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SHORT COMMUNICATION

Porous silicon microarray for simultaneous fluorometric immunoassay of the biomarkers prostate-specific antigen and human glandular kallikrein 2

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Abstract The authors have developed a porous silicon (P-Si) based duplex antibody microarray platform for simultaneous quantitation of the biomarkers prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2) in serum. Pore size-controlled P-Si surfaces have an extremely enlarged surface area that enables high-density immobilization of fluorescently labeled antibodies by physical adsorption. Automated microarraying of the antibodies provides a fast and reproducible duplex format of antibody arrays on the P-Si chips placed in the wells of a microtiter plate. The assay platform showed a 100 fg·mL^{-1} limit of detection for both PSA and hK2, and a dynamic range that extends over five orders of magnitude. After optimization of the density of both capture antibodies, neither the PSA nor the hK2 array showed cross-sensitivity to non-target proteins or other plasma proteins. The microarray was evaluated by titration of PSA and hK2, respectively, in the same serum samples. In our perception, this highly sensitive and selective platform holds promise for improved detection of tumor markers in an early diagnostic stage, but also to monitor the recurrence of prostate cancer.

Keywords Duplex antibody microarray · PSA · hK2 · Silicon anodization · Prostate cancer · Serum analysis

Introduction

The need for multiplex immunoassays, which allow simultaneous analysis of multiple biomarkers, is increasing due to its potential application of early diagnosis of disease [1–3]. Antibody or protein microarray techniques can be key tools to realize multiplex immunoassays since they ideally enable to immobilize a large number of probes or target proteins on the surface [4]. In most applications of protein/antibody microarrays the focus has been qualitative analysis such as profiling several hundreds or more proteins, globally analyzing protein phosphorylation, etc. [5, 6]. However, quantitative analysis of the microarray is crucially important for clinical application as a diagnostic tool since the quantification of biomarkers in body fluids can determine both occurrence and level of expression, which directly correlates to the stage of disease [2,

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7]. Hence, quantification of multi-target biomarkers in a highly sensitive manner is a major goal of microarray development to address the unmet needs for early diagnosis of diseases such as cancer.

Human tissue kallikreins (KLKs) located on chromosome 19, has since long gained attention due to their role as biomarkers for the screening, diagnosis, prognosis, and monitoring of various cancers including those of the prostate, ovarian, breast, testicular, and lung [8, 9]. Two of these human kallikreins, prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2), are currently used as valuable biomarkers of prostatic carcinoma [10]. Improving diagnosis accuracy of prostate cancer is crucially important since the long-term survival is close to 100 % in case of early detection (diagnosis before reaching a metastatic state) [11]. Prostate specific antigen (PSA) is the most well-known and frequently discussed biomarker, secreted from epithelial cells in the prostate gland, originating from leakage of pathological tissue to the vascular system, and has been shown to be proportional to the tumor burden [12, 13]. PSA is routinely used to measure the level in serum. Increasing levels of PSA in blood circulation can be caused by prostate cancer, but it also appears at benign prostate diseases since PSA is specific to prostate disorders but not to prostate cancer (PCa). Around 2–3 ng·mL^{-1} of PSA in plasma is a generally accepted diagnosis cut-off value, whereas higher values often merit further investigation, e.g. a prostate biopsy [14]. Although the current diagnostic method of PSA assaying captures almost all malignant cases, about 3 out of 4 cases are found to have an origin of other benign prostate disorders. Hence, population wide screening of only PSA is not a viable option due to the vast over diagnosis that this would lead to [10]. Therefore, requirement for additional prostatic tumor markers are increasingly high. Human kallikrein 2 (hK2) is also considered as a prostate cancer biomarker since the level of hK2 was found to be increased in serum from PCa patients relative to individuals with benign prostatic hyperplasia (BHP) [15]. It is also considered as a prognostic indicator for biochemical recurrence in men with PSA less than 10 ng·mL^{-1} [16]. Concentration of hK2 in human prostate is approximately 10–50 % of the PSA level and it is 50 to 100 fold lower than PSA concentration in blood serum [17, 18], making it a challenging biomarker to detect and quantitate.

