



Label free Impedimetric Immunosensor for effective bladder Cancer detection in clinical urine samples

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Abstract

Galectin-1 protein has been recently recognized as a valuable urinary biomarker for the diagnosis and prognosis of bladder cancer. Herein, we present a sensitive and specific impedimetric immunosensor for the quantitative and label free detection of Galectin-1 protein in clinical urine samples. The immunosensor consists of nine gold interdigitated microelectrodes (3×3 array), which can simultaneously monitor multiple immunoreactions by analyzing the normalized impedance variations at each microelectrode during immunosensing. To obtain enhanced sensitivities, we have utilized Galectin-1/ Al_2O_3 nanoprobe (Galectin-1 antibody conjugated to alumina nanoparticles) that can be selectively trapped on the microelectrode surface using positive dielectrophoresis (p-DEP). Preliminary studies highlight the feasibility of the proposed immunosensor for Gal-1 detection in T24 cell lysate spiked phosphate buffer saline and artificial urine samples with a limit of detection that is estimated to be in the pg/ml range. To verify its practical feasibility, we have tested the immunosensor for Galectin-1 detection in clinical urine samples obtained from normal patients and those diagnosed with bladder cancer. Analysis of the clinical tests shows that the median normalized impedance variation during immunosensing for 22 cancer patients and 26 normal patients is 27% and 10%, respectively, with an identified cutoff point of 19.5% above which the sensitivity and specificity of bladder cancer detection was 82.1% and 80.8%, respectively. Based on these results, the proposed immunosensor shows promise for bladder cancer diagnosis and prognosis in a point of care format, thus enabling improved public health monitoring.

Keywords Bladder Cancer · Impedance · Immunosensor · Dielectrophoresis · Clinical testing

1 Introduction

Bladder Cancer is one the most common types of malignant tumors affecting the urinary system. Patients diagnosed with bladder cancer have an extremely high risk of recurrence even after treatment with a threat of progression to an aggressive advanced stage (Chamie et al. 2013). Currently, the most commonly used detection methods include urine cytology and

cystoscopy (Sullivan et al. 2010). While urine cytology can non-invasively screen patients with a high risk of bladder cancer by looking for abnormal cells in urine, it is often used in addition to cystoscopy and other invasive internal examinations for improved accuracy. While cystoscopy is still the gold standard, it is painful for the patient and small papillary tumours can remain undetected which can result in disease recurrence. While molecular urinary tests are being widely developed, they have not yet had the required sensitivity and specificity to replace cystoscopy (Cheung et al. 2013). Thus, there is an urgent need for the development of point of care in-vitro diagnostics for effective bladder cancer detection and monitoring of recurrence and progression.

Among the various types of biosensors, immunosensors have attracted widespread interest for development of point of care tests as they can achieve rapid and sensitive detection of the specific binding of an antigen to its corresponding antibody (Moina and Ybarra 2012). Immunosensors are a class of affinity biosensors where the bio receptor (antibody) has a strong affinity and can selectively capture the desired analyte (antigen), and take advantage of the vast library of available

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antibodies (Sharma et al. 2016). Several transduction techniques have been proposed for achieving quantitative immunosensing such as enzyme linked immunosorbent assay or ELISA (Gutschow et al. 2014), fluorescent (Liu et al. 2017), electrochemical (Felix and Angnes 2017), piezoelectric (Guo et al. 2012) and magnetic (Pereira et al. 2010) among others. In particular, electrochemical immunosensors have several advantages like low-cost, simplicity, sensitivity and portability due to ease of integration with miniaturized electronics, thus making them arguably the most practical point of care diagnostic technique for quantitative protein detection (Wen et al. 2016). Among the different interrogation schemes utilized in electrochemical immunosensors, electrochemical impedance spectroscopy (EIS) is attractive as it can be used to directly and non-destructively monitor affinity-binding events without the need for additional amplification and labeling. When a target analyte is captured by the receptor functionalized electrode surface, it alters the electrical properties (e.g. capacitance, charge transfer resistance) of the electrode/solution interface, which can be analyzed using EIS (Chuang and Shaikh n.d.; Lisdat and Schäfer 2008). This label free detection system enables development of one-step, easy to use immunoassays with shorter detection time, thus making them highly suitable for point of care testing of various diseases (Selvam et al. 2017).

