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Improved porous silicon (P-Si) microarray based PSA (prostate specific antigen) immunoassay by optimized surface density of the capture antibody

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Abstract

Enriching the surface density of immobilized capture antibodies enhances the detection signal of antibody sandwich microarrays. In this study, we improved the detection sensitivity of our previously developed P-Si (porous silicon) antibody microarray by optimizing concentrations of the capturing antibody. We investigated immunoassays using a P-Si microarray at three different capture antibody (PSA - prostate specific antigen) concentrations, analyzing the influence of the antibody density on the assay detection sensitivity. The LOD (limit of detection) for PSA was 2.5ngmL^{-1}, 80pgmL^{-1}, and 800fgmL^{-1} when arraying the PSA antibody, H117 at the concentration 15μgmL^{-1}, 35μgmL^{-1} and 154μgmL^{-1}, respectively. We further investigated PSA spiked into human female serum in the range of 800fgmL^{-1} to 500ngmL^{-1}. The microarray showed a LOD of 800fgmL^{-1} and a dynamic range of 800 fgmL^{-1} to 80ngmL^{-1} in serum spiked samples.

Keywords

Antibody microarray; Porous silicon; Sandwich immunoassay; Prostate specific antigen

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1.Introduction

Microarray based immunoassays are currently undergoing intense developments for the detection of low abundant protein biomarkers in human biofluids such as serum, urine and CSF (cerebrospinal fluid). The microarray format can ultimately offer advantages in terms of a low amount antibody consumption, high sensitive readout, and multiplex performance. Such developments could hold promise of earlier diagnosis of disease, reducing the need for biopsy and providing post therapy monitoring of patients for recurrence [1, 2]. There are mainly two types of microarray-based immunoassays when analyzing a biofluid without performing any chemical modification or labeling of the sample:

1. The sandwich microarray antibodies are spotted on solid surfaces. The biofluid with the target analyte is subsequently incubated on the array for specific binding to the primary antibody. After addition of a secondary antibody that is allowed to bind the target, the sandwich complex is formed. Sandwich assays are widely used for diagnostics, frequently in 96-well formats. The translation of these assays into a miniaturized format is an attractive approach to minimize the consumption of sample and analyte. The most difficult step is to obtain a matched sandwich antibody pair [3, 4].

   As an alternative, an array of spotted biofluids (sample) can be probed with individual antibodies named reverse phase type immunoassay [5].

2. In reverse phase assay, many different samples (cell or tissue lysates) are immobilized in a microarray format and simultaneously analyzed for the presence of a single protein using a target-specific antibody. This enables label-free analysis of biological samples by simply arraying the biofluid and detecting the biomarkers with an antibody, e.g. by fluorescent labeling of the antibody or by catalyzed signal amplification and colorimetric readout [5, 6]. Such an assay has a potential for detection of autoantibodies in the classifications of different autoimmune disease [7], but due to its inherent properties it can not be used for analysis of low abundantly expressed biomarkers [8]

Sandwich immunoassays have become a major work horse in clinical diagnostics since they offer high detection sensitivity gained by the enrichment of the target proteins to the capturing antibody [9]. The assay specificity is greatly increased by using matched antibody pairs.

To increase the assay sensitivity, several amplification methods have been proposed that are linked to modifying the detection antibodies by e.g. dendritic amplification [10], catalyzed signal amplification with colorimetric readout [11,12] or detection with rolling-circle amplification [13]. On the other hand, enriching the concentration of the capture antibody may also enhance sensitivity yet maintaining a simple assay protocol. Increased density of the immobilized antibody on the each microarray spot can offer improved capturing capacity of target antigen, which in turn increases the number of antigen bound to the primary antibody and consequently more completed sandwich pairs are achieved at the end of the assay leading to increased detection signals [4,14].

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To enrich a capture antibody on the surface, the substrate or chip is of utmost importance. The substrate used in our current study is an in house developed three- dimensional porous silicon surface. It has proven to be highly compatible with protein microarray technology based on its spot quality, spot density and sensitivity [14]. As a model biomarker we used PSA (prostate specific antigen), which is the most commonly used biomarker for prostate disease, e.g. prostate cancer. PSA, a kallikrein-related peptidase, occurs in free (unbound) and bound (complex) forms secreted from the epithelial cell in the prostate gland [15]. Although PSA has its limitations to distinguish between malignant and benign prostate diseases, it still remains as a valuable biomarker capable of discriminating different prostate cancer stages and potential indicators of recurrence in patient after radical prostatectomy (RP) [16,17].