One important way to improve diagnostic accuracy of prostate cancer is to develop a highly sensitive and selective multiplex assay. PSA isoforms and complex form such as free PSA (fPSA) and α1-antichymotrypsin (ACT) bound PSA have been shown to increase clinical value in diagnosis and prediction of prostate cancer [19–21]. Especially, measuring the ratio of free PSA versus the level of PSA (total PSA) in circulation was determined to improve diagnostic accuracy of prostate cancer (PCa) differentiating cases of BPH (Benign Prostatic Hyperplasia) from PCa [22, 23]. However, duplex immunoassays such as addition of % fPSA to the existing tPSA were mostly developed using microtiter plate ELISA format in which two single-plex assays are performed in a microwell format. Real meaning of a duplex assay is to simultaneously detect two biomarkers. Järås et al., developed a microarray based duplex immunoassay format as a proof of concept where free and total PSA were monitored on a single microarray chip [24]. They showed the recording of sub-ng·mL^{-1} LOD of both free and total PSA with three orders of magnitude dynamic range. However, the sensitivity of the method was not sufficient to address low abundant biomarkers such as Human kallikrein 2 (hK2).

Various techniques have been developed, recording picogram to sub-picogram per mL level of PSA and hK2 respectively. In the case of PSA detection, signal amplification techniques using nanoparticle [25, 26], digital ELISA systems [27, 28], and also 3-D surface microarray systems [29] were developed. Microfluidic based electrochemical [30] and electromagnetic [31] platform has been developed to detect PSA in serum sample in rapid and high sensitive manner. Moreover, using time resolved fluorescent intensity technology such as DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) the limit of detection of hK2 was reported to be in the low pg·mL^{-1} with a 10^3 (3 pg·mL^{-1} to 3 ng·mL^{-1}) dynamic rage [32, 33]. Although several sensitive detection methods were developed for single-plex immunoassays, a highly sensitive PSA and hK2 duplex format has not yet been developed.

In this paper we describe the development of an antibody based porous silicon duplex immunoassay platform for highly sensitive and simultaneous detection of PSA and hK2 in human serum. The porous silicon substrate used herein is based on the in-house development of a surface morphology tailored at the micro- and nano-scale level [24, 29, 34]. The hydrophobic properties and 3-D surface morphology of P-Si provides sufficient spot confinement to increase the density of the affinity binder (i.e. the captured antibody) and thus leads to higher bioassay read-out sensitivity. It also offers great optical properties such as homogeneous fluorescent intensity spot profiles and low background fluorescence. The porous silicon surface is biocompatible, due to its hydrophilic nature at a molecular level while being hydrophobic at the microscale due to the surface morphology, which makes it amenable to immobilization of bio-specific binders with maintained affinity and selectivity [35]. This in turn enables analysis of complex sample such as blood, urine and CSF. By controlling pore size, P-Si allows to array the capturing antibodies for PSA and hK2 in high densities, which in turn made it possible to capture low abundant target biomarkers more easily [29, 34]. Moreover, unspecific binding and cross-reaction of the antibodies were drastically reduced due to the optimization of the surface density of each capture antibody. The platforms showed a high sensitivity in the duplex assay format reaching into the 100 fg·mL^{-1} limit of detection.