In this study, we present an electrochemical impedimetric immunosensor utilizing gold interdigitated microelectrodes for sensitive and specific detection of Galectin-1 protein in clinical urine samples. Urine samples of 26 normal patients and 22 bladder cancer patients (having advanced stage cancer ranging from stage 3 to stage 4) were tested using the proposed immunosensor. We have chosen Galectin-1 protein as the biomarker of interest for performing the clinical study since it is overexpressed in T24 cell lysate (Grade III bladder cancer cells) and can enable effective diagnosis and prognosis of advanced stage bladder cancer. In a previous cohort study, Wu et al. reported that Galectin-1 overexpression in bladder cancer tumors significantly predicted disease-specific survival at the univariate and multivariate levels (Wu et al. 2015). Rubinstein et al. also reported that cancerous tumor cells can secrete Galectin-1 protein to escape from T cell-dependent immunity by inducing the apoptosis of activated T cells, thus conferring the immune privilege to tumor cells (Rubinstein et al. 2004). The Galectin-1 protein is secreted onto the cell surface and into biological fluids including serum and urine.

The detailed fabrication protocol and preliminary feasibility of the proposed electrochemical immunosensor was presented in our previous study (Chuang et al. 2016a). The immunosensor utilizes interdigitated microelectrodes since they are known to improve sensitivity of two electrode systems due to advantages like fast attainment of steady state, low ohmic drop, rapid reaction kinetics, small sample volumes and improved signal to noise ratio (Radke and Alocilja 2005). To

enable effective immobilization of the antibodies on the microelectrode surface, we have synthesized Galectin-1/ Al_2O_3 nanoprobe (Galectin-1 antibodies conjugated to dielectric alumina nanoparticles via covalent silane coupling) which are then trapped on the electrode surface using positive dielectrophoresis (p-DEP). DEP is defined as the translational motion of a dielectric particle or biological cell in a suspended medium under the influence of a non-uniform AC electric field that acts on the particle driving it towards/against the direction (Radke and Alocilja 2005). Previously, we have used this technique for programmable three-step manipulation (focusing, guiding and trapping) of nanoprobe to create a lab on a chip device for multiplexed detection (Pethig 2010). The feasibility of the proposed impedimetric immunosensor for Galectin-1 detection was first tested using T24 bladder cancer cell lysate spiked phosphate buffer saline (PBS) and artificial urine samples. Finally, to ensure practical applicability for clinical diagnosis, we have analyzed the ability of the immunosensor to distinguish between negative (normal patient) and positive (bladder cancer patient) classes using clinical urine samples of a total of 48 patients registered at Chi-Mei Hospital, Tainan, Taiwan.

2 Experimental methods

2.1 Synthesis of Galectin-1/ Al_2O_3 nanoprobe

The Galectin-1/ Al_2O_3 nanoprobe were synthesized using a two-step method that involves surface modification of alumina nanoparticles (Al_2O_3 NPs) followed by conjugation with the Galectin-1 antibody and detailed synthesis protocol and characterizations have been reported previously (Chuang et al. 2016a; Nhan 2015). Al_2O_3 NPs with an average diameter of 50 nm were purchased from Evonik Degussa Taiwan Ltd. and surface functionalized with amino ($-\text{NH}_2$) groups using APTES (3-aminopropyltrimethoxysilane, MERCK). Typically, 3 g ethanol (95%), 1.5 g deionized water and 1.25 g of silane solution were mixed under magnetically stirring at room temperature for 3 h. Separately, 2.7 g Al_2O_3 NPs was dispersed in 100 g ethanol (95%) under constant stirring for 10 min. The two solutions were then mixed together and stirred at 70 °C for a further 3 h. The silane modified Al_2O_3 -NPs were collected by centrifugation at 10000 rpm for 15 min and baked at 60 °C for 12 h after which they are ready for antibody conjugation. Before conjugation to the Al_2O_3 -NPs, the Galectin-1 monoclonal antibodies (Abcam PLC) are first oxidized in a solution containing 0.1 M sodium acetate and 1 mM of sodium metaperiodate for 30 min with the solution pH maintained at 5.5. During this step, the hydroxyl ($-\text{OH}$) groups in the carbohydrate moieties of the Galectin-1 antibodies are oxidized to aldehyde ($-\text{CHO}$) groups, which then subsequently react with the amino groups present on the surface

of Al_2O_3 NPs via the formation of an amide bond. This covalent interaction enables well-oriented conjugation, where the fragment crystallizable (Fc) or tail region of the antibody is attached to the Al_2O_3 NPs while the fragment antigen binding (Fab) region is available for binding during immunoassay. After conjugation for 1 h via rotation, the obtained Galectin-1/ Al_2O_3 nanoprobles were centrifuged at 13500 rpm for 30 min at 4 °C to remove the supernatant before being redispersed in 0.1 M PBS solution.