The most commonly used diagnostic cut-off value for PSA in plasma is 2–3 $ngmL^{-1}$, higher values often merit further investigation, e.g. a prostate biopsy [15]. In order to detect prostate cancer (PCa) recurrence after radical prostatectomy by measuring PSA in plasma a three order of magnitude lower limit of detection is required [18]. To achieving such a demand, much higher sensitive detection methods are required. D. Liu et al. [19] used gold nanoparticle -based probe to increase detection sensitivity of PSA in serum. They selected five prostate cancer patients' serum and diluted until sub pictogram per milliliter using PBST buffer. LOD could be told sub to few $pgmL^{-1}$ in the paper. Another nanoparticle based PSA immunoassay (so called bio barcode system) was published by C.S. Thaxton et al. [20] to monitor prostate cancer recurrence. LOD of this system push down to 330 fg/mL and showed possibility of early detection of prostate cancer recurrence. Single molecular based digital ELISA systems are one of important direction toward ultra sensitive assay development. D.M Rissin et. al. realized the femtoliter chamber array platform, which occupies single bead with one-target molecules in one chamber [21,22]. This assay platform detected PSA in sera under 14 $fgmL^{-1}$ and also shown reliability of the assay for post-RP. Similar approach but increasing number of chambers enormously could be limit of detection less than 2 aM [22].

Although above methods are impressive in its low LOD, our developed P-Si based microarray platform has great advantage of its simplicity and robustness. P-Si surface does not require any chemical treatment such as amine, or epoxy coating for immobilization of antibody. Since physical adsorption is main motif to bind antibody on the surface, it does not require any laborious procedure such as incubation, humidity control and temperature control. It only takes few minutes to finish up antibody immobilization on the surface so that it is possible to reduce total assay time [23,24]. The P-Si microarray platform also dealt with 80 clinical plasma samples to quantify total and free PSA (duplex assay) [25]. The samples analyzed by this microarray obtained 0.14$ngmL^{-1}$ of LOD with dynamic range from 0.4 to 74.9 $ngmL^{-1}$ at total PSA and 0.76 $ngmL^{-1}$ of LOD with dynamic range from 0.87 to 295 $ngmL^{-1}$ in case of freePSA.

In this study, we improved detection sensitivity of our previously developed P-Si based antibody microarray by changing the concentration of the capturing antibody when spotting the microarray. Figure 1 schematically shows the effect of enriching the capture antibody in the immunoassay. Three different concentrations of capturing antibody were arrayed and

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evaluated in detecting PSA in buffer (PBS). The P-Si microarray, with optimized primary antibody concentration, was subsequently evaluated in detecting PSA spiked in human female serum. The reported data shows that the PSA detection sensitivity was improved about three orders of magnitude by increasing the concentration of capturing antibody in the P-Si microarray, with a LOD of 800fgmL^{-1}.

2. Materials and Methods

2.1 Porous silicon fabrication

Morphology and geometry of pores layers are affect physical property and characteristics of P-Si chip and they are strongly governed by a large number of etching parameters such as HF concentration, current density, anodization time, illumination, crystal orientation, silicon type, doping levels [23]. Generally good uniformity and mechanical stability of porous layer, low intrinsic fluorescent, and low wetting ability, i.e., a high liquid contact angle are demanded for porous support for protein immobilization [23,26]. The fabrication procedure of porous silicon in this paper was followed to optimum condition for immobilization of antibody as described previously [23]. In brief, silicon, 6–8 ohm·cm resistivity (boron doped p-type), <100> orientation, was purchased from Addision Engineering (San Jose, CA, USA). The wafer was placed in middle of an electrochemical-etching cell. The electrolyte solution consisted of 3.6 % hydrofluoric acid and 90.7 % dimethylformamide (Merck, Darmstad, Germany). The silicon was anodized for 70 min with backside illumination. Current density during anodization was 90mA/m^2 after which the silicon was washed in ethanol three times and diced into 3x3 mm pieces to fit a microtiter plate format (Corning Costar Corporation, Cambridge, MA, USA).

2.2 Proteins and Reagents

The monoclonal mouse antibody against PSA (H117) was produced as previously described [18]. The polyclonal sheep anti–PSA antibody and Alexa Fluor 488 labeled donkey anti–sheep antibody were purchased at Abcam, Cambridge, UK, (ab35355) and Jackson ImmunoReaserch, West Grove, PA, USA, (713-545-003), respectively. Prostate specific antigen (PSA) from human semen was obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.3 Analytical samples

Human female serum was obtained from a healthy blood donor, aliquoted, and stored −80 °C. The serum was spiked with PSA in a titration series ranging from 800 fgmL^{-1} to 80 ngmL^{-1}.

