Microfluidic electrochemical multiplex detection of bladder cancer DNA markers

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Research Highlights

- Free-base porphyrin tagged DNA is demonstrated to be a superior electrochemical sensor for DNA detection in microfluidic systems.
- A molecular beacon was used to reliably detect DNA sequences associated with bladder cancer within 20 minutes.
- The multiplexing microchannel device with 20 electrochemical sensors simultaneously detects different DNA sequences with a detection limit of 250 fM.

Keywords

Multiplex DNA detection; bladder cancer; microfluidic microchip sensor; porphyrin labelled DNA; voltammetry

Abstract

Bladder cancer (BC) is one of the most difficult cancers to diagnose, and is reportedly the most expensive cancer to treat and monitor due to the lack of accurate chemical sensors; only limited methods are available, which usually are restricted to cytology and cystoscopy. Thus a system is urgently needed to detect biomarkers that give an unambiguous diagnosis on BC, as it currently is among the top five cancers occurring. Here we present a multi-sensor microchip array which efficiently detects three specific bladder cancer DNA markers simultaneously with a detection limit of 250 fM, which is well below the amount of DNA markers found in urine samples. The detection is based on the electrochemical response of a free-base porphyrin marker which is embedded in a molecular beacon; this system can easily be adapted to detect other DNA markers and developed for field applications, including point-of-care diagnostics, and gives an unambiguous readout within 20 minutes.

1. Introduction

Bladder cancer (BC) is the fourth most common form of cancer [1], and cystoscopy is currently used as the gold standard for detection of BC. Besides from being invasive to the patient, this method is expensive and not very specific. Estimated overall costs for treatment and monitoring range between $90,000 and $200,000 per patient (pp) in the US (long term), due to the high rate of recurrence and disease progression which necessitates careful long-term monitoring [2, 3]. As a result of this, BC turns out to be the most expensive cancer to treat per patient. Therefore a non-invasive, affordable and reliable detection system is desired to reduce the screening cost, in order to ease economic burden on societies, and the detection of cancer specific DNA markers is a viable way in this direction. DNA methylation (5-MeC) is known to be a key epigenetic change in many different forms of cancer [4]. The hypermethylation of CpG islands, which occurs near the promoter region of a gene, is known to silence tumour suppressor genes and ultimately lead to the development of tumours. In cancer patients, free DNA in serum or urine is increased to a mean of 180 ng/mL compared to 13 ng/mL in healthy individuals [5], which is the low pM concentration range. A high content of methylated DNA has been reported in BC patients compared to healthy individuals [6, 7]. Three genes which are commonly methylated are epithelial cadherin (*E. Cad*), death associated protein kinase (*DAPK*)

and retinoic acid receptor beta (*RARβ*). These three genes are known as a tumour suppressor gene (*E. Cad*), a mediator of cell death (*DAPK*) and a mediator of cell growth gene (*RARβ*) respectively. Detection of these genes simultaneously in voided urine has been reported to diagnose BC with a 90.9% accuracy at a detection limit of 10 ng/mL [7]. However, this was combined with methylation specific PCR (MS-PCR), and multiple rounds of amplification steps are required to increase the amount of the target DNA for detection by a standard sensor. Therefore, what is required is a highly sensitive multiplex sensor, able to detect several DNA sequences in parallel, which eventually can be combined with MS-PCR but requiring only a single MS-PCR round.

Electrochemical sensors have attracted much attention due to their cheap production cost and potential to be miniaturised [8], and labelling a DNA probe with a redox marker allows for low detection limits [9]. We have shown previously that cobalt porphyrin-tagged DNA provides an ideal, stable probe to detect H5N1 virus specific DNA with a detection limit ~20 fM (accounting to ~1000 DNA molecules on the microelectrode) [10]. The use of an internal marker avoids addition of external redox markers and washing steps. We have also reported a biophotonic silicon sensor for BC biomarker detection of *DAPK*, *E. Cad* and *RARβ* where the focus was on singleplex detection, achieving a detection limit of 10 nM [11]. Here we report a multiplex electrochemical sensor with simple operational procedure and greatly improved sensitivity. A hairpin probe with a free-base porphyrin tag was immobilised on an array of gold sensors for the simultaneous detection of the three BC biomarkers. The results show that the sensor can detect all three markers in a single run with total operating time of 20 minutes and a detection limit of 250 fM.

