3 shows that the chemiluminescent intensity response increases continuously with increasing DNA concentration over a dynamic range of about five orders of magnitude. In general, the response signals would reach a plateau after 30 min. Therefore, 30 min was employed as the incubation time in subsequent experiments.

Optimization of analytical conditions

Parameters such as pH and ionic strength of the buffer were investigated in order to optimize the analytical performance. The influence of pH was studied between pH 8.8 and 10.5. When the pH of 50 mmol l^{-1} sodium hydrogencarbonate–carbonate buffer was 9.5, the avidin–phosphatase showed the maximum activity and this buffer was chosen for all subsequent studies. In order to observe the influence of the ionic strength of the solution on the enzyme activity, sodium chloride was added to the substrate buffer at concentrations of 100, 200 and 500 mmol l^{-1}. The enzyme was incubated for 30 min in the above solutions and the chemiluminescent intensity values were 322, 333 and 324, respectively. These results showed that the ionic strength had no significant influence on the enzyme activity. In addition, the dependence of the response signals on the substrate concentration was also observed. At AMPPD concentrations >100 μg ml^{-1}, the chemiluminescent intensity approached a constant value. At these concentrations, all the active sites of the enzyme are occupied by substrate molecules and the reaction rate is dependent only on the enzyme concentration. A 150 μg ml^{-1} concentration was chosen for subsequent studies.

The influence of the immobilization procedure on hybridization was studied and the response signals corresponding to different modification times of the gold sensing device are shown in Fig. 4. Initially the chemiluminescent intensity increased significantly with increasing immersion time in the solution of HS-ssDNA and reached a maximum after about 5 h. This suggested that the amount of complementary DNA coupled to the surface of the electrodes gradually increased through hybridization. With further immersion, however, the hybridization efficiency and the response signals decreased. This could be attributed to steric and electrostatic hindrance arising from the more tightly packed DNA monolayer. Similar phenomena have been reported for immobilization of oligonucleotides on a microtiter plate[35] and the characterization of DNA probes on gold surfaces using X-ray photoelectron spectroscopy.[36] Therefore, an immersion time of 5 h was adopted.

The influence of temperature on the hybridization efficiency was also studied. Different temperatures were used for the hybridization between the gold sensing device and 100 ng ml^{-1} of target probe. The hybridization efficiency was determined using the luminescence analyzer and the relative cheminescent intensities values are shown in Fig. 5. With increase in temperature the response signals reach a maximum at 25 °C and then gradually decrease. In order to study further whether this influence could be related to S–Au dissociation, different temperatures were used to incubate the gold sensing devices for 1 h prior to hybridization. It can be seen from Fig. 5 that this heat

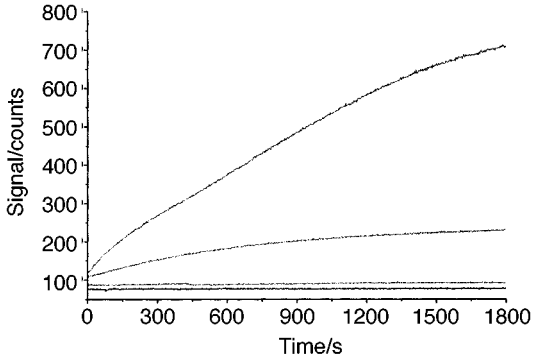

Fig. 2 The kinetics of the chemiluminescent signals. Concentration of the complementary oligonucleotides (from top to bottom): (1) 200; (2) 10; (3) 1.0 ng ml^{-1}; and (4) control DNA.

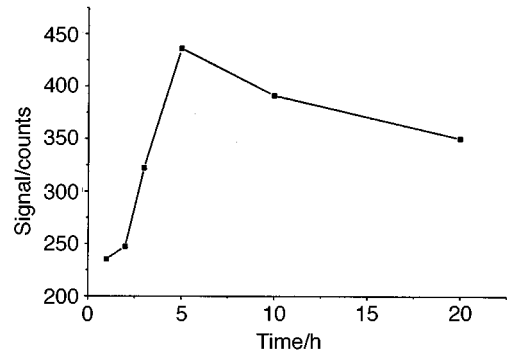

Fig. 4 Effect of modification time of the immobilized probe on chemiluminescent signals. Concentration of the complementary DNA: 100 ng ml^{-1}.

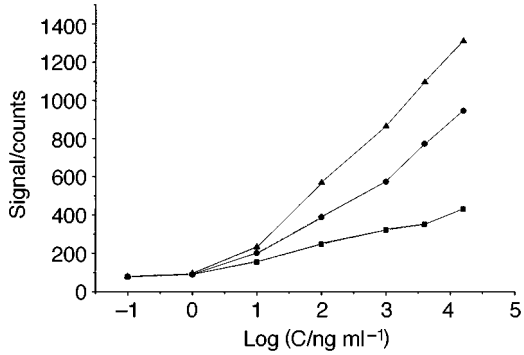

Fig. 3 Plot of the chemiluminescent signals *versus* concentration of the complementary nucleotide. The gold sensing devices were incubated in the substrate AMPPD solution for various times (from bottom to top): (a) 5; (b) 15; and (c) 30 min.