2.2 Bladder cancer cell lysate preparation

The human urinary bladder urothelial carcinoma cell lines (T24, Bladder Cancer Grade 3 and RT4, Bladder Cancer Grade 1) were obtained from the Bioresource Collection and Research Center, Hsinchu, Taiwan and cultured in McCoy's 5A medium [GIBCO Life Technologies Corporation] supplemented with 10% foetal bovine serum. The T24 and RT4 cells were lysed and harvested using a mammalian protein extraction buffer (GE Healthcare). Two cell lines were used to perform specificity studies since Galectin-1 protein overexpression is observed only in T24 cell lysate while RT4 cell lysate, used as the negative control, shows overexpression of other proteins like Lactate dehydrogenase b (LDH-B). The protein concentration in the cell lysate was determined using a Bio-Rad DC protein assay kit and the original concentration of the T24 cell lysate was 0.25 mg/ml.

To further confirm that the nanoprobles were successfully immobilized on the microelectrode surface by p-DEP and that

the Galectin-1 antibodies retain their activity, we have performed fluorescent detection using T24 cell lysates that are labelled with red fluorescent Cyanine (Cy3) dye (GE Healthcare). The Cy3 powder was mixed with 0.1 M sodium carbonate solution and 200 μg of T24 protein was added to 100 μl of dye solution. After being left for 30 min at room temperature, the labelled T24 proteins were added to PBS (0.125 mg/ml) and the sample solution was ready for immunoassay.

2.3 Artificial urine synthesis

The artificial urine was synthesized using a previously reported protocol (Brooks and Keevil 1997). Briefly, 1 g of peptone (L37), 0.005 g of Yeast extract, 2.1 g of sodium bicarbonate (NaHCO_3), 10 g of urea ($\text{CH}_4\text{N}_2\text{O}$), 5.2 g of sodium chloride (NaCl), 3.2 g of sodium sulphate (Na_2SO_4), 1.3 g of ammonium chloride (NH_4Cl), 0.37 g of calcium chloride (CaCl_2), 0.0012 g of iron sulphate (FeSO_4), 0.49 g of magnesium sulphate (MgSO_4), 0.95 g of potassium dihydrogen phosphate (KH_2PO_4) and 1.2 g of dipotassium phosphate (K_2PO_4) were mixed into a litre of DI water while the pH was adjusted to 7.2 using NaOH.

2.4 Immunosensor fabrication

A schematic of the systematic protocol for annular gold interdigitated microelectrode array fabrication and an image of the completed immunosensor chip are presented in Fig. 1a, b. A