The DNA probe used is based on a hairpin design, where the molecular beacon will respond to hybridisation with the target strand through a change in structure, and with it a change in signal (current) will show an unambiguous response to the matched target (Fig. 1). The probe itself is immobilised on the gold surface of the electrode by a total of four sulphur-gold bonds for added stability, arising from the addition of two dithio-serinol groups at the 5'-end of the probe DNA (see section 2. Materials and Methods for details of the sequences of the DNA strands).
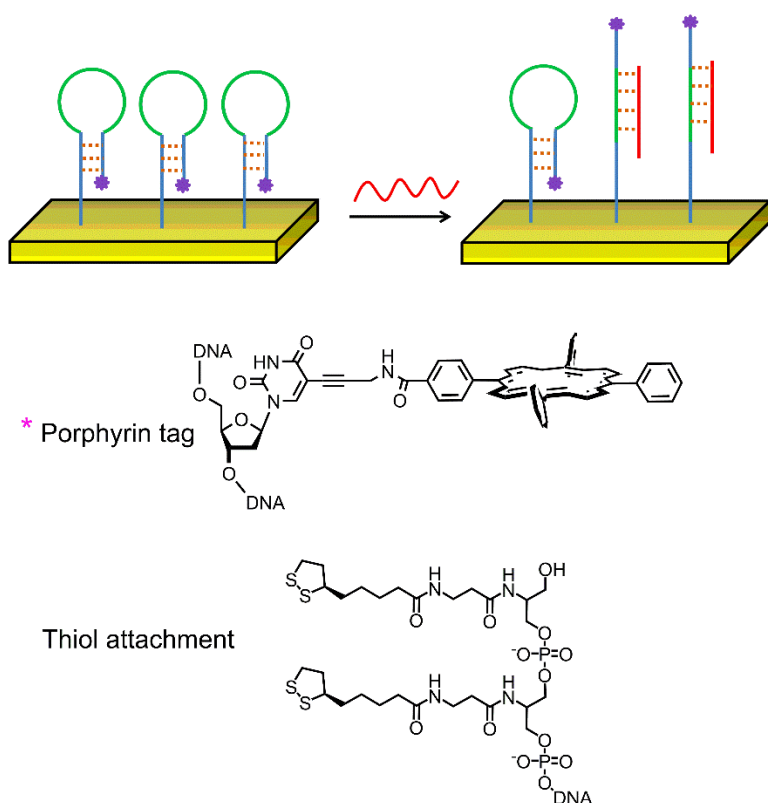
Fig. 1. Schematic of the working principle of the DNA hairpin probe, which is immobilised on gold micro-electrodes using dithiol groups. The porphyrin modifier acting as electrochemical sensor is shown; for sequences see Table 1.

2. Materials and Methods

2.1. Chemicals and Oligonucleotides

Sodium chloride, sodium citrate, 6-mercaptohexanol (MCH) (analytical grade) and Surine™ Negative Urine Control were obtained grade from Sigma-Aldrich (UK) and used as received. DNA synthesis phosphoramidites, solvents and reagents were obtained from Link Technologies (UK), CamBio (UK) or Tides Service Technology (GER).

The oligonucleotide sequences of *E. Cad*, *DAPK* and *RARβ* were synthesised using standard phosphoramidite solid phase DNA synthesis. Table 1 shows the sequences of DNA probes and targets used. The target sequences correspond to methylated cancer marker strands while the mismatched sequences correspond to the unmethylated strands, both as they would have been obtained after MS-PCR [11]. After MS-PCR, the unmethylated DNA sequence will contain a C to U transformation, whereas the 5-MeC will remain unchanged. The amplification of U as

T (and the concomitant G to A transformation in the complementary strand) then allows to distinguish methylated from unmethylated CpGs [12, 13]. Therefore the probes will contain C while the target will have G and mismatch will have A. DNA probes were synthesised based on a hairpin structure (Table 1, Fig. 1). The loop of the hairpin is complementary to the target cancer marker DNA sequence. The porphyrin was incorporated into DNA according to previously published procedures [14, 15]. Two dithio-serinol groups were added at the 5'-end for stable attachment of the probe to the gold surface. The strands were purified by HPLC using a gradient of HFIP-TEA (pH 7) buffer and methanol.

Table 1

Sequences of the hairpin and probe DNA strands (5' − 3'). The stem parts of the hairpin are underlined, the position of the transformed C is in italic, and the position of the porphyrin tag (**P**) is shown in bold.

