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ORIGINAL PAPER

Nanopore membrane-based electrochemical immunoassay

Jinseong Kim · Anuncia Gonzalez-Martin

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Abstract Using nanotechnology in the immunoassay field, Lynntech has developed a nanopore-based sensor with electrochemical signal transduction for the detection of biologically relevant molecular targets. Antibodies of interest were immobilized on the inside wall of nanopores and the antibody–antigen interaction was monitored by measuring the ionic conductance through the nanopores. We have used electrochemical impedance spectroscopy to monitor the changes in the ionic conductance due to the antibody–antigen interaction. To aid the development of a portable, fast immunoassay instrument, we have selected specific impedance frequency values that are very sensitive to the ionic conductance changes. Biomarkers of prostate cancer, prostate-specific antigen and hepsin, were successfully assayed by the nanopore membrane-based electrochemical immunoassay in both phosphate-buffered saline and plasma medium.

Keywords Electrochemical Immunoassay · Nanopore · Direct immunoassay

Abbreviations

Ab	antibody
Ag	antigen
DSP	dithiobis-succinimidyl propionate
PBS	0.01 M phosphate-buffered saline (0.138 M NaCl, 0.0027 M KCl, pH 7.4)
PCTE	polycarbonate Track Etch
PSA	prostate-specific antigen

J. Kim (✉) · A. Gonzalez-Martin
Lynntech, Inc.,
College Station, TX 77840, USA
e-mail: Jinseong.kim@lynntech.com
URL: www.lynntech.com

Introduction

Immunoassay is probably the most commonly used technology for the detection and quantification of biomolecules in the diagnosis and management of disease [1]. Since the advent of radioimmunoassay in the 1950s, the immunoassay technology has been developed in areas of quantification, methodology, sensitivity enhancement, automation, antibody engineering, etc. Even a variety of hazardous biological/chemical materials in environmental science and technology are measured by immunoassay-based methodology [2]. Among immunoassay methodologies, biosensors based on direct immunoassay have been attractive because of their potential benefit of rapid detection, real-time analysis, regeneration of the sensing device or inexpensive and disposable sensing device, no need for pretreatment, etc. [3].

On the other hand, nanotechnology has boomed recently because of the availability of new investigative tools, making possible to characterize chemical and mechanical properties at a single molecular level, discover novel phenomena and processes, and provide science with a wide range of tools, materials, devices, and systems with unique characteristics. One example of nanotechnology is the naturally formed α-hemolysin nanopore [4, 5]. This nanopore has been extensively used to study the transport of ions, polymers (e.g., DNA, RNA), and proteins at molecular level and inter- and intra-cellular signaling mechanisms [6]. Another example of relevant nanopore technology is the use of an ensemble of nanopores on a polycarbonate track-etched membrane or alumina templates to produce nanotubules and nanorods [7, 8].

Using nanotechnology in the immunoassay field, Lynntech has been developing a nanopore-based sensor for the detection of biologically relevant molecular targets in

samples including blood, saliva, and other body fluids, or for use in the research laboratory (purified samples) and clinical specimens. It uses electrochemistry as the detection method due to its ability to work with complex sample matrices, simple and low-cost instrumentation, and almost unrivalled detection limits [9]. Electrochemical immunoassays have the potential for the development of miniaturized devices, such as point-of-care diagnostic devices and onsite environmental monitoring, because both the detector and control instrumentation can be miniaturized, in addition to the technology tunable selectivity, high sensitivity, independence of turbidity, low cost and power requirement, and high compatibility with micromachining and microfabrication technologies [10].

We describe here the development of a nanopore membrane-based electrochemical immunoassay sensor. As illustrated in Fig. 1, selected antibodies are immobilized inside the nanopores of the membrane. There, the antibody–antigen interaction is monitored by measuring the ionic conductance through the nanopores. The antibody–antigen binding events at the nanopores partially block the ionic current through the nanopores and lead to significant changes on the ionic conductance (or its inverse, impedance). Recently, electrochemical immunoassay methods using nanoporous electrodes have been reported [11, 12]. In those methods, "nanowells" (rather than nanopores) were used, with no free flowing of ionic species through the nanopores. There, the antibody–antigen interaction was monitored by measuring modulation to the electro-ionic distribution of the interfacial electrical double layer [11] (as frequency shift of resonant frequency peaks) or diffusion of a redox couple [12] (as changes in the redox current measured on the base electrode surface). We have used electrochemical impedance spectroscopy to monitor the changes in the ionic conductance through the nanopores due to the antibody–antigen interaction. To aid the development of a portable, fast immunoassay sensor, we have selected specific impedance frequency values that are very sensitive to the ionic conductance changes. Biomarkers of prostate cancer, prostate-specific antigen (PSA) and hepsin [13], were successfully assayed by the nanopore membrane-based electrochemical immunoassay sensor.

