VIP Advanced Biosensing

DOI: 10.1002/anie.201006793

Direct Genetic Analysis of Ten Cancer Cells: Tuning Sensor Structure and Molecular Probe Design for Efficient mRNA Capture**

*Elizaveta Vasilyeva, Brian Lam, Zhichao Fang, Mark D. Minden, Edward H. Sargent, and Shana O. Kelley**

In order to facilitate the use of biomolecular markers in clinical medicine, devices are urgently needed that are highly sensitive, specific, cost effective, and automated.[1–3] Great strides have been made in this area with elegant sensing systems employing nanomaterials, microfluidics, and increasingly sophisticated device design.[4–7] The direct analysis of most types of clinical samples requires femtomolar detection limits to sense scarce analytes with an acceptably low level of false negatives. Very high levels of specificity are required to ensure low levels of false positives. From a practical perspective, equally important is a streamlined approach to sample workup, since the need for extensive sample processing can diminish the benefits of a sensor's innately high sensitivity and specificity. In the development of a sensing approach, several components can be tuned at the molecular and chemical level to optimize the performance of the system and refine requirements for sample processing. These components include the actual sensors used for detection, the probe molecules that specifically bind a given target, and the approach to sample workup (chemical, enzymatic, mechanical, fluidic, etc).

We recently reported a new approach to biomolecular detection utilizing a microchip-based platform for ultrasensitive nucleic acids detection.[8–10] Nanostructured microsensors deposited on the surface of the chip served as specific detectors of oligonucleotides,[8] mRNAs,[11] and microRNAs[12] when used in conjunction with an electrochemical reporter system (Figure 1).[13] Attomolar sensitivity was obtained with small oligonucleotides and microRNAs.[12]

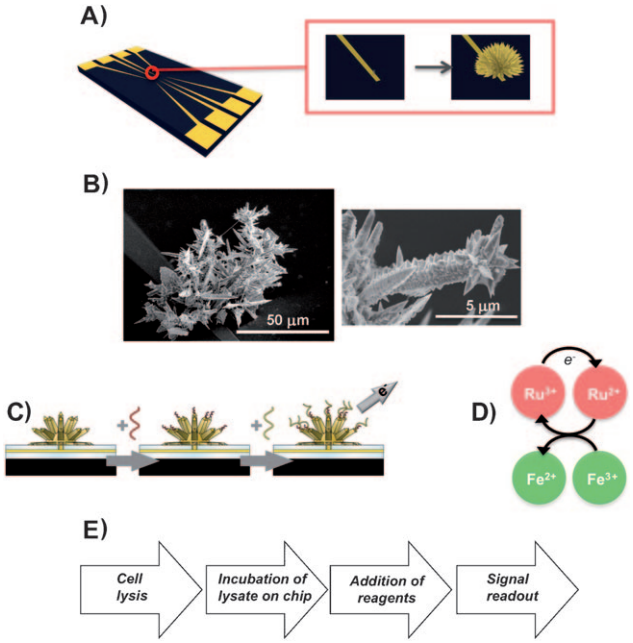

Figure 1. Chip-based sensors for detection of chronic myeloid leukemia cells. A) Chip layout. A gold pattern is deposited on the chip surface with 8 external contacts that are extended to narrow leads of a terminal width of 5 µm. A passivating layer of silicon dioxide is applied to the chip, and then 5 µm apertures are opened at the end of each lead to provide a microelectrode template. B) Scanning electron micrograph of a 100 µm sensor formed by using gold electrodepositon on the surface of the chip. C) Sequence of steps used to nucleic acids analysis. The sensors are first functionalized with probe molecules, and then hybridized with a target-containing solution. D) The introduction of the a Ru^{III}/Fe^{III} reporter group then permits hybridized material to be detected. E) Overall flow diagram of analysis trial.