Probe	DNA sequence
DAPK	Dithiol-<u>AAGGCGC</u>GGAGGATAGT_C_GGAT_C_GAGTTAA_C_GT_C_<u>GCGCC</u>**P**T
E. Cadherin	Dithiol-<u>AAGGCGC</u>TAATTTTAGGTTTAGAGGGTTAT_C_G_C_<u>GCGCC</u>**P**T
RARβ	Dithiol-<u>AAGGCGC</u>GGTTAGTAGTT_C_GGGTAGGGTTTAT_C_<u>GCGCC</u>**P**T
Target	
DAPK	GACGTTAACTCGATCCGACTATCCTCC
E. Cadherin	CGCGATAACCCTCTAAACCTAAAATTA
RARβ	GATAAACCCTACCCGAACTACTAACC
Mismatched sequence	
DAPK	AACATTAACTCAATCCAACTATCCTCC
E. Cadherin	CACAATAACCCTCTAAACCTAAAATTA
RARβ	AATAAACCCTACCCAAACTACTAACC

2.2 Surface Plasmon Resonance (SPR)

SPR was carried out using a Biacore® X100. Blank gold chips were used as received. _DAPK_, _E. Cad_ or _RARβ_ probes (1 µM) were immobilised onto the chip in buffer (0.1 M NaCl, 0.01 M

sodium citrate) over four manual runs of 1080 seconds. 1 mM MCH was added before the target DNA to reduce nonspecific binding. Target DNA was added using the immobilisation buffer (0.1M NaCl, 0.01 M sodium citrate) to the chip on a manual run of 1080 seconds.

2.3 Electrode Fabrication and Microfluidic Design

The array of the gold sensor microchip was designed to have 20 individual sensors and was fabricated based on the process previously discussed [16, 17]. Briefly, a SiO_2 layer was deposited on a silicon layer, and Ti/Au was added via PVD. Lithography was used to define the electrodes before wet etching to form the electrodes. The surface was then passivated before the bonding pads were opened.
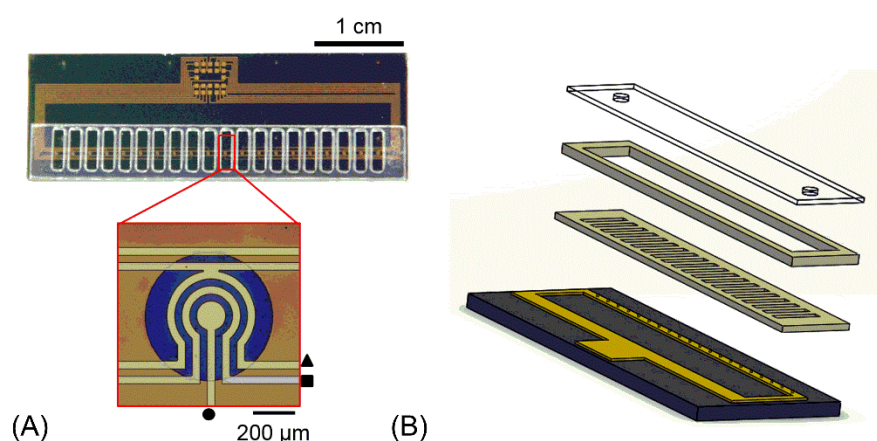

Fig. 2. Design of the microfluidic sensor. (A) Image of device used with an array of 20 sensors including a magnified image of an individual sensor with working electrode (●), reference electrode (■) and counter electrode (▲), scale bars indicate the length scale. (B) Schematic of the electrode device and the microfluidic channel design including assembly parts.

The microfluidic channel design consisted of three layers as shown in Fig. 1. The bottom two layers (45 mm x 7 mm) were made from double sided adhesive while the top layer was a thin sheet of plastic. Laser cutting was used to fabricate the double sided adhesive and the plastic sheet. The bottom layer was cut into an array of 20 individual sections, forming a chamber around each sensor pad. The middle section of the double sided adhesive was used as a spacer

to create a channel which connects all sensors. Circular holes (diameter 1.4 mm) were made in the top plastic layer to fit tubing for the fluid inlet and outlet. The adhesive spacer and the top plastic sheet were assembled once the probe immobilisation was done (see below). Confirmation that all sensors are exposed to an analyte solution was confirmed by injecting a dye solution *via* the inlet.

2.4 Immobilisation of Probe

Prior to assembly of the microfluidic channel, the microchips were cleaned by immersing in acetone and sonicating for 10 minutes, followed by rinsing with IPA and thorough washing with de-ionised water. The microchip was then treated with UV light and ozone for 20 minutes. The bottom layer of the fluidic channel was pasted onto the microchip to form separate chambers surrounding each sensor pad. The DNA probe (5 µL, 1 µM in 1M NaCl, 0.1M sodium citrate) was immobilised on the microchip for 5 hours. The sensor array allows for simultaneous detection of the three different probes, including mismatch sequence, and in triplicate to ensure reproducibility. With this, 18 sensor pads were used, leaving the first and last sensors blank for background measurement. Unbound probe was washed off the microchip using the buffer solution (1M NaCl, 0.1M Sodium citrate). To reduce nonspecific binding, the microchip was incubated with 1 mM 6-mercaptohexanol solution for 30 minutes. The adhesive spacer and top plastic sheet layers were then assembled to finish the microchip. Target DNA solutions of various concentrations were injected into the microfluidic channel and left to hybridise for 20 minutes before measuring.