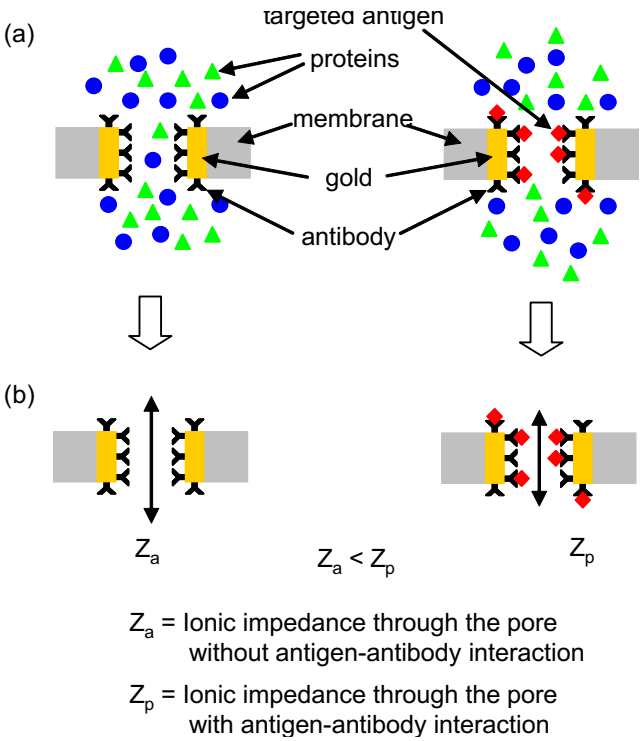

Fig. 1 Operational principles of the nanopore-based biosensor. **a** Selective affinity of antigens of interest to antibodies on nanopore, and **b** measurement of ionic impedance through the nanopores representing antibody–antigen interaction

Experimental section

Chemicals and materials

Nanopore membranes (GE Polycarbonate Track Etch (PCTE) membranes, 6-μm thick, 10, 30, and 50 nm pore sizes, 6×10^8 pores/cm^2 and Whatman™ Nuclepore™ Polycarbonate membranes, 6-μm thick, 100 nm pore size, 6×10^8 pores/cm^2) were purchased from GE Osmonics Labstore (Minnetonka, MN, USA) and Fisher Scientific (Pittsburgh, PA, USA), respectively. Tin chloride, trifluoroacetic acid, silver nitrate, sodium sulfite, sodium bicarbonate, sodium carbonate, sodium chloride, sodium phosphate monobasic, sodium phosphate dibasic, isopropanolic dithiobis-succinimidyl propionate, ammonia water, sulfuric acid, methanol, ethanol, isopropanol, and formaldehyde were purchased from Aldrich (St. Louis, MO, USA). A gold plating solution (Oromerse SO part B) was purchased from Technic (Cranston, RI, USA). Hepsin polyclonal antibody (affinity-purified) and hepsin blocking peptide (human hepsin sequence of amino acids 241–260) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Another pair of prostate-specific antigen and PSA antibody (rabbit hosted) was purchased from Fitzgerald Industries International (Concord, MA, USA). A rabbit serum was purchased from Pel-Freez Biologicals (Roger, AR, USA). A blocking solution (2% nonfat dry milk in phosphate-buffered saline (PBS)) was prepared daily prior to use to minimize nonspecific binding on the membrane [14].

Preparation of nanopore membrane-based biological sensor

Figure 2 depicts the procedure used for the fabrication of the nanopore membrane-based immunoassay sensors. Main steps are (1) to coat a gold layer on the inside wall of

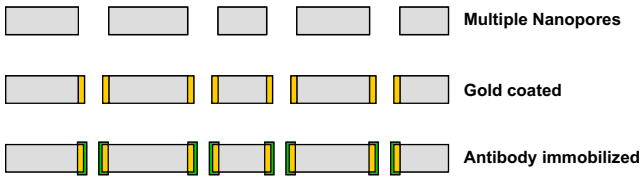

Fig. 2 Step-by-step procedure to fabricate nanopore-based biological sensor

nanopores in the polycarbonate membranes, (2) to immobilize antibodies on the gold layer, and (3) to block the membrane surfaces except for the antibody/antigen binding sites.