Materials and methods

Proteins and reagents

Recombinant human PSA (P3338) and hK2 (ATGP2175) were obtained from Sigma-Aldrich (http://www.sigmaaldrich.com) and ATGen (http://atgenglobal.com), respectively. The monoclonal mouse antibodies against PSA (5A6, 4P33) and against hK2 (6B7, ab40749) were purchased from HyTest (https://www.hytest.fi) and Abcam (http://www.abcam.com), respectively. The polyclonal sheep anti–PSA antibody and Alexa Fluor 488 labeled donkey anti–sheep antibody were purchased from Abcam, Cambridge, UK, (ab35355) and Jackson ImmunoReaserch (https://www.jacksonimmuno.com), West Grove, PA, USA, (713-545-003), respectively. The polyclonal goat anti–hK2 antibody (PAB7226) and Alexa Fluor 488 labeled donkey anti–goat (ab150129) antibody were purchased from Abnova (http://www.abnova.com) and Abcam, respectively. Phosphate buffered saline (PBS) and Tween-20 were purchased from Bio-Rad (http://www.bio-rad.com).

Human female serum spiked with PSA and hK2

Female single donor serum was purchased by SCIPAC (S122-1, http://www.bbisolutions.com), aliquoted, and stored – 80 °C. The serum was spiked with purified recombinant PSA and hK2 as standard. PSA and hK2 were spiked into female. 1 $mg{\cdot}mL^{-1}$ of PSA and/or hK2 was spiked into female serum sample preparing total concentration of the serum with 10 $\mu g{\cdot}mL^{-1}$. The samples were diluted by female sample serum to obtain a final concentration ranging from a 100 $fg{\cdot}mL^{-1}$ to a few 100 $ng{\cdot}mL^{-1}$.

Fabrication of porous silicon (P-Si)

The micro- & nano-morphology of the porous layer affects the fluid interaction and molecular adsorption properties of the P-Si chip and are strongly governed by a large number of etching parameters such as hydrofluidic acid (HF) concentration, current density, anodization time, illumination, crystal orientation, silicon type, doping levels [36]. The fabrication procedure of porous silicon in this paper was followed to optimize the conditions for antibody immobilization as previously described [33].

The P-Si was fabricated by anodic dissolution of silicon wafer in mixture of hydrofluidic acid (HF, 45 %) and dimethylformamide (DMF: Merck, Darmstad, Germany) (1:10 *v/v*) solution. The wafer was placed in middle of an electrochemical-etching cell. Current was passed through the silicon wafer to initiate and process the porous silicon wafer formation. The silicon, 6–8 ohm·cm resistivity (boron doped p-type), <100 > orientation, was purchased from Addison Engineering (San Jose, CA, USA). The silicon was anodized for 70 min with backside illumination. Current density during anodization was 90 $mA{\cdot}m^{-2}$ after which the silicon was washed in ethanol three times and diced into 3x3 mm pieces to fit a microtiter plate well format (Corning Costar Corporation, Cambridge, MA, USA).

Duplex sandwich antibody microarray

Figure 1 shows the P-Si duplex format for PSA and hK2. 3 × 3 mm porous silicon chip were place in the microarray (Scienion DW, Scienion AG). Around 300 pL droplets of both monoclonal mouse antibody for PSA (5A6) and hK2 (6B7) were sequentially dispensed using a piezoelectric dispenser with a spot to spot distance of 120 μm. The antibodies were immobilized on the chip surface by physical adsorption. The microarray has 10 × 1 0 (100 spots) array format, which half of arrays contain PSA antibody and the other half has hK2 antibody. Concentrations of PSA antibody and hK2 antibody are 60 $\mu g{\cdot}mL^{-1}$ and 100 $\mu g{\cdot}mL^{-1}$, respectively. The chip was then placed in the 96-well microtiter plate (Corning Costar Corporation, Cambridge, Massachusetts, USA). We choose black microtiter plate for preventing photo bleaching during assay. The chips, each containing 100 spots were blocked for 1 h in 100 μL blocking solution (5 % (*w/v*) non-fat dry milk in 10 mM PBS (Bio-Rad)) in order to prevent non-specific binding during the next incubation step. The microarray chips were washed 3 times in 100 μL PBS-tween (0.05 % Tween 20 in 10 mM PBS).