2.5 Electrochemical Measurements

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out using a three electrode configuration. Gold electrodes were used as pseudo reference and counter electrodes. The instrument used was Reference 600™ (Gamry Instrument, USA). CV was carried out with a scan rate of 50 mV/s in the range of 0.2 V to -0.8 V. The device signal output corresponds to the redox couple $P^{\cdot-}/P$. EIS measurements were recorded using a frequency range of 0.5 Hz – 500 kHz with an amplitude of 25 mV, using a redox probe solution (5 mM $[Fe(CN)_6]^{3-/4-}$, pH 7.4). Current measurements in the sensor was performed without redox probe; sample introduction was done either using citrate buffer (see above) which gave

a higher signal response than PBS buffer, or in untreated Surine™ Negative Urine Control; the response for both systems was identical. All experiments were performed in triplicate. The electrochemical response of the porphyrin tagged probe compared to the unmodified DNA probe is shown below in Fig. 3.
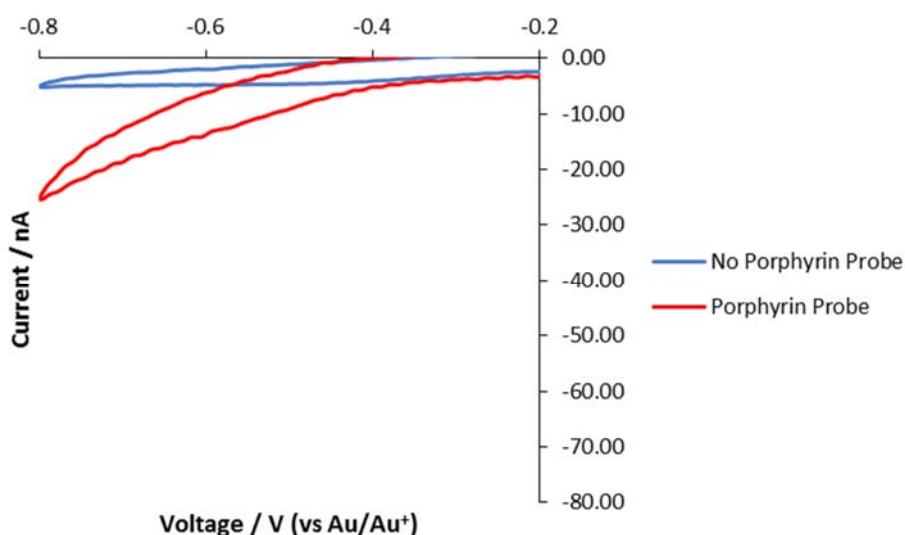

Fig. 3. Current measurement of the porphyrin tagged hairpin DNA probe (red line) in absence of target DNA strands. The blue line shows the response of the unmodified hairpin DNA probe which is essentially zero.

3. Results and Discussion

3.1. Probe characterisations

In order to confirm the working principle of the porphyrin hairpin probe and stable attachment to gold surfaces, binding of the target DNA sequences (which correspond to the methylated cancer markers) to the tagged DNA probes was confirmed by surface plasmon resonance (SPR) and electrochemical impedance spectroscopy (EIS) [18, 19]. The SPR signal increased with increasing amount of target sequence, as expected (Fig. 4A,B); mismatched sequences containing four G to A mutations showed a fivefold reduced signal. The reusability of the target probe is also demonstrated by SPR, where injection of a 50 mM NaOH solution results in rapid and complete removal of the target DNA strand (Fig. 4C, NaOH injection is indicated by black arrows). Subsequent injection of a new target solution (indicated by red arrows in Fig. 4C) shows that the target can again be hybridised to the probe, and increasing concentrations of