Electroless gold coating on PCTE membranes was carried out according to a standard approach described in the literature [15, 16]. Once the inside walls of nanopores in the membranes were coated with gold, antibodies of interest were immobilized on the gold using isopropanolic dithio-bis-succinimidyl propionate (DSP) in isopropyl alcohol [17]. A disulfide linkage in the middle of DSP rapidly chemisorbed to gold surfaces and two ester functional groups of DSP were cleaved to bind to amine groups of antibodies during incubation in a phosphate-buffered saline [17]. Then, the nanopore membrane with the immobilized antibodies was immersed in a blocking solution using a modification of a published procedure [18]. At this point, the nanopore membranes with the immobilized antibodies were ready for immunoassay. For that, the prepared membranes were dipped into sample solutions containing a given concentrations of antigen(s) for about 30 min. Then, the membranes were rinsed with PBS to remove unbound biomolecules and inserted in the electrochemical cell.

Electrochemical cell and instrumentation

To measure the ionic impedance through the nanopore membrane, an electrochemical cell was built, consisting of two polycarbonate blocks (each of which had an internal volume of 0.05 mL) with inlet and outlet ports, and an opening of 0.5 mm diameter, facing each block. The inside of each block contained a platinum wire electrode. In between the two polycarbonate blocks, a nanopore membrane was inserted. Then, the cell was sealed except for the inlet and outlet ports and the electrolyte (0.25 M PBS with 0.8% saline) was flown inside the cell. The ionic impedance of the electrolyte through the nanopores was measured using a PAR potentiostat/galvanostat Model 273 and a Solartron Frequency Response Analyzer Model 1260 in a two electrode configuration (the working electrode in one block and the auxiliary electrode in the other block). Measurements were taken at 50 mV AC, open circuit potential. In our initial studies, the frequency was sweep from 10 to 10 Hz. To aid the development of a portable, fast immunoassay sensor, we selected a specific impedance frequency value (10 kHz) that was very sensitive to the ionic conductance changes due to the antibody–antigen interaction. When conducting single frequency studies, the average of ten measurements is reported.

Results and discussion

Frequency selection

As indicated above, we selected 10 kHz as the impedance frequency value to aid the development of a portable, fast immunoassay sensor. This frequency showed to be very sensitive to the ionic conductance changes due to the antibody–antigen interaction, and we could obtained average signals (based on ten readings) in less than a second.

Figure 3 shows the Nyquist and the Bode impedance plots before and after exposure to a target antigen. It can be observed that the biggest changes in the impedance signals ($|Z|$) occurred at the frequency range around 10 kHz. The goal of this paper is to demonstrate that the use of a single impedance frequency can be used for the development of a nanopore membrane-based electrochemical immunoassay. On the other hand, the complete impedance plot contains important information regarding the electrode/antibody/antigen interfaces and can be obtained by proper fitting to an electronic equivalent circuit. This analysis will be discussed in a subsequent manuscript.

Selection of nanopore size

Since membranes with different nanopore sizes are available, we conducted experiments to select the best nanopore membrane for the immunoassay sensor. It is expected that impedance changes will be larger with smaller nanopore sizes because of bigger disturbance of the ionic conductivity through the pore by the antigen/antibody interaction. On the other hand, the pore size needs to be large enough to allow the binding of the antibody to the pore wall and to permit antigens reaching the antibodies. The electrolytic impedance of 0.25 M PBS with 0.85% saline through nanopore membranes of four different pore sizes (100, 50, 30, and 10 nm) was measured after each of the following steps: gold plating, antibody immobilization, addition of blocking agents, and antigen–antibody conjugation. Figure 4 shows the impedance values for membranes containing either hepsin or PSA antibodies. The biggest changes in the impedance values were observed for the 10 nm pore size membranes for both hepsin and PSA. There was a large increase of the impedance when antigens were bound to the specific antibodies. This shows that the antibody/antigen interaction