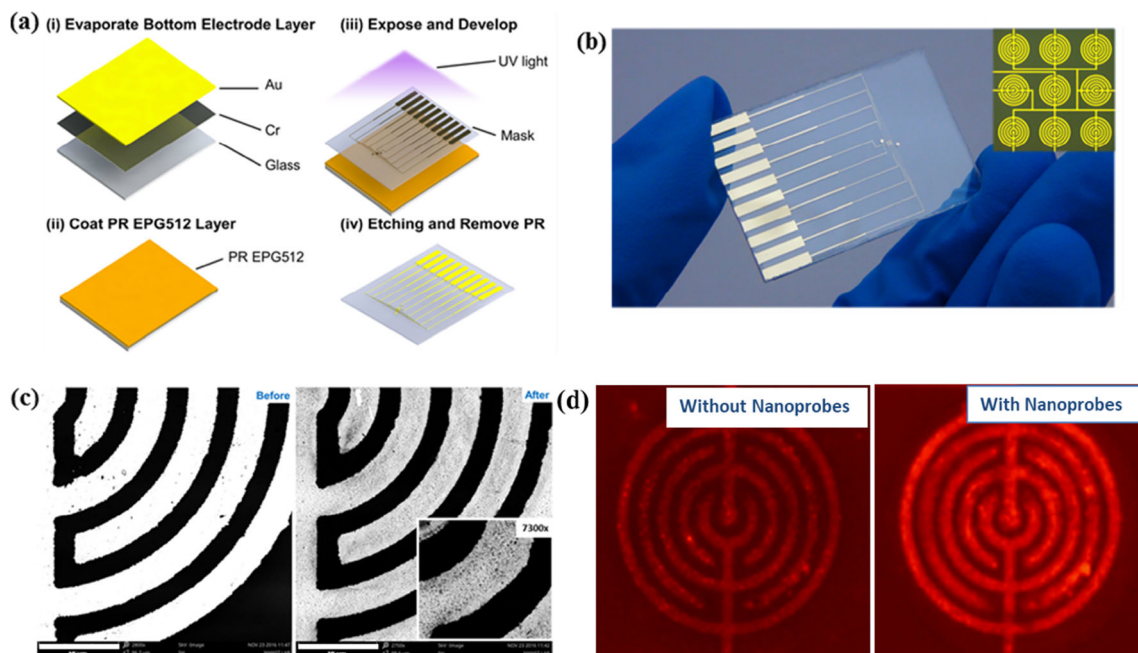


Fig. 1 (a) Schematic of the immunosensor fabrication process. (b) An image of the finished immunosensor and (inset) an optical microscopy image of the 3×3 gold annular microelectrode array. (c) SEM image of the gold microelectrode before and after DEP trapping of nanoprobles

where the white scale bar represents 30 μm (d) Fluorescence image of the microelectrode surface (without and with dielectrophoretically trapped nanoprobles) after immunosensing with Cy3 labelled T24 cell lysate spiked PBS sample solution

$40 \times 60 \text{ mm}^2$ microscope glass slide is first cleaned using acetone and methanol followed by copious rinsing with deionized water and drying in a vacuum oven at 80 C for 1 h. Next, electron beam physical vapor deposition (EBPVD) technique is used to sequentially evaporate 300 \AA of chromium as an intermediary layer and 700 \AA of gold as the electrode layer on the glass slide. To pattern the gold microelectrodes, a standard photolithographic protocol consisting of resist spin coating, masked UV exposure and developing is utilized. The final step involves wet etching to remove cured photoresist from the patterned microelectrode surface. The fabricated immunosensor chip consists of a 3×3 interdigitated gold microelectrode array with a finger width and gap of 10 \mu m each and a total sensing area of 0.64 mm^2 . Next, the prepared Galectin-1/ Al_2O_3 nanoprobe are trapped on the gold microelectrode surface using p-DEP force by applying an AC signal of 10 V_{pp} at 100 KHz for 30 min using a function generator (AFG3022, Tektronix). The immobilization of nanoprobe can be confirmed by the Scanning Electron Microscopy (SEM) images of the microelectrode surface shown in Fig. 1c. Also, fluorescence detection performed using an optical microscope (Olympus, BX51) shows significantly enhanced fluorescence intensity after immunosensing with Cy3 labelled T24 cell lysate on the nanoprobe modified microelectrode surface as compared to immunosensing performed on a bare gold microelectrode surface as shown in Fig. 1d, thus further confirming successful immobilization of the nanoprobe. After nanoprobe trapping, the microelectrode surface is incubated with bovine serum albumin (BSA) to block all remaining active sites for eliminating residual binding capacity and reducing non-specific interactions that may affect the observed impedance response. The immunosensor is now ready for operation and the baseline impedance is measured (Z_0) using an LCR meter (Wayne Kerr Electronics, WK 6420) with data acquisition controlled using a computer-based LabVIEW program to scan the impedance in a frequency range of 1 kHz to 100 kHz .

2.5 Immunosensor operation

The preliminary feasibility of the proposed immunosensor for Galectin-1 detection was tested using T24 cell lysate spiked PBS and artificial urine samples. T24 cell lysate contains not just Galectin-1 but also a range of other proteins derived from the kidney and urinary tract that are present in clinical urine samples. Using T24 cell lysate instead of pure Galectin-1 protein can better mimic urine samples of patients suffering from bladder cancer. Feasibility studies were conducted using 6 dilutions (0.25, 0.125, 0.0625, 0.03125, 0.01563 and 0.0078 mg/ml) of T24 cell lysate in PBS and artificial urine. Next, clinical testing was performed using human urine samples of 26 normal patients and 22 bladder cancer patients collected from Chi-Mei Hospital, Taiwan, to confirm