target give a correspondingly higher SPR response. The stable attachment of the *RARβ* probe onto the sensor array surfaces and its specific response to the target DNA sequence is shown by EIS; Fig. 4D shows the Nyquist plot of the measurements using 5 mM [Fe(CN)6]3-/4- (pH 7.4) as a redox probe. The increase in the charge transfer value, shown by the diameter of the semi-circle (Rct) where the values are 21,382 Ω (blank), 35,634 Ω (probe) and 68,721 Ω (target), corresponds very well to the selective formation of the duplex with the target DNA [20].
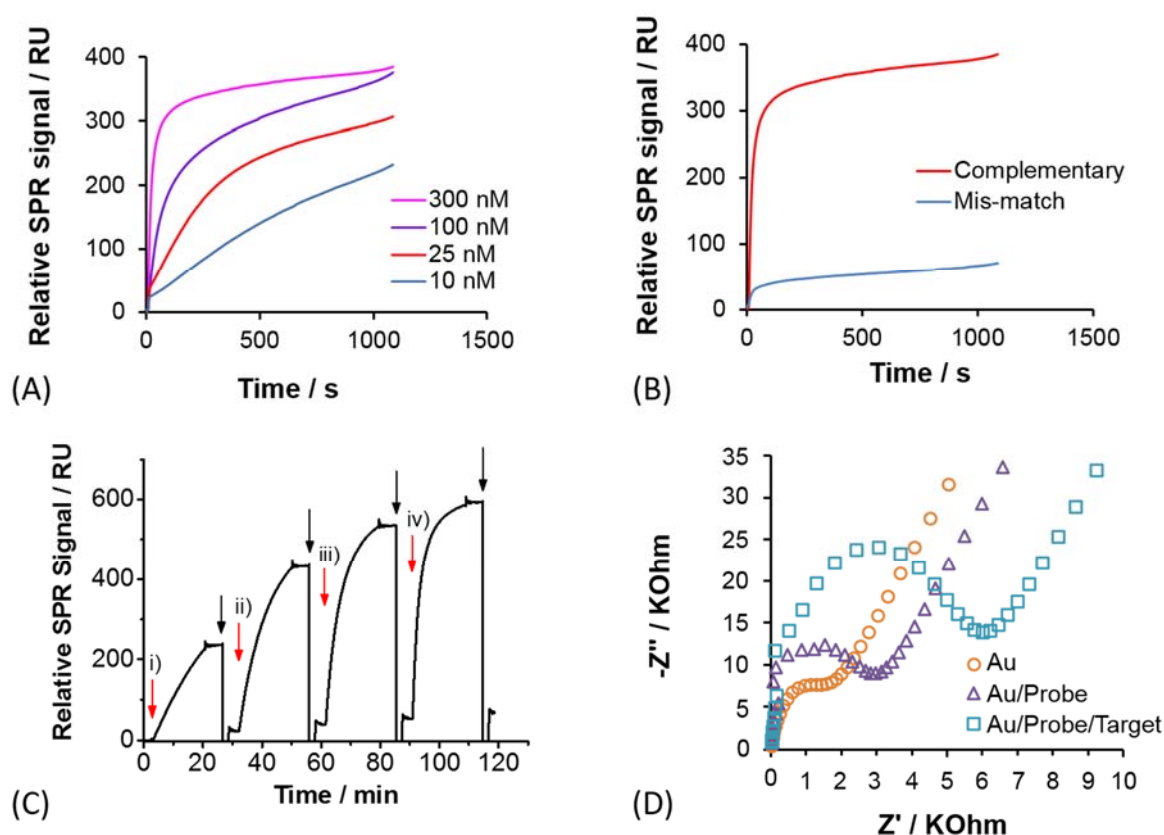

Fig. 4. (A) SPR sensograms for porphyrin tagged *RARβ* probe, showing hybridisation with the target DNA at different concentrations; (B) SPR sensograms comparing hybridisation of complementary and mismatched DNA at 300 nM; (C) SPR sensogram showing regeneration and reuse of the DNA probe, where black arrows indicate injection of 50 mM NaOH solution for regeneration of the probe, and red arrows indicate injection of the target probe at i) 10 nM, ii) 25 nM, iii) 100 nM and iii) 300 nM; (D) Nyquist plot from EIS confirming stable attachment of the probe on the surface with specific response to the target DNA sequence.

3.2. Multiplexed detection of BC biomarkers

To analyse multiplex detection of the three BC cancer markers, the different probes were immobilised individually on the electrodes as described above. First, a solution containing 300 nM of the *RARβ* target was added to the microfluidic sensor array and incubated for 20 minutes before CV measurements were carried out. The results are shown in Fig. 5A-C. In Fig. 5A and B the weak non-specific response of the *DAPK* and *E Cad* probes to the *RARβ* target is shown. The current difference ΔI is 15−20 nA. Fig. 5C shows the current response when the probe and target are matched, which is much larger than with non-matching probes, and ΔI is in the range of 40−50 nA. The difference in current is large enough to unambiguously distinguish between matched and mismatched DNA targets.

In principle, the large increase in distance of the porphyrin to the electrode surface upon hairpin opening should give a signal decrease. However, we have seen previously that porphyrins in a duplex environment are buried within the hydrophobic major groove of the dsDNA [10], thus are hardly accessible for the electrolyte and giving low current. On the other hand, porphyrins on a single stranded DNA gave a much larger current as they are solvent exposed. In this respect, here the hairpin is a "signal-off" state because is in effect in a duplex environment, whereas when hybridised with the target the porphyrin is attached to a single strand overhang and is therefore in the "signal-on" state.