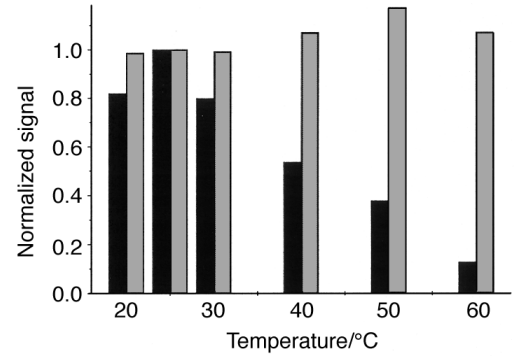

Fig. 5 Effects of hybridization temperature (black bars) and heat process (shaded bars) on the gold sensing device.

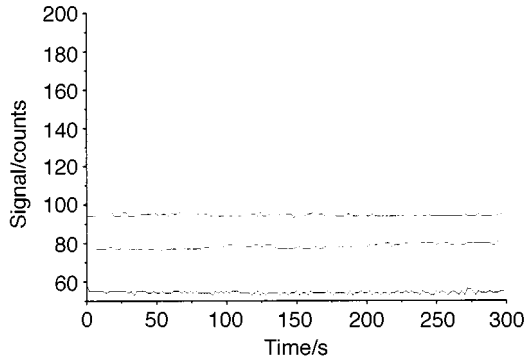

Fig. 6 Chemiluminescent detection of the specific DNA fragment. Concentration of the fragment (from top to bottom): (1) 120 fmol; (2) 60 fmol; and (3) blank.

process exerts less influence on the performance of the gold sensing devices. As a result, the effect of temperature on the hybridization efficiency could be attributed to the dissociation of the hybridized oligomer. Under the above conditions, the detection limit for the complementary DNA is 15 pM (signal-to-noise ratio = 3). The six electrodes modified with the same HS-ssDNA were employed to assay 100 ng ml^{-1} oligonucleotide and the average relative standard deviation was 8.7%.

Analytical application

In order to study further the application possibilities of this chemiluminescent biosensor in nucleic acid assays, a sandwich-type hybridization was developed to analyze specific DNA fragments. In sandwich hybridization [Fig. 1(b)], the DNA of interest would not need to be labeled with biotin in the analytical procedure. The specific nucleic acids could not only be captured by the immobilized probe of the biosensors, but also coupled to alkaline phosphatase through hybridization with the labeled probe B. The 566 bp DNA fragments were employed to be analyzed as a model. Two DNA samples were analyzed by this method and the results are shown in Fig. 6. The amounts of the DNA fragments were about 58.4 ± 5.1 and 122.0 ± 4.6 fmol, respectively. The detection sensitivity in terms of moles is in agreement with those provided by labeled oligonucleotides.

Conclusions

We have developed a new biosensing approach to the nucleic acid detection and this system has been successfully used for the determination of specific DNA fragments relative to hepatitis B DNA. The method has been shown to have a wide response range and low detection limits. In addition, the chemiluminescent detection system is fairly simple and easy to manipulate without the introduction of excitation light. Furthermore, this assay is suitable for automation and may hold promise in constructing multicomponent DNA arrays. Meanwhile, it can be considered a useful tool for the quantitative detection of nucleic acids for diagnostic and research purposes.