PSA and hK2 single-plex sandwich immunoassay

The prepared microarray chips were incubated with 15 μL each of PSA or hK2 spiked serum solutions for 1 h. After incubation, the chips were washed again 3 times in 100 μL PBS-Tween. The next step was to incubate the chips with 15 μL of polyclonal sheep anti-PSA antibody or polyclonal goat anti-hK2 antibody. The washing steps were repeated before exposing the chips to incubation of secondary antibody (PSA sandwich assay for Alexa Fluor 488 labeled donkey anti–sheep antibody and hK2 sandwich assay for Alexa Fluor 488 labeled donkey goat–sheep antibody). After 1-h incubation, the chips were washed three times by PBS-Tween and dried with pressurized air before microscopic observation.

PSA and hK2 duplex sandwich immunoassay

The duplex sandwich immunoassays were performed as the single-plex assay described as above, but with additional as follow. Mixture spiked serum sample of PSA and hK2 were prepared various concentration. The detection antibodies and secondary antibody for PSA and hK2 were also mixed with their total concentration 1 μg in the micro array mL^{-1}. The microarray chip were incubated with mixture serum of 15 μL for 1 h and washed by PBS-Tween three times. Prepared mixture antibody were added and incubated on the chip. The chips

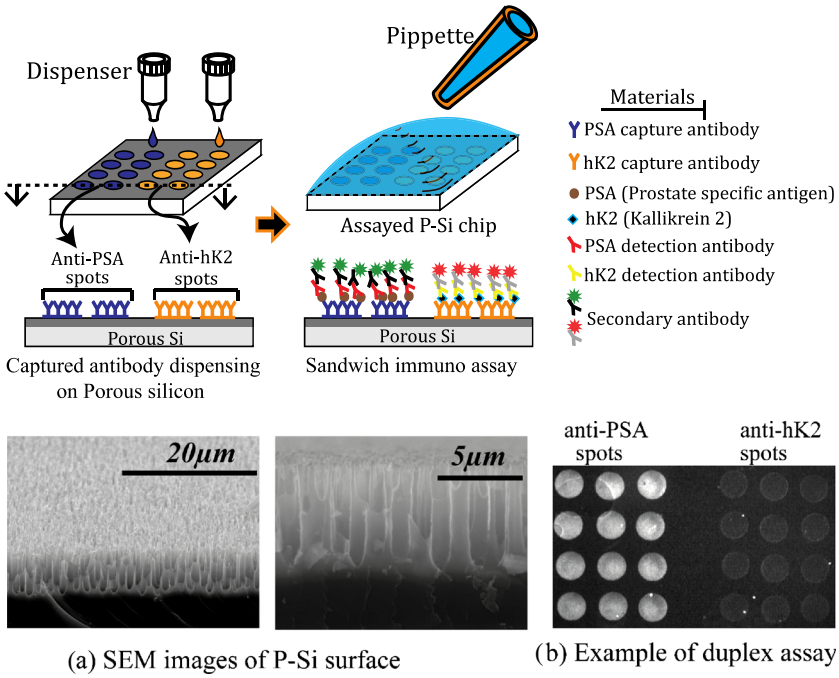

Fig. 1 Schematic of P-Si chip immunoassay procedure (top), starting with dispensing of PSA and hK2 captures antibodies onto the porous silicon surface. Non-contact dispensing type microarray (Scienion DW) was used to array the PSA and hK2 antibodies, and PSA and hK2 spiked serum sample was added. Subsequently, detection antibodies (polyclonal detection and fluorescent labeled secondary antibody) provided the fluorescent readout signal. The scanning electron micrographs show a sequential zoom into a typical surface (bottom figure **a**). The macro-pores of micrometer size are clearly seen, combined with a micro and nano-morphology (pore size around sub-μm to μm). A typical readout of the duplex assay is seen in **b**)

finally were washed three times by PBS-Tween and dried with pressurized air before microscopic observation.