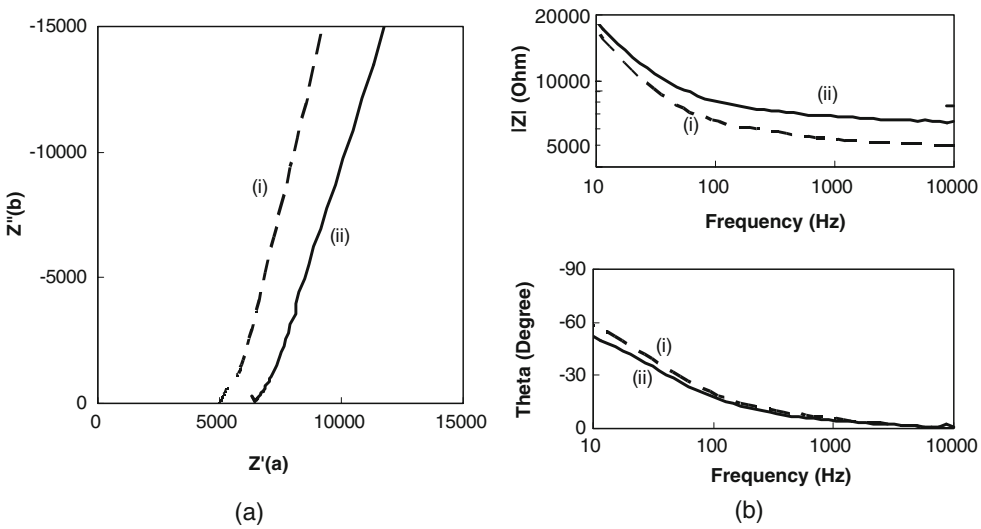

Fig. 3 Electrochemical impedance spectroscopy plots: **a** Nyquist and **b** bode plots for the nanopore with immobilized hepsin antibodies in 0.25 M PBS (*i*) before and (*ii*) after exposure to hepsin antigens

significantly decreases the ionic conductivity (i.e., increases the impedance) through the nanopore. On the other hand, non-specific binding of blocking agents showed only a slight increased the impedance.

Reproducibility and concentration dependence

For the reproducibility and concentration dependence tests, identical nanopore membranes (10 nm pore size) with immobilized hepsin antibodies were prepared. The hepsin antibody concentration in the solution used for the immobilization process was 10 ppm. The ionic impedance of 0.25 M PBS with 0.85% saline through each membrane was measured three times before and three times after the conjugation of the hepsin peptide (antigen) with the antibody. The hepsin peptide concentration in solution was 1 ppm, 100 ppb, or 10 ppb. For each concentration, three or four nanopore membranes were used. Figure 5 shows the signal dependence on the hepsin antigen concentration. The average of signals (where signal is defined as the difference of the ionic impedance at 10 kHz after and before exposure to the antigen) had a log–log dependence on the hepsin antigen concentration in the tested range (0.01 to 1 ppm). Each point is the average of signals obtained with three to four nanopore membranes. This test demonstrates the potential of using this technique for the quantitative analysis of targeted biomarkers.

The detection limit for hepsin antigen is estimated to be given either by the instrument resolution or the test reproducibility. The minimum impedance value 3 times above noise