Solutions containing a mixture of two or all three target strands were then tested. As can be seen from the response pattern shown in Fig. 5D-F, large CV peak intensity differences from each probe were observed only in the presence of their specific targets, and multiple targets can accurately be detected in a single run. Thus the microfluidic sensor is highly specific and can be applied for multiplexing of the three BC biomarkers, and also other DNA sequences.
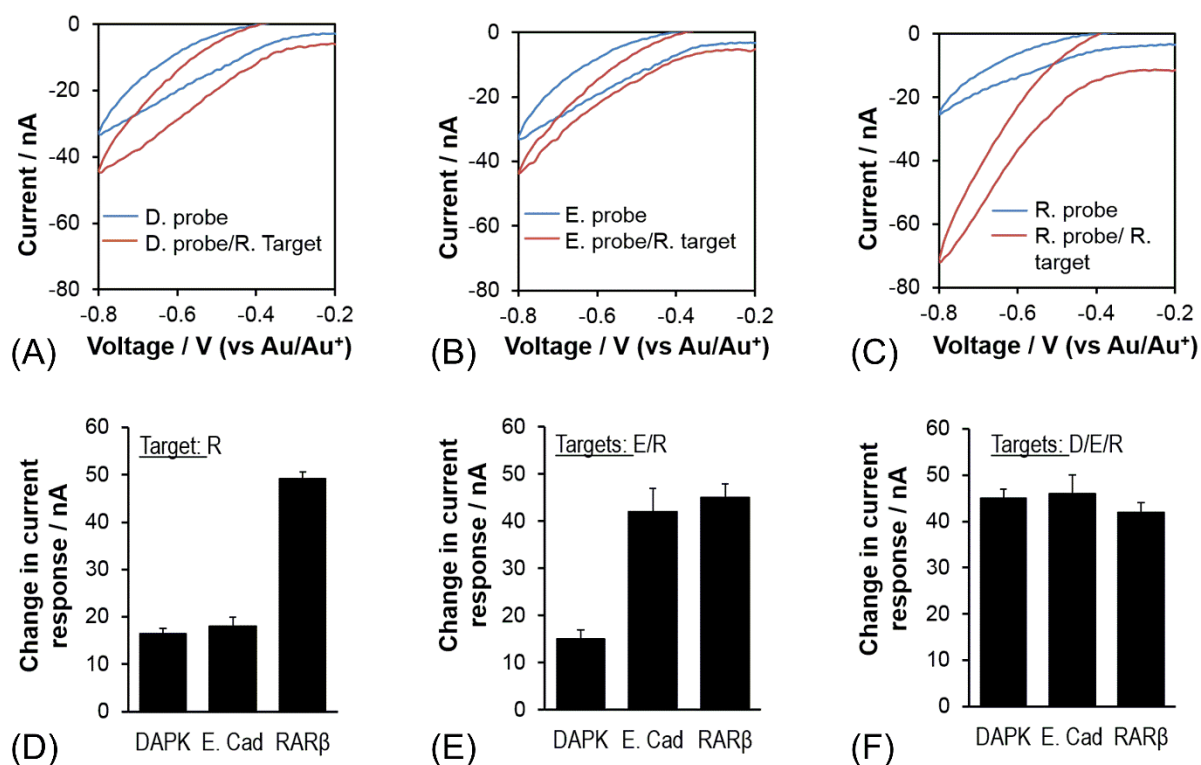
Fig. 5. Electrochemical analysis of the porphyrin-tagged hairpin DNA showing the selectivity of the different probes on the different sensors in the microchip array: difference in current response (ΔI) between mismatched [(A), (B)] and complementary (C) target DNA sequences; relative change in current (ΔI) using target solutions with one (D), two (E) and three (F) matching target strands to the corresponding probes (D = *DAPK*, E = *E Cad*, R = *RARβ*.

3.3. Determination of the limit of detection

To determine the limit of detection (LOD), a dynamic range of concentrations from 100 nM down to 200 fM was tested on the sensor microchip. The results are shown in Fig. 6 using the *E. Cad* probe as a representative example. The data follow a linear relationship in current difference *vs* the target concentration over the entire range, which spans six orders of magnitude (10^{-7} to 10^{-13} M). The microchip can hence be used to determine the concentration of the target DNA over a large dynamic concentration range with excellent accuracy. Additionally, a high concentration of 100 nM of mismatched DNA was tested, which is well above what is found in samples of BC patients which is in the pM range. The response was within the error range of the matched target at 200 fM, and significantly lower than for the matched DNA at 250 fM. This value can be regarded as a lower limit for the detection of the target DNA. The sensor

therefore allows for the detection of marker genes at the relevant biological level, which are in the picomolar range.