Higher limits of detection were observed when heterogeneous samples of RNA from cancer cells were analyzed for specific mRNAs: nanograms of RNA, corresponding to millions of cancer cells, were required for analysis.[11]

Here, we report a series of discoveries that have enabled the genetic analysis of as few as 10 cancer cells. To achieve this record-breaking level of sensitivity, we generated large-footprint sensors that were able to capture large, slow-moving analytes. We also developed an improved class of probe molecules: amino acid/nucleic acid chimeras (ANAs) that were necessary in order to detect a specific cancer biomarker. ANAs overcome three fundamental limitations that we found arise when using neutral probe molecules: poor solubility, aggregation, and poor monolayer quality. The resulting sensor

[*] E. Vasilyeva, Prof. S. O. Kelley
Department of Biochemistry, University of Toronto
Toronto, ON (Canada)
E-mail: shana.kelley@utoronto.ca
B. Lam, Prof. S. O. Kelley
Department of Chemistry, University of Toronto (Canada)
Z. Fang, Prof. S. O. Kelley
Department of Pharmaceutical Sciences
University of Toronto (Canada)
Prof. M. D. Minden
Ontario Cancer Institute, University Health Network
Toronto, ON (Canada)
Prof. E. H. Sargent
Department of Electrical and Computer Engineering
University of Toronto (Canada)

[**] We wish to acknowledge the Canadian Institute of Health Research, NSERC, and the Ontario Institute for Cancer Research for their generous support of this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201006793.

system reported herein displays excellent sensitivity and specificity. Remarkably, it achieves this excellent performance even when analyzing *unpurified* samples—the only sample processing step required is cell lysis.

We identified the *bcr-abl* gene fusion that is specific to chronic myeloid leukemia (CML) as an interesting model system to hone the capabilities of our chip-based sensors so that small numbers of cancer cells could be analyzed. The *bcr-abl* mRNA transcript is a RNA molecule that is 8500 nucleotides long.[13] Targeting mRNA is attractive given that most mRNAs exist as linear sequences and therefore denaturation is not required. Multiple copies of mRNAs are typically present within a cell, providing built-in amplification at the cellular level, improving the prospects for the direct analysis of small numbers of cells. However, the large sizes of mRNAs present a challenge for chip-based sensing as the slow diffusion of such large molecules can impede rapid analysis. We took the view that to aid in the detection of large molecules, it would be important to increase the reach of the sensor into the solution volume, thus providing greater interaction between the target molecules in solution and the probe molecules tethered to the sensor surface. Using existing diffusional models,[14,15] and taking into consideration the copy number of the *bcr-abl* fusion mRNA (ca. 3000 copies per cell)[16] and its rate of diffusion, we determined that if we were able to increase spatial footprint of our sensors to approximately 100 μm, we would be able to detect as few as 10 CML cells within analysis times approaching 30 min.

Our previous work on chip-based microsensors focused on palladium sensors that were 5–10 μm in diameter.[8] To extend the reach of these sensors into solution, it was necessary to use gold as the electrode material. While electroplated palladium did not produce grain sizes that allowed growth of the sensor into solution, the electroplating of gold produced spiky structures with large substructures extending many μm into solution when low plating potentials were used (see Figure S1 in the Supporting Information). After extensive variation of plating conditions, sensors with the desired 100 μm footprint were obtained (Figure 1 B) by applying a potential of 0 mV for 175 s in a solution of 20 mM H_2AuCl_4 in 0.5 M HCl.

In order to test these sensors against the *bcr-abl* mRNA target, probes that would specifically bind to the junction region between the two fused genes were required. Initial efforts to produce a functional probe for this region (Figure 2) included the synthesis of DNA and PNA probes. When challenged with mRNA from the K562 cell line, which contains the most common form of the *bcr-abl* gene fusion, hybridization was not detected with either probe. Hybridization analysis was carried out by monitoring currents in a solution containing a Ru^{III}/Fe^{III} electrocatalytic reporter system.[16] The solution used in these trials contained 1 ng μL^{-1} of total mRNA, which contained > 600000 copies of the target per μL, or approximately 1 pM—a concentration that should have been readily detectable based on our calculations. The DNA probe-modified sensors exhibited high background signals and small decreases in current upon introduction of the mRNA solution. The PNA-modified sensors exhibited lower background currents, but here too, only small decreases in current were observed upon hybridization with mRNA.