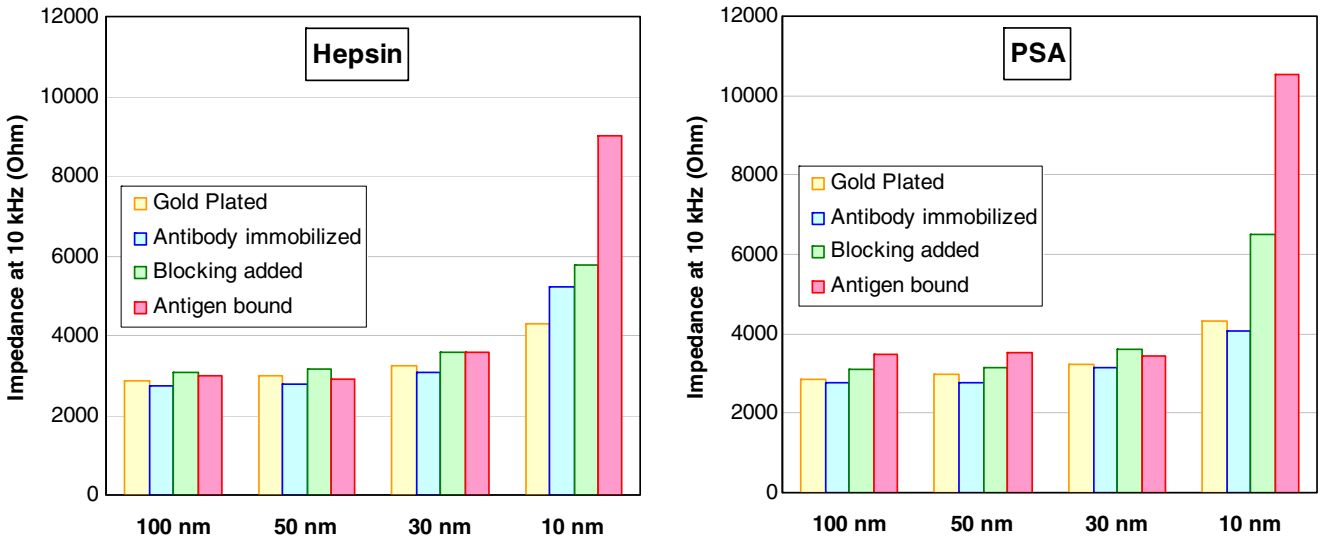

Fig. 4 Ionic impedance of 0.25 M PBS with 0.85% saline through nanopore membranes of different pore sizes (100, 50, 30, and 10 nm) after gold plating, antibody immobilization, addition of blocking agents, and antigen bounding at the nanopores. For the hepsin test, hepsin antibodies were immobilized at the nanopores and the membrane was exposed to hepsin antigens in the last step. For the PSA test, PSA antibodies were immobilized at the nanopores and the membrane was exposed to the PSA antigens in the last step

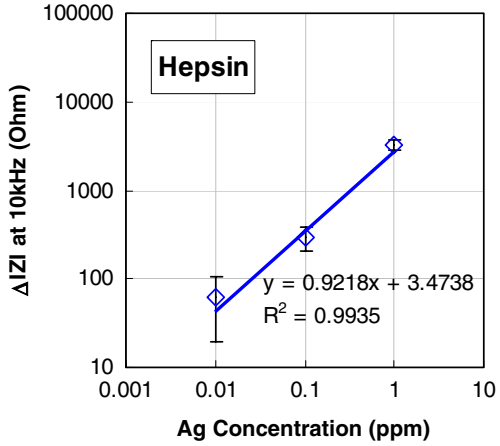

Fig. 5 Dependence of the signal on the hepsin antigen concentration at the hepsin antibody nanopore sensor. The signal represents the change of ionic impedance at 10 kHz due to the antigen–antibody interaction. Each point is the average of signals obtained with three or four nanopore membranes. The standard deviation for each average point was 68%, 31%, and 12% at 0.01, 0.1, and 1 ppm, respectively

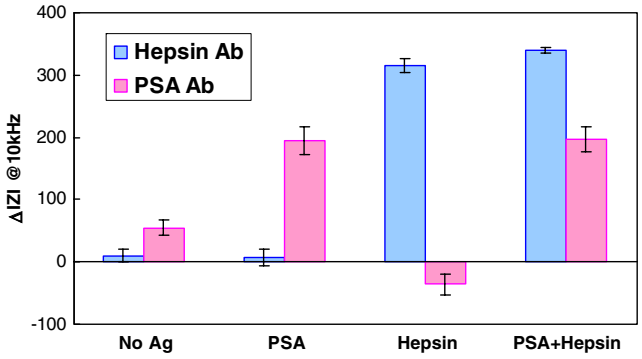

Fig. 6 Specificity test of hepsin antibody and PSA antibody in rabbit serum with no antigen, exposure to hepsin antigen only, exposure to PSA antigen only, and exposure to both types of antigens. Each experiment is the average of the impedance change using three membranes

level is about 10 Ω. Since the plotted values is the difference of the impedance values before and after exposure to the antigen, the limit value would be $((10\ \Omega)^2+(10\ \Omega)^2)^{1/2}$, i.e., 14 Ω, corresponding to 3 ppb. On the other hand, reproducibility is reflected in the standard deviation at 0.01 ppm (43 Ω), which corresponds to 6 ppb. Therefore, the detection limit for hepsin antigen is estimated to be 6 ppb.

The hepsin antigen used here is a hepsin-blocking peptide (2.5 kDa) and not the human hepsin (45 kDa). Hepsin is a putative membrane-associated serine protease [19], not a serum-based enzyme, so a reference value of the normal level of human hepsin in blood is not available. The concentration of hepsin in studies related to the hepsin activity is about 1 nM (i.e., 45 ppb) [20], well inside the tested detection range of the nanopore membrane-based electrochemical immunoassay sensor.