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References

1 M. Yang, M. E. McGovern and M. Thompson, *Anal. Chim. Acta*, 1997, **346**, 259.
2 J. Wang, G. Rivas, X. Cai, E. Palecek, P. Nielsen, H. Shiraishi, N. Dontha, D. Luo, C. Parrado, M. Chicharro, P. A. M. Farias, F. S. Valera, D. H. Grant, M. Ozsoz and M. N. Flair, *Anal. Chim. Acta*, 1997, **347**, 1.
3 K. M. Millan, A. J. Spurmanis and S. R. Mikkelsen, *Electroanalysis*, 1992, **4**, 929.
4 K. M. Millan and S. R. Mikkelsen, *Anal. Chem.*, 1993, **65**, 2317.
5 K. M. Millan, A. Saraullo and S. R. Mikkelsen, *Anal. Chem.*, 1994, **66**, 2943.
6 K. Hashimoto, K. Ito and Y. Ishimori, *Anal. Chim. Acta*, 1994, **286**, 219 .
7 J. Wang, X. Cai, B. Tian and H. Shiraishi, *Analyst*, 1996, **121**, 965.
8 J. Wang, X. Cai, G. Rivas, H. Shiraishi, P. A. M. Farias and N. Dontha, *Anal. Chem.*, 1996, **68**, 2629.
9 S. Palanti, G. Marrazza and M. Mascini, *Anal. Lett.*, 1996, **29**, 2309.
10 Y. Mishima, J. Motonaka and S. Ikeda, *Anal. Chim. Acta*, 1997, **345**, 45.
11 J. Wang, X. Cai, G. Rivas, H. Shiraishi and N. Dontha, *Biosens. Bioelectron.*, 1997, **12**, 587.
12 J. Wang, G. Rivas, X. Cai, N. Dontha, H. Shiraishi, D. Luo and F. S. Valera, *Anal. Chim. Acta*, 1997, **337**, 41.
13 K. Hashimoto, K. Ito and Y. Ishimori, *Anal. Chem.*, 1994, **66**, 3830.
14 F. Caruso, E. Rodda, D. N. Furlong and V. Garing, *Sens. Actuators B*, 1997, **41**, 189.
15 C. Nicolini, V. Erokhin, P. Facci, S. Guerzoni, A. Ross and P. Paschkevitsch, *Biosens. Bioelectron.*, 1997, **12**, 613.
16 F. Caruso, E. Rodda and D. N. Furlong, *Anal. Chem.*, 1997, **69**, 2043.
17 J. Wang, P. E. Nielsen, M. Jiang, X. Cai, J. R. Fernandes, D. H. Grant, M. Ozsoz, A. Beglieter and M. Mowat, *Anal. Chem.*, 1997, **69**, 5200.
18 Y. Okahata, M. Kawase, K. Niikura, F. Ohtake, H. Furusawa and Y. Ebara, *Anal. Chem.*, 1998, **70**, 1288.
19 C. E. Jordan, A. G. Frutos, A. J. Thiel and R. M. Corn, *Anal. Chem.*, 1997, **69**, 4939.
20 A. J. Thiel, A. G. Frutos, C. E. Jordan, R. M. Corn and L. M. Smith, *Anal. Chem.*, 1997, **69**, 4948.
21 F. Kleinjung, S. Klussmann, V. A. Erdmann, F. W. Scheller, J. P. Furste and F. F. Bier, *Anal. Chem.*, 1998, **70**, 328.
22 L. Henke, P. A. E. Piunno, A. C. McClure and U. J. Krull, *Anal. Chim. Acta*, 1997, **344**, 201.
23 F. Kleinjung, F. F. Bier, A. Warsinke and F. W. Scheller, *Anal. Chim. Acta*, 1997, **350**, 51.
24 B. G. Healey, R. S. Matson and D. R. Walt, *Anal. Biochem.* 1997, **251**, 270.
25 L. Wodicka, D. Dong, M. Mittmann, M.-H. Ho and D. J. Lockhart, *Nature Biotechnol.*, 1997, **15**, 1359.
26 J. Cheng,E. L. Sheldon, L. Wu, A. Uribe, L. O. Gerrue, J. Carrino, M. J. Heller and J. P. O'Connell, *Nature Biotechnol.*, 1998, **16**, 541.
27 C. E. Edman, D. E. Reymond, D. J. Wu, E. Tu, R. G. Sosnowsk, W. F. Butler, M. Nerenberg and M. J. Heller, *Nucleic Acids Res.*, 1997, **25**, 4907.
28 T. Livache, B. Fouque, A. Roget, J. Marchand, G. Bidan, R. Teoule and G. Mathis, *Anal. Biochem.*, 1998, **255**, 188.
29 D. Proudnikov, E. Timofeev and A. Mirzabekov, *Anal. Biochem.*, 1998, **259**, 34.
30 D. Guschin, G.Yershov, A. Zaslavsky, A. Gemmell, V. Shick, D. Proudnikov, P. Arenkov and A. Mirzabekov, *Anal. Biochem.*, 1997, **250**, 203.
31 I. Bronstein, J. C. Voyta and B. Edwards, *Anal. Biochem.*, 1989, **180**, 95.
32 H. Arakawa, M. Maeda and A. Tsuji, *Anal. Biochem.*, 1991, **199**, 238.
33 S. Girotti, M. Musiani, E. Ferri, G. Gallinella, A. R. Zerbini, G. Gentilomi and S. Venturoli, *Anal. Biochem.*, 1996, **236**, 290.
34 N. J. Geddes, E. M. Paschinger, D. N. Furlong, F. Caruso, C. L. Hoffmann, and J. F. Rabolt, *Thin Solid Films*, 1995, **260**, 192.
35 N. H. L. Chiu, T. K. Christopoulos and J. Peltier, *Analyst*, 1998, **123**,1315.
36 T. M. Herne and M. J. Tarlov, *J. Am. Chem. Soc.*, 1997, **119**, 8916.

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