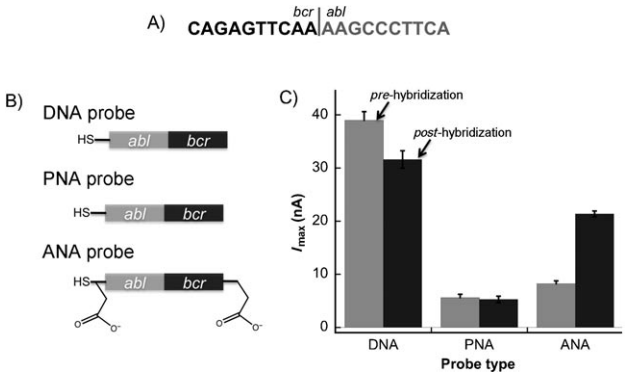

Figure 2. Testing of probes for the CML *bcr-abl* gene fusion. A) The junction region between the *bcr* gene (dark gray) and the *abl* gene (light gray) within the mRNA expressed that generates the *bcr-abl* kinase. B) Structures of DNA, PNA, and ANA (amino acid/nucleic acids chimera) probes. C) Testing of DNA, PNA, and ANA probes for hybridization of mRNA isolated from the K562 cell line that carries the *bcr-abl* gene fusion. The solutions used to challenge each probe contained 1 ng of RNA per μL of solution. See Figure S2 in the Supporting Information for representative electrochemical data used. Each electrochemical assay reported was carried out in > 6 independent trials and error bars represent standard error.

PNA probes, which carry no molecular charge at neutral pH, offer many advantages when used for biosensing. One key advantage is the increase in binding affinity for target DNA or RNA sequences.[17] Moreover, when sensing schemes are used that rely on changes in surface charge upon the binding of a nucleic acid to a sensor, lowered background signals, and consequently improved limits of detection, can be obtained because of the absence of charge in the probe. Our electrocatalytic reporter system, which relies on the attraction of ruthenium ions to the sensor surface by nucleic acids captured by immobilized probe molecules, has been shown to benefit from this effect.[18]

In the present case, where there are stringent requirements on the specific sequence employed, PNA probes performed poorly. As discussed above, the probes were found not to participate in hybridization when immobilized on our sensors; moreover, we often observed precipitation within the solution of the original probe. From these observations, it was apparent that the sequence was prone to aggregation. We concluded that the specific sequence mandated by this particular biosensing application was not usable when synthesized as a neutral molecule.

We sought to develop a strategy to take the prescribed *bcr-abl* probe sequence and generate a novel probe molecule that would exhibit the needed solubility and monolayer-forming properties. We hypothesized that the introduction of negatively charged amino acids at the termini of the sequence would beneficially alter the probe's properties.

This subtle modification—the inclusion of two aspartic acid units—rectified the behavior of our probe in that it was now highly soluble in aqueous solution, and exhibited much better performance when used to detect the *bcr-abl* mRNA. Low background currents were observed at probe-modified

sensors, and significant increases occurred upon hybridization of K562 mRNA containing the fusion (Figure 2C).

With our optimized sensors and probes in hand, we investigated the limit of detection and dynamic range when the system was challenged with purified cellular mRNA. A control probe that was only half-complementary to the target (*e13a2*) was used alongside *bcr-abl* probe (*e14a2*) and showed no signal change even at the highest concentration tested, indicating that the assay had excellent specificity. Detectable signal was observed as low as 1 pg μL^{-1} of total mRNA (Figure 3) and the signal increased linearly over four decades

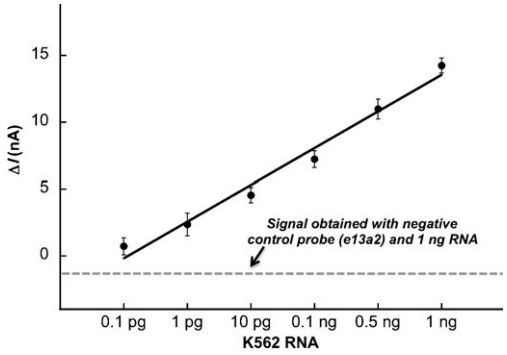

Figure 3. Sensitivity of sensors modified with an ANA probe towards the mRNA from the K562 cell line that carries the *bcr-abl* gene fusion. The specificity of the readout was confirmed using a control probe (*e13a2*). The low level of negative signal change observed when this probe was tested against 1 ng RNA is indicated by the dotted line.

of target concentration. The pg μL^{-1} detection limit observed corresponds to mRNA from 30 cells. This result is comparable with a commercially available polymerase chain reaction (PCR) assay designed to specifically detect CML; the assay has a similar detection limit.[19] A direct comparison of data generated with this assay is compared to microelectrode sensing in Figure S3. This chip-based system is the first ever to exhibit PCR-like sensitivity towards large, slowly diffusing targets like mRNAs.