feasibility for practical diagnostic application. The urine samples were stored in a $-40 \text{ }^\circ\text{C}$ lab freezer immediately after collection. Before immunosensing, each urine sample tube was placed in a water bath at a temperature of $37 \text{ }^\circ\text{C}$ followed by centrifugation at 5000 rpm for 5 min to obtain the supernatant, which is used as the sample solution. The sample volume used for all tests (spiked PBS, spiked artificial urine and clinical urine samples) is 100 \mu l that was dropped on the microelectrode surface for 30 min to perform immunosensing. Next, a washing step is utilized to remove any unbound proteins and the impedance after immunosensing is measured (Z_1). All impedance measurements (bare electrode, after nanoprobe trapping and after immunosensing) were performed in deionized water and the absolute impedance variation (ΔZ) during immunosensing can be mathematically expressed as $\Delta Z = |Z_1 - Z_0|$. We have tried to eliminate any differences in the initial impedance at different electrodes due to their differing lengths by normalizing the impedance response during immunosensing (ΔZ) with the initial impedance observed after nanoprobe trapping at that particular electrode (Z_0). This normalized impedance variation ($\Delta Z/Z_0$) is calculated at an operating frequency of 10 KHz at which we observe the maximum stable response during immunosensing.

3 Results and discussion

3.1 Preliminary feasibility of immunosensor for Galectin-1 detection

Before proceeding to clinical testing using human urine samples, the preliminary feasibility of the proposed immunosensor for sensitive and specific Galectin-1 protein detection was tested using T24 cell lysate spiked PBS and artificial urine samples as shown in Fig. 2. PBS was chosen since its osmolarity and ionic concentration match those of human body while artificial urine provides similar conditions to those found in human urine. The bode plots (impedance vs frequency) for each step during surface modification and immunosensor operation are shown in Fig. 2a. It can be seen that the impedance of the bare electrode increased after trapping of dielectric Galectin-1/ Al_2O_3 nanoprobe and further increased after immunosensing with Galectin-1 protein present in the cell lysate. This is because capacitive effects dominate the impedance response since all EIS measurements are performed in low conductivity deionized water at a frequency of 10 KHz . The microelectrode surface modification with dielectric nanoprobe followed by antibody-antigen interaction during immunosensing can be visualized as increasing the effective thickness of the electrode/electrolyte parallel plate capacitor, thus resulting in a decrease in the interfacial capacitance and consequently an increase in the observed