Fluorescent readout and analysis

The fluorescent spots images were observed using a Nikon eclipse TE2000-U. The mean intensities of the spots were measured and quantified by an open source image-processing tool, Image J (http://rsbweb.nih.gov/ij/). Among all microarray spot images, we selected 9 (3x3 array) spots on each P-Si chip for quantification of the data since they were imaged in one single screen shot. A total of 18 spots were chosen for data analysis since all experiments were performed on two independent chips. The spot intensities (S) were measured and quantified across the area. The local background (B) was sampled in the same way and subtracted from the spot signals, generating mean spots intensities (S-B) as presented in the graphs. The limit of detection (LOD) was defined as the lowest detectable signal from 3-sigma standard deviation above the mean spot intensities of the negative control (N).

Results and discussion

PSA and hK2 immunoassay in a duplex format

The optimized concentration of capture antibodies for the duplex microarray was selected to minimize cross-reaction and un-specific binding and provide optimal conditions for PSA and hK2 detection in serum. For minimizing cross-reaction, we therefore quantified the cross-reaction between the PSA antibody (5A6) and the hK2 secondary antibody (PAB7226) with respect to their corresponding target proteins. Detailed process and result were reported on supplementary materials in "Antibody optimization for removing cross-reaction of antibodies" section (also see Fig S-1 in supplementary materials).

We carried out each PSA and hK2 immunoassay using the selected duplex antibody configuration (60 $\mu g \cdot mL^{-1}$ in the micro array of PSA capture antibody and 100 $\mu g \cdot mL^{-1}$ in the micro array of hK2 capture antibody). Each antigen was spiked into serum in a concentration ranging from 100 $fg \cdot mL^{-1}$ in the micro array to 100 $ng \cdot mL^{-1}$ in the micro array and the immunoassay was also accompanied by a negative control (No antigen spiked serum). Figure 2a shows a titration

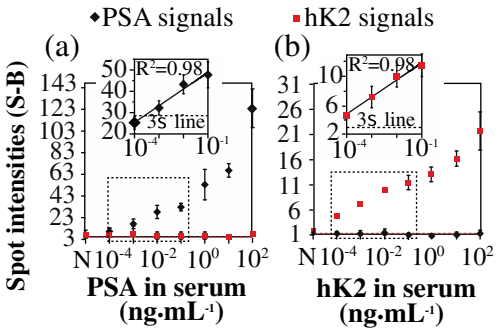

Fig. 2 Titration of PSA spiked into serum (100 $fg \cdot mL^{-1}$ to 100 $ng \cdot mL^{-1}$) was performed and immunoassayed and the fluorescent signals from the PSA antibody and the hK2 antibody arrays were plotted, Fig. 2**a**. Similarly, we immunoassayed hK2-spiked into serum (from 100 $fg \cdot mL^{-1}$ to 100 $\mu g \cdot mL^{-1}$) and recorded each spot signal Fig. 2**b**. The LOD was found to be sub $pg \cdot mL^{-1}$ using the 3σ definition and the dynamic range was approximately six (from 100 $fg \cdot mL^{-1}$ to 100 $ng \cdot mL^{-1}$) orders of magnitude