While PCR is a highly sensitive technique for sequence detection, it often requires extensive sample processing and nucleic acid purification to eliminate interferents that inhibitors of the enzymes used for amplification. Given that our detection system does not rely on any enzymatic reactions, we hypothesized that it might be much more tolerant of unpurified samples. To test whether it is possible to detect fusion mRNA from an unpurified sample, we lysed K562 cells suspended in buffer using an electric field.[20] We achieved rapid lysis without the use of added reagents. Lysates were generated containing 10 to 1000 cells; the results of their incubation with *bcr-abl* probe-modified sensors are illustrated in Figure 4A. The negative control probe again showed no signal increase, confirming the specificity of the assay. The detection of 10 cells—present as an unpurified lysate—indicates that these sensors are highly sensitive and robust.

We then proceeded to analyze leukocytes from CML patients using the same lysis-only sample preparation procedure and the sensor and assay described above. Leukocytes

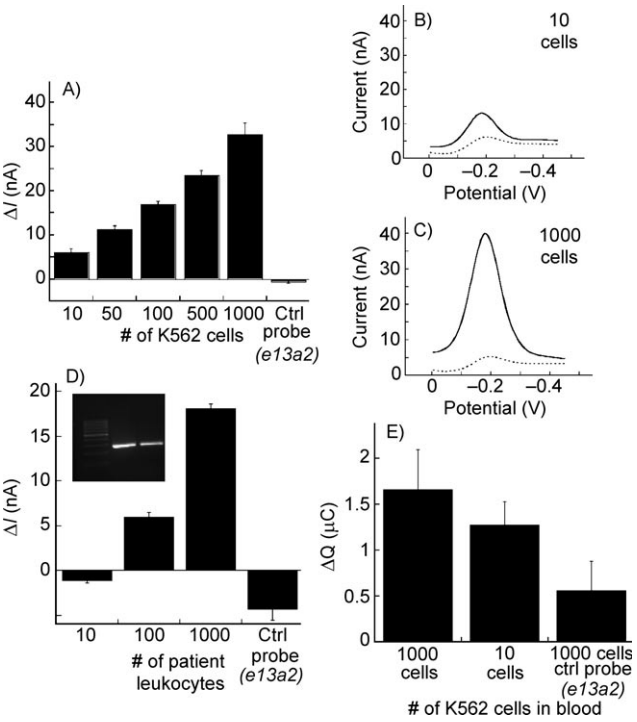

Figure 4. Performance of ANA-modified sensors when challenged with crude cell lysates. A) K562 lysates. Signals obtained before and after incubation of probe-modified microelectrodes with lysates of K562 cells were compared by monitoring the limiting reductive current in a Ru^{III}/Fe^{III} electrocatalysis solution. A negative control probe (*e13a2*) was used to assess specificity. Low levels of background were also observed when VCaP and DU145 cells were tested that lack the fusion. B,C) Representative differential pulse voltammograms showing the change in signal when lysates containing 10 and 1000 cells were introduced. Dotted lines represent the signal collected before hybridization, and the solid lines are signals collected after hybridization. D) Detection of the *bcr-abl* gene fusion in CML patient leukocytes. Lysates were generated from 10–1000 cells, and signal changes monitored after 30 min. The presence of the *bcr-abl* fusion was confirmed using PCR (inset). Primers specific to each gene were used to analyze a fragment of the fusion, and used with K562 cells (center band) and patient cells (right band). E) Detection of the *bcr-abl* gene fusion in whole blood. A lysate of K562 cells was mixed with blood lysate and incubated with sensors displaying a fully complementary or half-complementary (control) probe for 30 min.

were suspended in buffer for lysis, and the lysate was incubated with probe-modified sensors for 30 min. Absence of a positive signal with the half-complementary negative control again confirmed the specificity of hybridization. Here the detection limit approached 100 cells; the fact that this detection limit is elevated relative to that obtained with the pure K562 cells relates to the fact that the patient sample contained both CML cells and normal leukocytes, with only the former cell type carrying the gene fusion.

Our ultimate challenge to the system lay in analyzing CML samples in whole blood. Analysis of complex samples is always challenging due to their heterogeneity: in particular, direct analysis in blood is impeded rapid degradation of nucleic acids by nucleases, and by the fouling of surfaces by the components of blood. We sought to challenge our system

with blood spiked with CML cells to determine whether the analysis of whole blood samples would be feasible.

We observed an offset of the background signal when we used blood only. We therefore introduced the use of a control probe to follow this shift. By referencing our measurements for the target-probe case to the control-probe case, we were able to detect as few as 10 K562 cells in the presence of a 5000000-fold excess of nucleated blood cells (Figure 4E).

