

# Simultaneous electrochemical detection of both PSMA (+) and PSMA (–) prostate cancer cells using an RNA/peptide dual-aptamer probe†

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**Using an RNA/peptide dual-aptamer probe, both PSMA (+) and PSMA (–) prostate cancer cells were simultaneously detected by electrochemical impedance spectroscopy. This approach can be applied as a general tool for early diagnosis of prostate cancer.**

Early diagnosis of prostate cancer has been a matter of great concern as its occurrence has increased.<sup>1</sup> A prostate biopsy test, a microscopic examining process, is one of the key diagnosis tools for prostate cancer,<sup>2</sup> yet this method is not used for all suspected cases because it would result in many unnecessary biopsies as well as acute pain for patients and a large social cost. Several tests such as a digital rectal examination (DRE), a blood test for prostate specific antigen (PSA), and transrectal ultrasonography (TRUS) are generally conducted prior to the biopsy test for the initial diagnosis.<sup>3</sup> However, such initial tests provide relatively high erroneous results due to their indirect processes.<sup>4</sup> Thus, it is necessary to investigate a new direct preliminary testing method which can provide more correct and sensitive results.

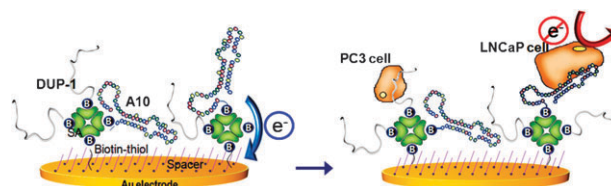
Prostate cancer has two types of cell lines, PSMA (+) and PSMA (–) cells, based on the expression of the prostate specific membrane antigen (PSMA, a prostate cancer marker protein).<sup>5</sup> Studies on PSMA (+) prostate cancer<sup>6</sup> have been accelerated in diagnosis and drug targeting after its specific anti-PSMA RNA aptamer (A10) was identified.<sup>7</sup> However, the late discovery of PSMA (–) specific ligand, DUP-1 peptide aptamer, has limited the investigation of diagnosis and drug targeting for the PSMA (–) cells.<sup>8</sup> Furthermore, the simultaneous detection of both cell types has not been reported yet. Based on this need to develop new techniques for both types of prostate cancer including the PSMA (–) cell line, we have designed a dual-aptamer probe, conjugating A10 RNA aptamer to DUP-1 peptide aptamer, for a direct electrochemical impedance spectroscopy (EIS) sensor.

Electrochemical detection methods can offer a sensitive, selective, economical, and miniaturized device for biological

applications.<sup>9</sup> Of the various electrochemical methods, EIS analysis allows for detection of capacitance, reactance and resistance changes originating from surface species.<sup>10</sup> Thus, this EIS method is highly suitable for measuring the surface appearance of an electrode.

Aptamer-based detection methods are attractive because of their high sensitivity and selectivity to the target molecule, and their good stability in various physical and chemical environments. In this study, two aptamers specific to prostate cancer cells, an anti-PSMA RNA aptamer<sup>7</sup> (A10, GGGAGGAC-GAUGCGGAUCAGCCAUGUUUACGUCACUCCUUG-UCAAUCCUCAUCGGC, underlined nucleotide represents the modified pyrimidines of 2'-F UTP and 2'-F CTP) for the PSMA (+) cell line and a DUP-1 peptide aptamer<sup>8</sup> (FRPNRAQDYNTN) for the PSMA (–) cell line, were conjugated to streptavidin to build the dual-aptamer probe. Prior to the EIS measurement, the specificity of aptamers to prostate cancer cells was confirmed by a cell labeling experiment with streptavidin modified Q<sub>655</sub> dots (Fig. S1†). Dual-aptamer modified Q<sub>655</sub> dots were bound to both PSMA (+) cell, LNCaP cell lines, and PSMA (–) cell, PC3 cell lines, but not to non-related HeLa cells. A small amount of the Q<sub>655</sub> was bound to PNT2 cells, a prostate normal cell line, and this reflects weak expression of PSMA.<sup>11</sup> The labeling experiments clearly show that the dual-aptamer probe could then be generally targeted to both LNCaP and PC3 cells.

As presented in Fig. 1, both biotin-modified A10 and DUP-1 aptamers combine with streptavidin to form the dual-aptamer probe on the surface of the Au working electrode (details in the electronic supplementary information†). When prostate cancer cells were applied into sample solution, impedance would be increased by difficulty of charge transfer through physical blocking by bound cells to the aptamer probe on the electrode surface.