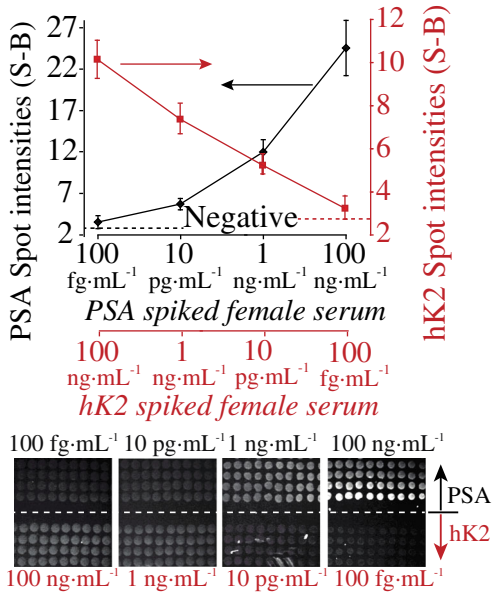

Fig. 3 Concurrent titration series of PSA and hK2 and recorded fluorescence signals from the four mixed samples of PSA and hK2 spiked into female serum. Four cases of serum samples were analysed with PSA and hK2 concentrations from 100 fg·mL^{-1} to 100 ng·mL^{-1} and 100 ng·mL^{-1} to 100 fg·mL^{-1}, respectively. The lowest spot signals (100 fg·mL^{-1} of each antigen) were distinguished against the negative control, while the counter antigen concentrations were 100 ng ·mL^{-1}

series of PSA spiked serum sample on the duplex antibody microarray. LOD can be defined in between 100 fg·mL^{-1} to 1 pg·mL^{-1} (less than 500 fg·mL^{-1}) by definition of the 3σ rule (the lowest detectable signal at 3σ above the mean spot intensities of the negative control). It should be noted that the spot signals of the hK2 antibody array are constant at the negative control baseline for all concentrations of PSA spiked in serum, which confirmed no un-specific binding between the hK2 capture antibody and the PSA antigen at a 100 μg·mL^{-1} concentration of the hK2 antibody. Microarray images are also shown in supplementary materials (Fig S-2).

In most cases, the spot reproducibility within the chips displayed CVs less than 25 %. The linear regression of the mean spot intensities versus PSA concentration corresponded to a coefficient of determination (R^2) equal to 0.98. The dynamic range was defined as the lowest detectable signal of PSA at a 3σ level above the negative control to the highest intensity before the spot signal was saturated. We performed the PSA assay in higher concentration from 10 ng·mL^{-1} to 10 μg·mL^{-1} to define the dynamic range (See supplementary data Fig S-3a). Spot signals were saturated at PSA levels above 100 ng·mL^{-1}, and hence the dynamic range of the PSA P-Si microarray immunoassay was determined to be around 5 orders of magnitude (from few tens of pg·mL^{-1} to 100 ng·mL^{-1}).

The corresponding titration was subsequently made for hK2 spiked into serum, at levels of 100 fg·mL^{-1} to 100 ng·mL^{-1}, Fig. 3b. The negative control and the PSA spots displayed the same low base line signal over the entire hK2 concentration range, indicating a negligible un-specific binding of hK2 to the PSA capture antibody (spotted at 60 μg·mL^{-1}). The LOD of hK2 was found to be 100 fg·mL^{-1} based on 3σ definition. Spot reproducibility and linear regression were similar to that of the PSA assay with a spot CV less than 25 % and a coefficient of determination (R^2) of 0.986. The dynamic range of the hK2 assay was found to be around six orders of magnitude, a few hundred fg·mL^{-1} to 100 ng·mL^{-1}, above which the fluorescence signal was saturated (See Fig S-3b in supplementary materials).

The P-Si duplex microarray format with its optimized capture antibody concentrations displayed a high sensitivity with a broad dynamic range for both PSA and hK2. The assay also displayed a negligible cross-reaction interference of the target molecules versus their non-target antibodies.

Simultaneous immunoassay using PSA and hK2 spiked serum

Simultaneous PSA and hK2 detection was finally performed using our optimized duplex P-Si microarray format. A mixture of PSA and hK2 was spiked into female serum and four samples were prepared with the PSA and hK2 concentrations as given in Table 1. Two negative control samples were also prepared, Table 1. 15 μL of each mixed sample were incubated on the P-Si duplex antibody microarray platform. After washing the P-Si chips were incubated with detection and secondary antibody, sequentially. The detection and secondary antibodies were prepared for the duplex assay with their total concentration fixed to 1 μg·mL^{-1}. After the final washing step, the P-Si chips were analyzed by fluorescence microscopy.