We reported in previous work using unlabelled DNA probes, which were immobilised on a silicon sensor, that a LOD in the region of 10 nM was achieved.[11] In the current chip design, a much higher sensitivity and thus great improvement is demonstrated. The distinctively lower LOD in the femtomolar range gives a clear advantage of using an electrochemical sensor compared to a silicon sensor which works using optical methods.
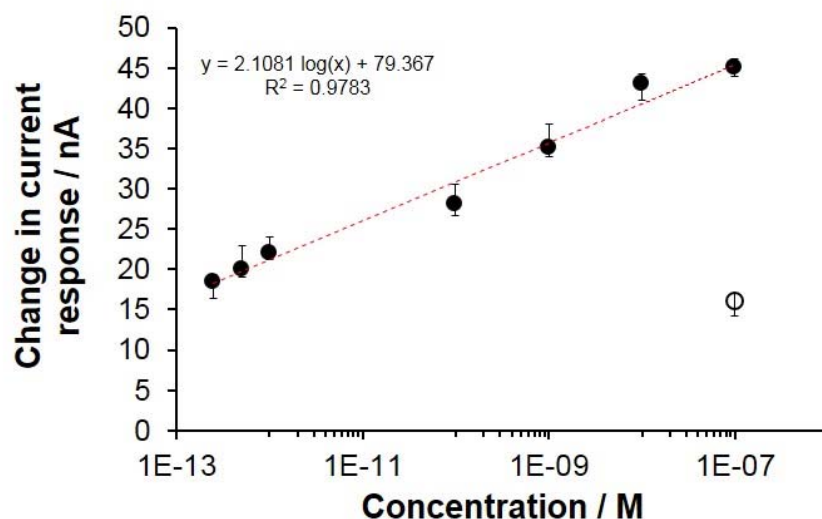

Fig. 6. Relative difference in current (ΔI, normalised peak current relative to baseline probe value in the absence of target) at variable target concentrations obtained from *E. Cad* target and probe. The full circles represent complementary DNA probe, whereas the open circle measurement corresponds to the response of a mismatched sequence (100 nM).

4. Conclusions

We have demonstrated that a porphyrin tagged hairpin-DNA probe can be used to create a selective and sensitive multiplex sensor. The attachment of the highly versatile porphyrin tag removes the need of a redox probe solution, allowing a sample to be directly used in analysis. Three different DNA markers, which are specific for bladder cancer, can simultaneously be detected on a single microchip containing up to 20 sensors. This design allows for measuring the target DNA in multiple repeats quickly, thus eliminating false readouts. The low detection limit of 250 fM is below the range of the clinically relevant concentrations where the cancer marker genes generally can be found in a patient's urine sample, and could therefore allow for early diagnosis. Certainly the design is not limited to 20 sensors and could easily be extended to form a true multiplexing device. Methylation specific PCR will still be required to differentiate between methylated and unmethylated gene markers. However, research has already been done to allow this step to be carried out on-chip [21, 22]. Combining both selective detection and methylation specific PCR should be the next focus of research. Our microchip design is a further significant step towards a point-of-care device for cancer detection, which otherwise requires costly and invasive methods, and will help to reduce the increasing burden on health care. The novelty of our system therefore lies in the combination of a unique and highly sensitive porphyrin marker with a multisensory microfluidic sensor, which gives unambiguous readout within minutes and without the requirement for additional electrochemical reagents.

References

[1] P. Negraes, F. Favaro, J. Camargo, M. Oliveira, J. Goldberg, C. Rainho, et al., DNA methylation patterns in bladder cancer and washing cell sediments: a perspective for tumor recurrence detection, BMC Cancer, 8 (2008) 238.

[2] C. Yeung, T. Dinh, J. Lee, The Health Economics of Bladder Cancer: An Updated Review of the Published Literature, PharmacoEconomics, 32 (2014) 1093-104.

[3] K.D. Sievert, B. Amend, U. Nagele, D. Schilling, J. Bedke, M. Horstmann, et al., Economic aspects of bladder cancer: what are the benefits and costs?, World J Urol, 27 (2009) 295-300.

[4] W.M. Rideout, P. Eversole-Cire, C.H. Spruck, C.M. Hustad, G.A. Coetzee, F.A. Gonzales, et al., Progressive increases in the methylation status and heterochromatinization of the myoD CpG island during oncogenic transformation, Mol Cell Biol, 14 (1994) 6143-52.