2.4 Sandwich immunoassay

The porous silicon wafer was diced into 3×3 mm chips to fit into microtiter platewells. Droplets of approximately 300 pl monoclonal mouse antibody (H117) were dispensed in a 9×9 array format onto a porous silicon chip at a spot to spot distance of 250μm using an in-house developed piezoelectric micro-dispenser [24,27]. Three different concentrations of capturing antibodies (15μgmL^{-1}, 35μgmL^{-1}, and 154μgmL^{-1}) were arrayed on each P-Si chip and PSA was assayed in PBS buffer to evaluate the influence of antibody concentration. The corresponding assays were subsequently performed on PSA spiked

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human female serum samples. The mean spot intensities, calculated from 12 spots for each sample, were used to derive a calibration curve for PSA in the concentration range 800 $pgmL^{-1}$ to $80ngmL^{-1}$.

The antibody-activated chips were loaded in 96 well plates for the immunoassay. To remove loosely bound antibody, the chips were washed three times by 10 mM PBS. After blocking in 100μl 5% (w/v) non-fat dry milk in PBS (Bio-Rad, Hercules, CA, USA) for 1 hour to prevent non-specific binding, the chips were washed 3 times in PBS-Tween solution (0.05% Tween 20 in 10mM PBS) and 100 μl of PSA spiked human female serum pipetted into each well and incubated for 1 hour. After an additional washing step, the chips were incubated with 100μl of polyclonal sheep anti-PSA antibody (1/1000 dilution of its original concentration) as the secondary antibody. Following a washing step, $1\mu gmL^{-1}$ of 100 μl Alexa Fluor 488 labelled anti- sheep polyclonal detector antibody was added to the chips and incubated for 1 hour. Finally, the chips were washed 3 times and dried in room temperature. Fluorescence readout was performed by a BX51WI microscope with laser confocal unit (Olympus, Japan). For reproducibility, all assay were performed in two independence chips.

2.5 Morphology analysis by FESEM

The morphology of the porous silicon surface was analysed by a field emission scanning electron microscope (FESEM; JEOL JSM-6700F), Figure 2. Before SEM analysis, a 10-nm layer of platinum was deposited on the samples.

2.6 Measurement

The intensity of each spot was quantified by an open source image processing tool kit, ImageJ (http://rsbweb.nih.gov/ij/). 9 spots of each P-Si chip were chosen to quantification of data, which means totally 18 spots were used for data analysis since all experiment were performed within two independent chips. The intensity of each spot image was measured and averaged across its circular area. Local background signal was collected the same way and subtracted from the spot signals, generating mean spot intensities as presented in the graphs. The LOD (limit of detection) was defined as the lowest detectable PSA concentration corresponding to at least two standard deviations above the mean spot intensities of the negative control (N) signals.

3. Results and Discussion

3.1 Surface morphology of porous silicon (P-Si) and microarray format

The 3-D morphology of the micro/nano porous silicon surface layer offers a high capacity of antibody immobilization. Figure 2-a shows macroporous layers with a characteristic size of sub micro −1μm on the silicon surface by FESEM images. Captured antibody (H117) was microarrayed onto the 3-D porous silicon surface using an in-house developed piezoelectric microdispenser. Figure 2-b shows an example of spots and the homogenous intensity profile of a microarray obtained by the micro-/nanoporous surface morphology.

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3.2 Assay performance against concentrations of capturing antibody