Figure 3 displays microarray spot intensities of four sets of PSA and hK2 mixture samples. The duplex microarray signals of PSA and hK2 are clearly shown in each antibody array images of Fig. 3. The intensities of the PSA spots increase in proportion to concentration of PSA (black line) while those of hK2 diminish in accordance with the decreasing of hK2 levels in each sample (red line). Two negative experiments were also performed to crosscheck for un-specific binding. All spot signals of the four spiked samples were higher than that of the negative control, hence concluding that the duplex assay was

Table 1 Four PSA and hK2 spiked serum sample and their concentration

	Sample 1	Sample 2	Sample 3	Sample 4	Negative 1	Negative 2
PSA	100 fg·mL^{-1}	10 pg·mL^{-1}	1 ng·mL^{-1}	100 ng·mL^{-1}	0 fg·mL^{-1}	100 ng·mL^{-1}
hK2	100 ng·mL^{-1}	1 ng·mL^{-1}	10 pg·mL^{-1}	100 fg·mL^{-1}	100 ng·mL^{-1}	0 fg·mL^{-1}

Table 2 Figure of merit of comparable PSA/hK2 immunoassay

Material & Methods	LOD / Dynamic ranges	Comments	Ref
Gold nanoparticle based ELISA	0.1 $pg{\cdot}mL^{-1}$ / 0.1 $pg{\cdot}mL^{-1} \sim 100$ $ng{\cdot}mL^{-1}$	High sensitivity, broad dynamic rages but lack of multiplexing	[25]
Nanoparticle bio-barcode	0.33 $pg{\cdot}mL^{-1}$ / 0.33 ~ 33 $pg{\cdot}mL^{-1}$	Fast assay, Potential of multiplexing but complicate process	[26]
Digital ELISA	60 $ag{\cdot}mL^{-1}$ / 60 $ag{\cdot}mL^{-1} \sim 6$ $pg{\cdot}mL^{-1}$	Ultra-sensitivity, Multiplexing but only allow low concentration assay	[27, 28]
DELFIA (Lanthanide labeled probe)	3 $pg{\cdot}mL^{-1}$ / 3 $pg{\cdot}mL^{-1} \sim 3$ $ng{\cdot}mL^{-1}$	Commercially available, good sensitivity but lack of multiplexing	[32, 33]
Porous silicon	0.1 $pg{\cdot}mL^{-1}$ / 0.1 $pg{\cdot}mL^{-1} \sim 100$ $ng{\cdot}mL^{-1}$	High sensitive, multiplexing and broad dynamic ranges	This work

not affected by un-specific binding against non-target components. The duplex antibody microarray displayed an ability to simultaneously measure both PSA and hK2 in serum at a highly sensitive level of sub pictogram per mL LOD, possibly opening the route to early detection of prostate cancer relapse.

Conclusion

The surface morphology and hydrophobic properties of micro/nanoporous silicon presents a large surface area 3D matrix for efficient surface adsorption based immobilization of antibodies. Our P-Si duplex antibody microarray platform has a sensitivity for PSA detection comparable to other ultra-sensitive detection methods such as nano-particle based detection [25, 26], single molecular digital ELISA systems [27, 28] and displays a better sensitivity of hK2 detection compared to optimized ELISA based systems for hK2 detection [32, 33]. Table 2 shows figure of merits of comparable PSA/hK2 immunoassay.

This platform also enables simultaneous measurement of both PSA and hK2 in serum at the same level of sensitivity by optimizing the capture antibody concentration. This optimization also minimized the levels of cross-reaction and unspecific binding and thus makes it possible to perform the duplex assay in a highly sensitive and selective manner.

Future efforts will be targeted to real clinical sample cohorts preferably classified according to age and stage of disease, including before and after prostatectomy. The presented duplex PSA and hK2 may be accompanied with additional biomarkers providing an increased multiplex level and further improved diagnostic precision. The developed platform however relatively long assay time (at least four hours) and complicate process. Combining with electrochemical sensing techniques and microfluidics [30, 31] the platform can improve total assay speed and realize system automation.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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