[5] S. Jahr, H. Hentze, S. Englisch, D. Hardt, F.O. Fackelmayer, R.-D. Hesch, et al., DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells, Cancer Res, 61 (2001) 1659-65.

[6] P.-C. Chen, M.-H. Tsai, S.K. Yip, Y.-C. Jou, C.-F. Ng, Y. Chen, et al., Distinct DNA methylation epigenotypes in bladder cancer from different Chinese sub-populations and its implication in cancer detection using voided urine, BMC Medical Genomics, 4 (2011) 1-11.

[7] M.W.Y. Chan, L.W. Chan, N.L.S. Tang, J.H.M. Tong, K.W. Lo, T.L. Lee, et al., Hypermethylation of Multiple Genes in Tumor Tissues and Voided Urine in Urinary Bladder Cancer Patients, Clin Cancer Res, 8 (2002) 464-70.

[8] H. Radecka, J. Radecki, Development of Electrochemical Sensors for DNA Analysis, in "DNA in Supramolecular Chemistry and Nanotechnology", Ed. Eugen Stulz and Guido Clever: Wiley Blackwell, p. 139-157; 2015.

[9] D. Kang, X. Zuo, R. Yang, F. Xia, K.W. Plaxco, R.J. White, Comparing the Properties of Electrochemical-Based DNA Sensors Employing Different Redox Tags, Anal Chem, 81 (2009) 9109-13.

[10] I. Grabowska, D.G. Singleton, A. Stachyra, A. Gora-Sochacka, A. Sirko, W. Zagorski-Ostoja, et al., A highly sensitive electrochemical genosensor based on Co-porphyrin-labelled DNA, Chem Commun, 50 (2014) 4196-9.

[11] Y. Shin, A.P. Perera, J.S. Kee, J. Song, Q. Fang, G.-Q. Lo, et al., Label-free methylation specific sensor based on silicon microring resonators for detection and quantification of DNA methylation biomarkers in bladder cancer, Sensor Actuat B-Chem, 177 (2013) 404-11.

[12] J.G. Herman, J.R. Graff, S. Myöhänen, B.D. Nelkin, S.B. Baylin, Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands, Proc Natl Acad Sci, 93 (1996) 9821-6.

[13] Y. Zhang, V. Bailey, C.M. Puleo, H. Easwaran, E. Griffiths, J.G. Herman, et al., DNA methylation analysis on a droplet-in-oil PCR array, Lab Chip, 9 (2009) 1059-64.

[14] A. Brewer, G. Siligardi, C. Neylon, E. Stulz, Introducing structural flexibility into porphyrin-DNA zipper arrays, Org Biomol Chem, 9 (2011) 777-82.

[15] L.-A. Fendt, I. Bouamaied, S. Thöni, N. Amiot, E. Stulz, DNA as Supramolecular Scaffold for Porphyrin Arrays on the Nanometer Scale, J Am Chem Soc, 129 (2007) 15319-29.

[16] T.S. Pui, P. Kongsuphol, S.K. Arya, T. Bansal, Detection of tumor necrosis factor (TNF-α) in cell culture medium with label free electrochemical impedance spectroscopy, Sensor Actuat B-Chem, 181 (2013) 494-500.

[17] P. Kongsuphol, H.H. Ng, J.P. Pursey, S.K. Arya, C.C. Wong, E. Stulz, et al., EIS-based biosensor for ultra-sensitive detection of TNF-α from non-diluted human serum, Biosens Bioelectron, 61 (2014) 274-9.

[18] H. Nguyen, J. Park, S. Kang, M. Kim, Surface Plasmon Resonance: A Versatile Technique for Biosensor Applications, Sensors, 15 (2015) 10481.

[19] O. Zagorodko, J. Spadavecchia, A.Y. Serrano, I. Larroulet, A. Pesquera, A. Zurutuza, et al., Highly Sensitive Detection of DNA Hybridization on Commercialized Graphene-Coated Surface Plasmon Resonance Interfaces, Anal Chem, 86 (2014) 11211-6.

[20] Y.T. Long, C.Z. Li, H.B. Kraatz, J.S. Lee, AC impedance spectroscopy of native DNA and M-DNA, Biophys J, 84 (2003) 3218-25.

[21] J. Yoon, M.K. Park, T.Y. Lee, Y.-J. Yoon, Y. Shin, LoMA-B: a simple and versatile lab-on-a-chip system based on single-channel bisulfite conversion for DNA methylation analysis, Lab Chip, 15 (2015) 3530-9.

[22] T.Y. Lee, Y. Shin, M.K. Park, A simple, low-cost, and rapid device for a DNA methylation-specific amplification/detection system using a flexible plastic and silicon complex, Lab Chip, 14 (2014) 4220-9.