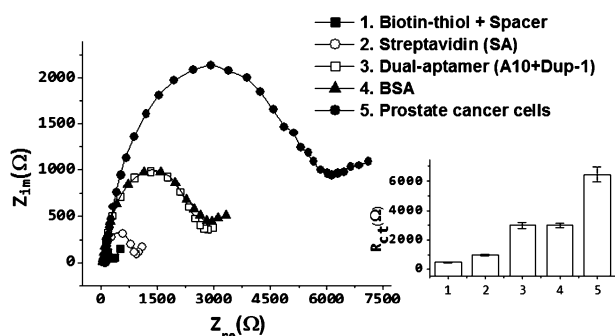
**Fig. 1** Schematic illustration for detection of prostate cancer cells. The modification of the Au electrode surface has been performed in the following sequence: (a) biotin-thiol + spacer, (b) streptavidin (SA), (c) A10 + DUP-1, (d) BSA, and (e) prostate cancer cells. Inhibition of the charge transfer was maximized by binding of prostate cancer cells to its specific aptamers on the surface of the electrode.

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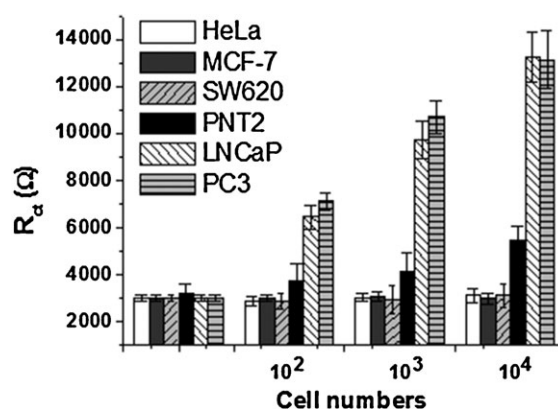


**Fig. 2** Nyquist plots for each immobilization step: SAM layer modification on the Au working electrode with biotin-thiol and spacer-thiol (■), streptavidin immobilization step (○), dual-aptamer, the A10 RNA aptamer and the DUP-1 peptide aptamer application step (□), BSA-treatment step (▲), and  $1 \times 10^2$  prostate cancer cells (PC3) binding step (●) (Inset: Plot for  $R_{ct}$  values of each step). Detailed conditions for EIS analysis are in the electronic supplementary information.†

The surface decoration of the working electrode was performed in several steps. At first, the primary modification of biotin on the working electrode was carried out using 0.1 mM biotinylated thiol ( $\text{HSCH}_2(\text{CH}_2)_9\text{CH}_2\text{NH}_2\text{CO-biotin}$ ) with 50  $\mu\text{M}$  spacer, 6-mercapto-1-hexanol ( $\text{HSCH}_2(\text{CH}_2)_4\text{CH}_2\text{OH}$ ) by immersion in 200  $\mu\text{L}$  ethanol-chloroform (50/50) solution with shaking for 14 h at RT.<sup>12</sup> Secondly, 200  $\mu\text{L}$  of 0.1  $\text{mg mL}^{-1}$  streptavidin with 50  $\mu\text{g mL}^{-1}$  BSA were applied to the primary modified working electrode in 200  $\mu\text{L}$  PBS (pH 7.4) for 30 min, and then various ratios of biotinylated A10 aptamer (10–15  $\mu\text{M}$ ) and DUP-1 aptamer (10–20  $\mu\text{M}$ ) were applied. Finally, BSA (0.2  $\text{mg mL}^{-1}$ ) was added to the electrode for 30 min to prevent the non-specific interactions for effective sensing. Prostate cancer cells could then be selectively bound to the aptamer probe. Using EIS analysis, these steps can be readily detected by analyzing the change in charge transfer resistance ( $R_{ct}$ ) identified from the diameter of the semicircle in the Nyquist plots, which describe the resistance for transferring the electrons of the  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  ions (Fig. 2).

When both A10 and DUP-1 aptamers are conjugated through streptavidin, it is clear that the ratio of the two aptamers is critical for impartial targeting. Thus, various ratios (A10:DUP-1) of 1.5:1, 1:1, 1:1.5, and 1:2 were tested. The critical A10:DUP-1 ratio of 1:2 was found to successfully target both types of prostate cancer cells by EIS measurements (Fig. S2†).