This study documents the highly sensitive, specific analysis of nucleic acid biomarkers in complex samples. It relies on direct lysis of the cells under study within a medium, for example blood, that contains a large excess of noncomplementary molecules. Specifically, we were able detect mRNA derived from 10 cells present as an unpurified, unprocessed lysate. The optimization of the sensing electrode geometry, and the development of improved probe chemistry, were necessary for this advance.

Experimental Section

Gold(III) chloride (99.9%), hexaammineruthenium(III) chloride, potassium ferricyanide, magnesium chloride, and 6-mercapto-1-hexanol (97%), 10× TBE buffer, Ultra Pure Agarose, dimethylformamide, piperidine, TFA, *m*-cresol, TIPS, diethyl ether, acetonitrile, and DTT were purchased from Sigma–Aldrich Canada Ltd.; 70% perchloric acid, sulfuric acid, ACS-grade acetone, and isopropyl alcohol were obtained from EMD. PNA monomers were purchased from Link technologies, Knorr resin was purchased from NovaBiochem; HATU and *N*-methylmorpholine were purchased from Protein Technologies, Inc., and RedSafe from FroggaBio; K562 cell line was obtained from ATCC, 25 mL suspension flasks were purchased from Sarstedt, Iscove's Modified Dulbecco culture medium and fetal bovine serum were obtained from Invitrogen; CML patient samples were provided by Princess Margaret Hospital, whole blood was obtained at Princess Margaret Hospital blood laboratory.

Peptide nucleic acid oligonucleotides were synthesized using solid-phase synthesis approach on a Prelude automated peptide synthesizer (Protein Technologies, Inc.). Control ANA Probe corresponding to *e13a2*: NH_2-Cys-Gly-Asp-TGAAGGGCTTCTTCCT-TATT-Asp-$CONH_2$ and *bcr-abl* ANA probe corresponding to *e14a2*: NH_2-Cys-Gly-Asp-TGAAGGGCTTTTGAACTCTG-Asp-$CONH_2$. PNA probes contained the same sequence but lacked the Asp residues. DNA probe was purchased from ACGT and a thiol-containing linker was added in-house: TGAAGGGCTTTT-GAACTCTG-linker-SH. All probe molecules were stringently purified using an Agilent 1100 series HPLC. Concentration was determined by measuring absorbance at 260 nm.

The chips were produced at the Canadian Photonics Fabrication Center as described in reference [8]. The one modification made in the processing was the enlargement of the aperatures for sensor growth to 5 μm. Chips and microelectrodes were prepared as described in reference [8]. Electrodeposition of gold microelectrodes were accomplished by dipping the chip into the plating solution (20 mM H_2AuCl_4 in 0.5 M hydrochloric acid) and applying constant of 0 mV for 175 s.

A solution containing 5 μM thiolated PNA probe in 50 mM sodium chloride was added to the sensors and left in a dark humidity chamber overnight at room temperature for self-assembly of a monolayer. A solution of 10 μM MCH was then added to each chip for 1 h at room temperature. The chip was then washed twice with 50 mM NaCl.

Electrochemical signals were measured in a solution containing 10 μM $[Ru(NH_3)_6]^{3+}$ and 2 mM $[Fe(CN)_6]^{3-}$ in 1× PBS. Differential pulse voltammetry signals before and after hybridization were collected with a scan rate of 100 mV s^{-1} and scanned from 0 mV to −350 mV. Results were quantified by subtracting peak currents in DPV scans as follows, $\Delta I = I_{\text{after hybridization}} - I_{\text{before hybridization}}$.

K562 cells were cultured in 25 mL suspension cell flasks with vent caps in Iscove's Modified Dulbecco's Medium/10% fetal bovine serum. The cells were grown in a humidified incubator (70–95%) at 37.0 °C with CO_2 (5%).

K562 cells were collected and centrifuged at 600 rcf for 5 min at 4 °C. The media was then removed and the cells were washed with equal volume of 1× PBS. The cell pellet was then resuspended in 1× PBS and used for lysis. CML patient sample frozen stocks were thawed quickly at 37 °C in a water bath. Immediately, the cells were added to 10 mL of fresh media supplemented with 10% FBS and centrifuged at 400 rcf for 5 min at 4 °C. The pellet was was resuspended in 1× PBS for further use. Primary samples were collected following informed consent according to an REB approved protocol.

Total mRNA was isolated from cells using Dynabeads (Invitrogen). Quality of the mRNA sample was tested using 1% agarose gel electrophoresis.