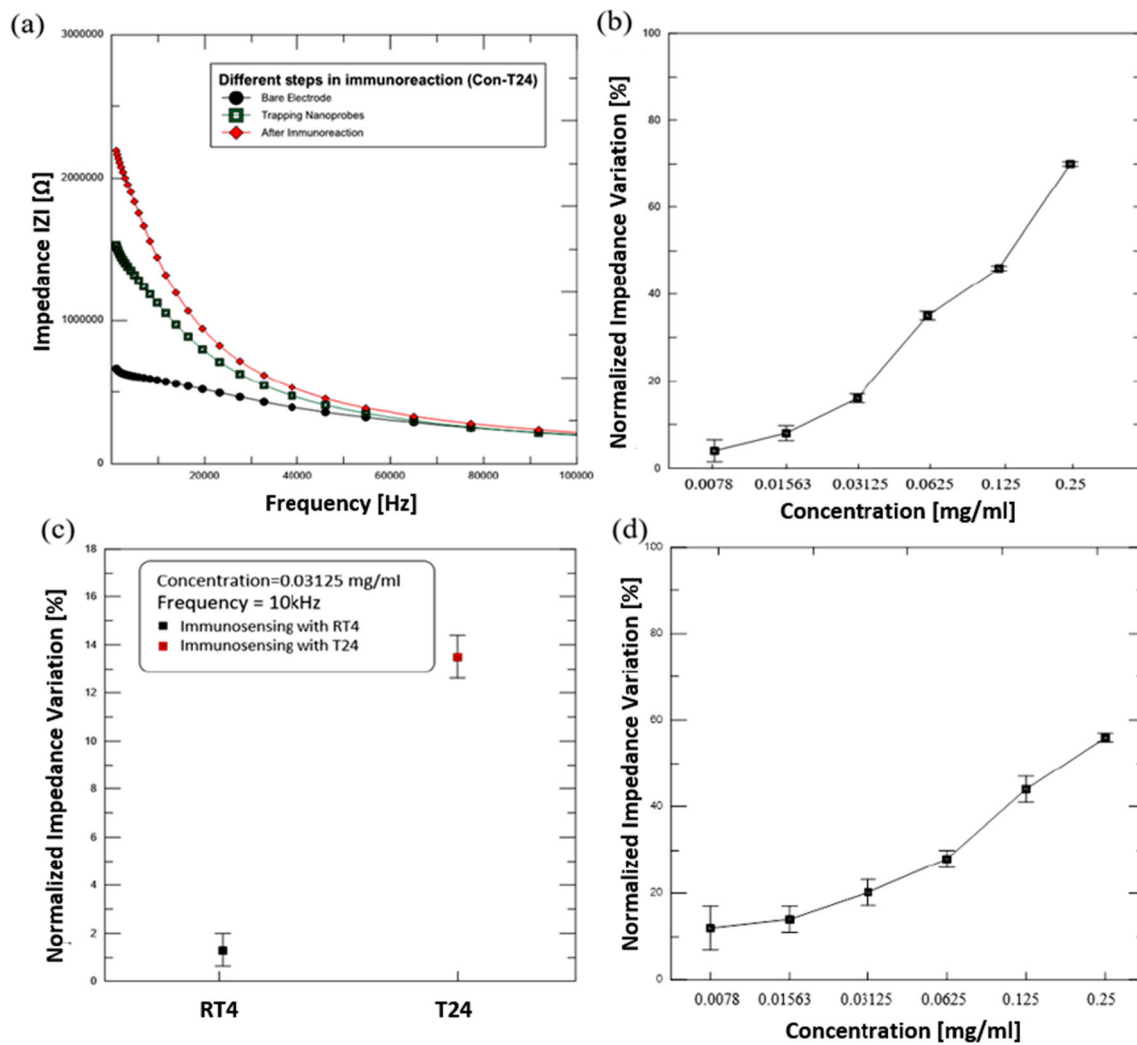


Fig. 2 (a) Bode plots for each step during microelectrode surface modification and immunosensor operation. (b) Normalized impedance variation for different concentrations of T24 cell lysate spiked PBS sample solutions. (c) Normalized impedance variation for Galectin-1

impedance at each step. We have also performed detailed equivalent circuit modelling in our previous study to fit the experimental data and predict the system elements that effect the impedance response of the immunosensor based on interdigitated microelectrodes modified with nanoprobes (Chuang et al. 2016b).

The normalized impedance variation increases with the concentration of cell lysate present in PBS as shown in Fig. 2b with an observed limit of detection of about 0.0078 mg/ml. However, since cell lysate is a complex fluid containing a multitude of proteins (about 10 (Moina and Ybarra 2012)/cell) (Milo 2013) including Galectin-1, the actual LOD for Galectin-1 detection is estimated to be in the pg/ml range. Furthermore, to ensure that the impedance variation is specific for Galectin-1 immunosensing, we have performed detection using lysates of two different bladder cancer cell lines, namely T24 and RT4. The normalized impedance variation after

specificity testing using cell lysates from two different bladder cancer lines, namely T24 and RT4. (d) Normalized impedance variation for different concentrations of T24 cell lysate spiked artificial urine sample solutions

immunosensing with T24 cell lysate spiked PBS (0.03125 mg/ml) is about 17% which is significantly higher than that observed for the RT4 cell lysate spiked PBS (0.0325 mg/ml) of about 1–2% as shown in Fig. 2c. This is because T24 (grade III bladder cancer cells) will produce post cancerous Galectin-1 proteins while RT4 (grade I bladder cancer cells) will show an over expression of other proteins like Lactate dehydrogenase b (LDH-B). This shows that Galectin-1 present in the T24 cell lysate is specifically detected and forms an immune-complex with the Galectin-1 antibodies present on the immunosensor surface. We also tested the feasibility of the immunosensor to perform detection using T24 cell lysate spiked artificial urine samples. The normalized impedance variation as shown in Fig. 2d increases with increasing concentration of T24 cell lysate in spiked artificial urine with a similar trend and LOD as that observed for the T24 cell lysate spiked PBS samples. These preliminary results

confirm the feasibility of the proposed immunosensor for sensitive and specific detection of Galectin-1 protein.