Specificity

One of the most important features of immunoassay is the specificity of the bioconjugation, which should be also present in the nanopore membrane-based electrochemical immunoassay sensor. Three (3) identical nanopore membranes (10 nm pore size) were gold plated and the hepsin antibodies were immobilized from a 10 ppm hepsin antibody solution. Each membrane was first immersed in a PSA antigen solution (0.1 ppm). After that, the membranes were immersed into a hepsin antigen solution (0.1 ppm). The ionic impedance was measured before and after immersion in the PSA antigen solution and after immersion in the hepsin antigen solution. As shown in Table 1, there was not significant change in the ionic impedance when the membrane with immobilized hepsin antibodies was exposed to PSA antigens. However, the impedance signal substantially changed when the membrane with immobilized hepsin antibodies was exposed to hepsin peptide (the corresponding antigen), as expected. In a similar experiment, a membrane with immobilized PSA antibodies was exposed to the hepsin peptide, with no significant change in the impedance values. However, when it was exposed to PSA antigens, the ionic impedance signal substantially changed. This results clearly demonstrated that the nanopore-based immunoassay sensor sustain the feature of specificity for immunoassay.

Testing in rabbit serum

The specificity of nanopore-based biological sensor was tested in rabbit serum as a preliminary pre-clinic testing. A

Table 1 Specificity tests of (a) hepsin antibody and (b) PSA antibody to hepsin and PSA antigens

	Hepsin antibody nanopore membrane		PSA antibody nanopore membrane	
Antigen exposure	Solution of 0.1 ppm PSA	Solution of 0.1 ppm hepsin	Solution of 0.1 ppm PSA	Solution of 0.1 ppm hepsin
$\Delta\lvert Z\rvert$ at 10 kHz[a]	−22±9	236±23	220±26	−61±16

$\Delta|Z| = |Z|_{\text{after antigen exposure}} - |Z|_{\text{before antigen exposure}}$

[a] The ionic impedance of 0.25 M PBS with 0.85% saline through nanopore membranes (10 nm pore size) with immobilized antibody was measured at three or more membranes

series of identical nanopore membranes (10 nm pore size) were prepared with either immobilized hepsin antibodies or immobilized PSA antibodies from a 10 ppm antibody solution. The membranes were immersed in one of four different samples: (1) rabbit serum with no antigen, (2) rabbit serum with 0.01 ppm PSA antigen, (3) rabbit serum with 0.01 ppm hepsin antigen, and (4) rabbit serum with both 0.01 ppm PSA and 0.01 ppm hepsin antigens. Prior to use, the rabbit serum was ten times diluted with PBS. After the membrane sensors were incubated and rinsed, the ionic impedance of 0.25 M PBS with 0.85% saline through each membrane was measured. As shown in Fig. 6, there were substantial changes in the impedance signal when the correct antibody–antigen conjugation took place. Very similar changes were obtained when the targeted antigens were present in solution by themselves or in the presence of another type of antigens. This experiment demonstrates the feasibility of using the nanopore membrane-based electrochemical immunoassay in actual sera or plasma, which are matrices much more complex than PBS.

Conclusion

This work demonstrates the feasibility of using the nanopore membrane-based electrochemical immunoassay sensor for the detection of target biomolecules. This novel technology implements emerging nanotechnology into well-established antibody-based assay using electrochemical signal transduction. We used this sensor to detect biomarkers of prostate cancer, PSA and hepsin. We selected 10 kHz as the impedance frequency value to aid the development of a portable, fast immunoassay sensor. This frequency showed to be very sensitive to the ionic conductance changes due to the antibody–antigen interaction. Antibodies were successfully immobilized on gold-coated nanopores in polycarbonate membranes. The best resolution in the antibody/antigen interaction was obtained using nanopore membranes of 10 nm pore size. Signals for hepsin antigen detection were linearly dependent on the hepsin antigen concentration in the range from 0.01 to 1 ppm, based on a log–log relationship. The feature of immunoassay—specificity—was successfully demonstrated by exposing nanopore membranes with an attached antibody type to the target antigen or another type of antigen, where either type of antigen was present in PBS solution or in actual sera or plasma. This last medium was also used to demonstrate the immunoassay specificity towards a target antigen in the present of a second type of antigen. In all cases, no significant signal change was observed in the absence of the target antigen. Signal change was large and alike when the target antigen was present either in the absence or presence of the second type of antigen. The specificity was successfully demonstrated with hepsin and PSA antigens in complex media such as rabbit sera.

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