Cells lysis (K562, patient samples, whole blood) was achieved using an electrical lysis chamber (see ref. [20]). Pt wires used to produce the electric field were inserted into PDMS (polydimethylsiloxane) membrane. Resuspended cells were loaded into a syringe pump. In case of whole blood, it was diluted 100 times in 1× PBS and 1 mL was loaded into a 5 mL syringe. Lysis was achieved at a flow rate 25uL min^{-1}, 400 V, and 1 mA current.

Hybridization solutions contained either total mRNA, or unpurified cell lysate in 50 mM NaCl. Electrodes were incubated with the target sequences at 37 °C in a humidity chamber in the dark for 30 min and were washed prior to electrochemical analysis. Hybridization volume was typically 30 μL.

Received: October 28, 2010
Revised: January 2, 2011
Published online: April 6, 2011

Keywords: biosensor · microchips · microelectrodes · nucleic acids

[1] J. Bell, *Nature* **2004**, *429*, 453.
[2] D. R. Walt, *Science* **2005**, *308*, 217.
[3] A. D. Weston, L. Hood, *J. Proteome Res.* **2004**, *3*, 179.
[4] C. S. Thaxton, R. Elghanian, A. D. Thomas, S. I. Stoeva, J. S. Lee, N. D. Smith, A. J. Schaeffer, H. Klocker, W. Horninger, G. Bartsch, C. A. Mirkin, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18437.
[5] N. A. Mousa, M. J. Jebrail, H. Yang, M. Abdelgawad, P. Metalnikov, J. Chen, A. R. Wheeler, R. F. Casper, *Sci. Transl. Med.* **2009**, *1*, 6.
[6] D. M. Rissin, C. W. Kan, T. G. Campbell, S. C. Howes, D. R. Fournier, L. Song, T. Piech, P. P. Patel, L. Chang, A. J. Rivnak, E. P. Ferrell, J. D. Randall, G. K. Provuncher, D. R. Walt, D. C. Duffy, *Nat. Biotechnol.* **2010**, *28*, 595.
[7] E. Pavlovic, R. Y. Lai, T. T. Wi, B. S. Ferguson, R. Sun, K. W. Paxco, H. T. Soh, *Langmuir* **2008**, *24*, 1102.
[8] L. Soleymani, Z. Fang, E. H. Sargent, S. O. Kelley, *Nat. Nanotechnol.* **2009**, *4*, 844.
[9] L. Soleymani, Z. Fang, X. Sun, H. Yang, B. J. Taft, E. H. Sargent, S. O. Kelley, *Angew. Chem.* **2009**, *121*, 8609; *Angew. Chem. Int. Ed.* **2009**, *48*, 8457.
[10] L. Soleymani, Z. Fang, S. O. Kelley, E. H. Sargent, *Appl. Phys. Lett.* **2009**, *95*, 143701.
[11] Z. Fang, L. Soleymani, G. Pampalakis, M. Yoshimoto, J. A. Squire, E. H. Sargent, S. O. Kelley, *ACS Nano* **2009**, *3*, 3207.

[12] H. Yang, A. Hui, G. Pampalakis, L. Soleymani, F.-F. Liu, E. H. Sargent, S. O. Kelley, *Angew. Chem.* **2009**, *121*, 8613; *Angew. Chem. Int. Ed.* **2009**, *48*, 8461.
[13] F. vanRhee, A. Hochhaus, F. Lin, J. V. Melo, J. M. Goldman, N. C. P. Cross, *Blood* **1996**, *87*, 5213 – 5217.
[14] P. E. Sheehan, L. J. Whitman, *Nano Lett.* **2005**, *5*, 803 – 807.
[15] P. R. Nair, M. A. Alam, *Nano Lett.* **2008**, *8*, 1281.
[16] M. A. Lapierre, M. M. O'Keefe, B. J. Taft, S. O. Kelley, *Anal. Chem.* **2003**, *75*, 6327.
[17] O. Brandt, J. D. Hoheisel, *Trends Biotechnol.* **2004**, *22*, 617.
[18] Z. Fang, S. O. Kelley, *Anal. Chem.* **2009**, *81*, 612.
[19] Z. Jobbagy, R. van Atta, K. M. Murphy, J. R. Eshelman, C. D. Gocke, *J. Mol. Diagn.* **2007**, *9*, 220.
[20] H. Y. Chang, A. K. Bhunia, C. Lu, *Biosens. Bioelectron.* **2006**, *22*, 582.