3.2 Clinical testing using human urine samples

To validate the applicability of the proposed immunosensor for clinical diagnosis, we have tested the immunosensor for detection of bladder cancer in clinical urine samples (48 patients consisting of 26 normal patients and 22 patients with bladder cancer). A larger normalized impedance variation implies a higher urinary expression of Galectin-1, thus resulting in a higher probability of the patient suffering from bladder

cancer (Fig. S1). The median of normalized impedance variation for normal and bladder cancer patients was 10% and 27%, respectively, as shown in the box plots in Fig. 3a. When considering the results of two populations (disease and disease free), it is very rare to observe a perfect separation between the groups and generally the distribution of the test results will overlap as shown in Fig. 3b when a cut-off point of 19.5% was used. This is expected in most real case scenarios, which will not only produce true positives (TP) and true negatives (TN), but also false positives (FP, patients who pass the test but actually have the disease) and false negatives (patients who fail the test but are actually healthy). Based on the

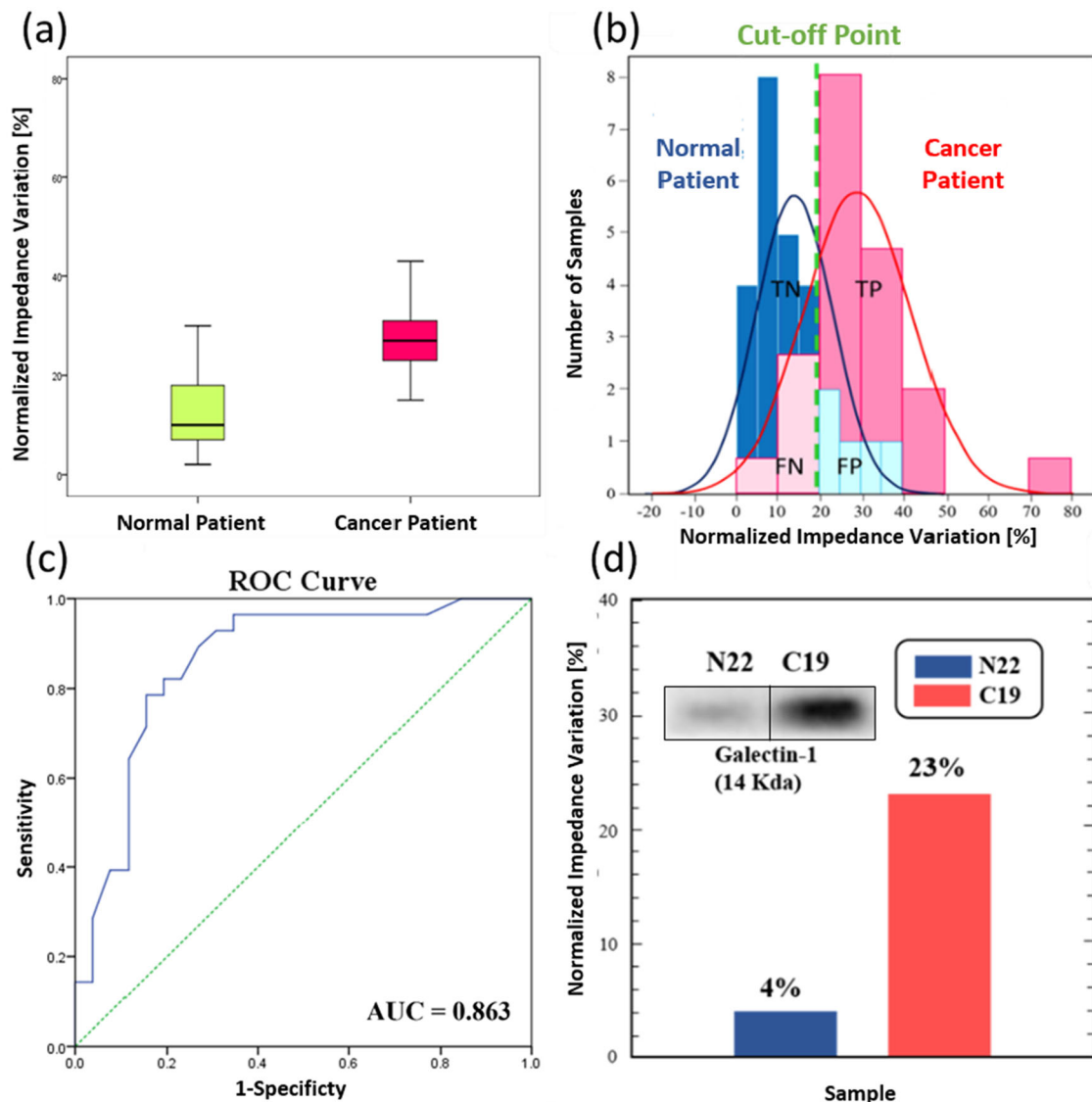


Fig. 3 (a) Box plots of normalized impedance variation and (b) Normal distribution of test results obtained for normal and bladder cancer patients. (b) ROC curve of sensitivity vs (1-specificity) for different chosen cut-off points. (c) Comparison of the impedance response of the immunosensor with western blot analysis (inset) for samples N22 and C19 representing normal patient 22 and bladder cancer patient 19, respectively. The

western blot image has been cut and only the bands observed at 14 kDa which correspond to the molecular weight of Galectin 1 are shown. A detailed description of the western blot protocol and the complete image of the observed bands at different molecular weights are provided in the supplementary section (Fig. S2)

observed TP, TN, FP and FN values, the sensitivity and specificity can be calculated at each cut-off point. We have also obtained the Receiver Operating Characteristic (ROC) curve in Fig. 3c by plotting the true positive rate (sensitivity) as a function of the false positive rate (1 - specificity). Each point on the ROC curve represents a sensitivity/specificity pair that corresponds to a particular cut-off point or decision threshold for a positive result. The sensitivity and specificity vary for different cut-off points and are inversely related to each other as shown in the supplementary section (Table S1) for cut-off points between 1 and 77. We have used these values based on the range of normalized impedance variation values observed during immunosensing using urine samples of the 48 patients. We have chosen an optimal cut-off point of 19.5% at which the calculated sensitivity was 82.1% and the specificity was 80.1%. For a test displaying