In general, a surface bound immunoassay becomes more sensitive, if the affinity of the capture antibody increases and/or if the surface density of capture antibody molecules increases [4,14]. To optimize our P-Si microarray we evaluated the assay sensitivity by arraying the PSA capture antibody, H117, at three different concentrations and performed the PSA immunoassays in buffer solution. In Figure 3 the microarray fluorescence readout versus the PSA concentration is presented for three different antibody concentrations (15, 35 and 154 μgmL^{-1}). The increased concentration of the capture antibody assay yielded a lowered PSA LOD from 2.5 $ngmL^{-1}$ at an antibody concentration of 15 μgmL^{-1} to a LOD of 800 $fgmL^{-1}$ at an antibody concentration of 154 μgmL^{-1}. It was however noted that at an antibody concentration of 154 $ugmL^{-1}$ the negative control (N) displayed an elevated signal above zero caused by unspecific binding between capturing antibody and its binding partner (such as secondary or the detector antibody). It was also noted that the dynamic range was extended from 10^{0}–10^{2} $ngmL^{-1}$ to 10^{-3}–10^{2} $ngmL^{-1}$ as the concentration of the antibody was increased. The signal intensities were enhanced in proportion to the concentration of the capturing antibody, which followed the expectations of improved LOD with increased surface density of the capture antibody. Table 1 shows coefficient of variance and mean spot intensities of the figure 3. In most case, spots reproducibility within the chips has good reliability (CVs ~ 10–20%). However, three cases such as two negative controls in capturing antibody concentration of $15\mu gmL^{-1}$ and $35\mu gmL^{-1}$, and 8 $pgmL^{-1}$ of PSA when the capturing antibody was 35 μgmL^{-1} are significantly high CV (~ 70 to 85 %). That is caused by no or less signal difference between spot images and background. In comparison to our earlier study using a nanoparticle enhanced microarray assay [24], the P-Si microassay`s reported herein displayed an improved LOD of two orders of magnitude (800 $fgmL^{-1}$ when arraying the capture antibody, H117, at a concentration of 154 μgmL^{-1} as compared to 0.07 – 0.14 $ngmL^{-1}$ of the earlier nanoparticle assay)

3.3 PSA microarray assays in female human serum

The P-Si microarray assay was subsequently evaluated in human serum. Three P-Si microarrays with antibody concentrations of 35 μgmL^{-1}, 77 μgmL^{-1} and 154 μgmL^{-1} were primarily tested in PSA-spiked human female serum at three different PSA concentrations; $800pgmL^{-1}$, $8ngmL^{-1}$ and $80ngmL^{-1}$. The choice of antibody concentration was based on the data in Figure 3. The corresponding titration series of PSA in female serum was recorded for microarrays with three different antibody concentrations: 35, 77 and 154 μgmL^{-1}, Figure 4. In case of the 35 μgmL^{-1} antibody concentration, spots signals could not be clearly distinguished against the negative controls (N) at a PSA level of 8 $ngmL^{-1}$. CVs are varied 10 to 20 % that is similar to that of assay in PBS buffer (figure 3). Over 80 % CV value appears in negative control when capturing antibody concentration is $15\mu gmL^{-1}$ that is caused by less resolution between signal and background. At a H117 concentration of 154 $ugmL^{-1}$ the negative control also increased significantly in accordance with observations in Figure 4. Therefore, 77 $ugmL^{-1}$ of H117 was selected as an optimal capture antibody concentration for PSA assay in serum. It was also noted that the signal intensities of PSA-spiked serum samples were significantly lower (about 50%) than those obtained with PSA in PBS buffer. This result agrees with the 30–40 % loss of immunoaccessible PSA seen in earlier studies using purified PSA added to serum. [18,27,28]

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3.4 Improvement of the detection limit for determination of PSA

To evaluate the performance of the optimized PSA assay, titration of PSA spiked serum was conducted at a PSA concentration range from 800fgmL^{-1} to 800 pgmL^{-1}. We chose an antibody concentration of 77 μgmL^{-1} since it showed good assay readout with a minimum of negative (N) control, Figure 4. Figure 5 shows the titrations of PSA in serum. Limit of detection, LOD, was found to be around 800 fgmL^{-1} based on a criterion of two standard deviations above the negative control (N). The optimized microarray PSA assay displays a broad dynamic range of five orders of magnitude from 800fgmL^{-1} to 80 ngmL^{-1}, which now opens for detection of patients with early tumor relapse after radical prostate ectomy.

4. Conclusions

In this work, we demonstrate the importance in optimizing the capture antibody concentration in surface bound immunoassays. An increased capture antibody concentration enables our P-Si microarray to detect sub/low pico-gram per milliliter of PSA in undiluted human serum with a broad dynamic range of five orders of magnitude. The sensitivity of our protein microarray for PSA detection covers relevant diagnostic cut off points (from 0.6 ngmL^{-1} to 4ngmL^{-1}) generally used as indicators of potential malignant prostate disease. The microarray format, an improved LOD (800fgmL^{-1}) and a wide dynamic range (800 fgmL^{-1} to 80 ngmL^{-1}) allows for earlier detection of recurrent disease in prostate cancer patients.