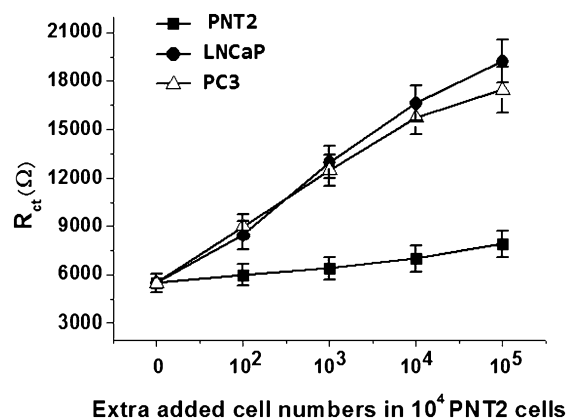
Five cancer cell lines including prostate cancer cells were prepared for the controlled experiments: LNCaP from PSMA (+) prostate cancer, PC3 from PSMA (–) prostate cancer, HeLa from cervical cancer, SW620 from colon cancer, and MCF-7 from breast cancer. Additionally, PNT-2, a non-cancerous cell line, was prepared to investigate the effect of weak expression of PSMA in a normal prostate cancer cell line. Each cell was cultured in conditions of RPMI-1640 (Hyclone) supplemented with 10% FBS, 100  $\text{U mL}^{-1}$  Penicillin G, and 100  $\text{mg mL}^{-1}$  streptomycin at 37 °C in 5%  $\text{CO}_2$  humid incubator. The cultured cells were trypsinized before preparing a suspension state in serum free media. The cell numbers were then counted, and  $1 \times 10^2$ ,  $1 \times 10^3$ , and  $1 \times 10^4$  cells in 400  $\mu\text{L}$  RPMI-1640 medium were used in EIS analysis.



**Fig. 3** A plot of  $R_{ct}$  values of the Nyquist plot for the detection of different numbers of the six cell types: HeLa, SW620, MCF-7, PNT2, LNCaP, and PC3.

As LNCaP (dashed white column in Fig. 3) or PC3 (horizontal lined gray column) cells increased to  $1 \times 10^2$ ,  $1 \times 10^3$  and  $1 \times 10^4$  in aptamer-modified electrode immersed solution (400  $\mu\text{L}$ ),  $R_{ct}$  coincidentally increased, which indicates a direct interaction between cells and the specific aptamers on the working electrode. For both LNCaP and PC3 cells, this sensor successfully detected to  $1 \times 10^2$  cells irrespective of PSMA-expression. However,  $R_{ct}$  values were not changed by sequential increments of non-prostate cancer cells, HeLa, SW620, and MCF-7. On the other hand, weak increases of  $R_{ct}$  were shown for the PNT2 (black column) cell line, indicating that PNT2 expresses PSMA in a small quantity.<sup>11</sup> Binding of prostate cancer cells was indirectly confirmed by physically detaching the cells from the electrode. Micrographs and the cell numbers for the detached cells are presented in Fig. S3.† It provides exact evidence for binding of prostate cancer cells on the dual-aptamer conjugate modified gold electrode.

In addition, we examined the specific detection of prostate cancer cells when PNT2 was co-incubated as a background material (Fig. 4). Since the PSMA is also expressed in the PNT2 cell line, discriminative detection of prostate cancer cells is required for practical application as a sensor. Resuspended LNCaP and PC3 cells ( $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ , and  $1 \times 10^5$ )



**Fig. 4**  $R_{ct}$  values of the Nyquist plot for extra added cells, PNT2 (■), LNCaP (●), and PC3 (△), in  $1 \times 10^4$  PNT2 cells contained in sample solutions with different numbers: 0,  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ , and  $1 \times 10^5$  cells.

were additionally supplemented in a high concentration of PNT2 normal cells ( $1 \times 10^4$ ) in 400  $\mu$ L RPMI 1640 medium. When different numbers of LNCaP and PC3 cells were incubated with the electrode, the  $R_{ct}$  values significantly increased with both LNCaP cells and PC3 cells, even though a high concentration of PNT2 cells was present in cell suspensions. However,  $R_{ct}$  values were slightly increased with negligible change when only PNT2 cells were additionally applied, in comparison to those of prostate cancer cells. Distinction of  $R_{ct}$  values between prostate cancer and normal cells was clearly shown even if  $10^2$  cells of either LNCaP or PC3 were present in  $10^4$  normal cells (Fig. 4). This suggests that this dual-aptamer impedance sensor can be used for the sensitive diagnosis of prostate cancer even if a small amount of prostate cancer cells ( $\sim 1\%$ ) exists among a great quantity of normal cells such as in surgically removed suspicious prostate tissues for a biopsy test. The biopsies can be prepared with ease of patients' pain because only a really small amount of cells is needed for this diagnosis method based on its sensitivity. It can provide accurate results from more biopsy specimens from a patient.

In this study, we synchronously detected both prostate cancer cells, PSMA (+) and PSMA (–) cell lines, with a high specificity using a single dual-aptamer conjugate system by means of EIS. This cell-based, direct, and sensitive method can be widely applied for preliminary diagnosis. In addition, these sensitive electrochemical approaches with the smart dual-aptamer probe can be expected to be recognized as a non-painful biopsy test.

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