perfect discrimination and accuracy (no overlap between disease and disease free populations), the ROC curve passes through the top left corner with an area under curve (AUC) value of 1 (100% Sensitivity and Specificity). AUC is a combined measure of sensitivity and specificity and is a good indicator of the overall performance of a diagnostic test. It can be interpreted as the probability of a randomly chosen diseased patient to be more likely to be diseased than a randomly chosen disease free patient (Hanley and McNeil 1982). The minimum AUC of 0.5 should be considered a chance level while an AUC of 0 implies that the test incorrectly classifies all the patients. In this study, an AUC value of 0.863 was observed, thus showing acceptable discrimination between disease and disease free patients for a point of care setting.

To confirm the reliability of the immunosensor response obtained during clinical testing, we have performed a comparative analysis using a conventional western immunoblotting technique. As seen in the inset in Fig.3d, a strong band is observed at 14 kDa (molecular weight of Galectin-1) for cancer patient sample C19 while a faint, almost invisible band is observed for normal patient sample N22. These results agree with those obtained using the immunosensor where the normalized impedance variation for N22 and C19 was 4% and 23%, respectively. Consequently, the observed clinical trial results demonstrate the feasibility of the immunosensor for Galectin-1 detection in human urine samples.

4 Conclusions

In summary, we have developed an electrochemical impedimetric immunosensor for real time, quantitative and label free detection of bladder cancer biomarker Galectin-1 in clinical urine samples. Preliminary studies in buffer and artificial urine samples demonstrate the feasibility of the immunosensor for sensitive and specific detection of Galectin-1 protein. Clinical testing using human urine samples

shows that the immunosensor response could be used to differentiate between normal and bladder cancer patients with an acceptable sensitivity and specificity. Clinical trials over a larger population are still needed to further validate the efficacy of the immunosensor for bladder cancer detection. Consequently, the proposed electrochemical immunosensing platform shows promise for point of care bladder cancer diagnosis and can be further extended to enable multiplexed detection of a wide range of immunoreactions.

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Author contributions statements M.O. Shaikh and C.H. Chuang wrote the main manuscript text, M.O. Shaikh and T.C. Huang prepared all the figures and performed ion concentration tests and clinical tests. T.F. Wu assisted with nanoprobe preparation and results discussion.

Compliance with ethical standards

Competing interests The author(s) declare no competing interests.

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