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Highlights

We develop Porous Silicon based microarray for PSA detection in human serum

We optimize density of capturing antibody for improving sandwich immunoassay of PSA

Porous silicon has high capacity of capturing of antibody

The microarray offers 800fgmL^{-1}(0.6amol) of PSA in human serum

It also has broad detection dynamic ranges ((800 fgmL^{-1} to 80 ngmL^{-1})

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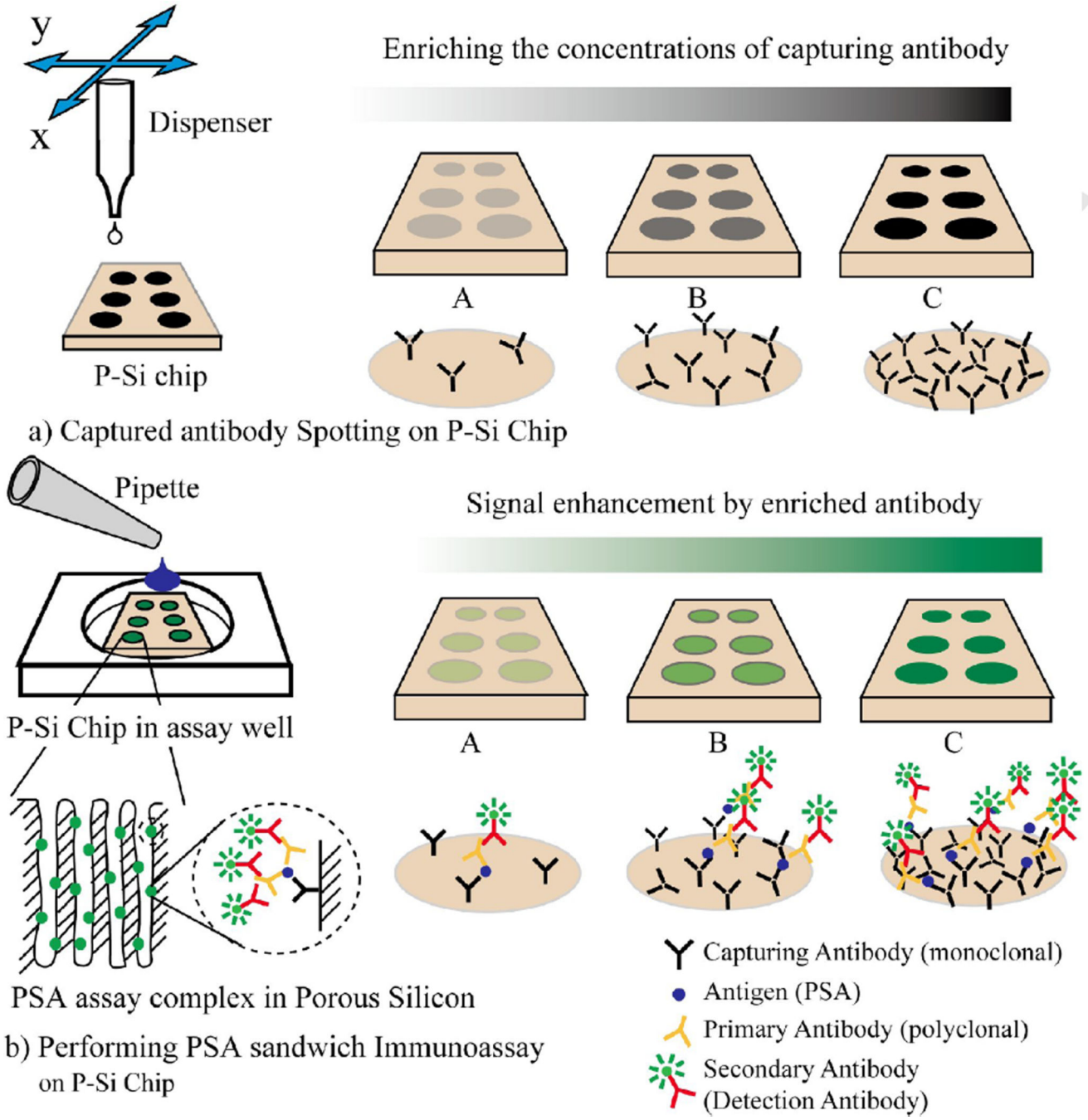

Figure 1.

Capturing antibody was spotted on the solid support (P-Si chip). (a) By changing the capture antibody concentration the surface density of the spotted antibody increased. (b) Enhanced microarray signal readouts were obtained at higher density of capturing antibody microarray.

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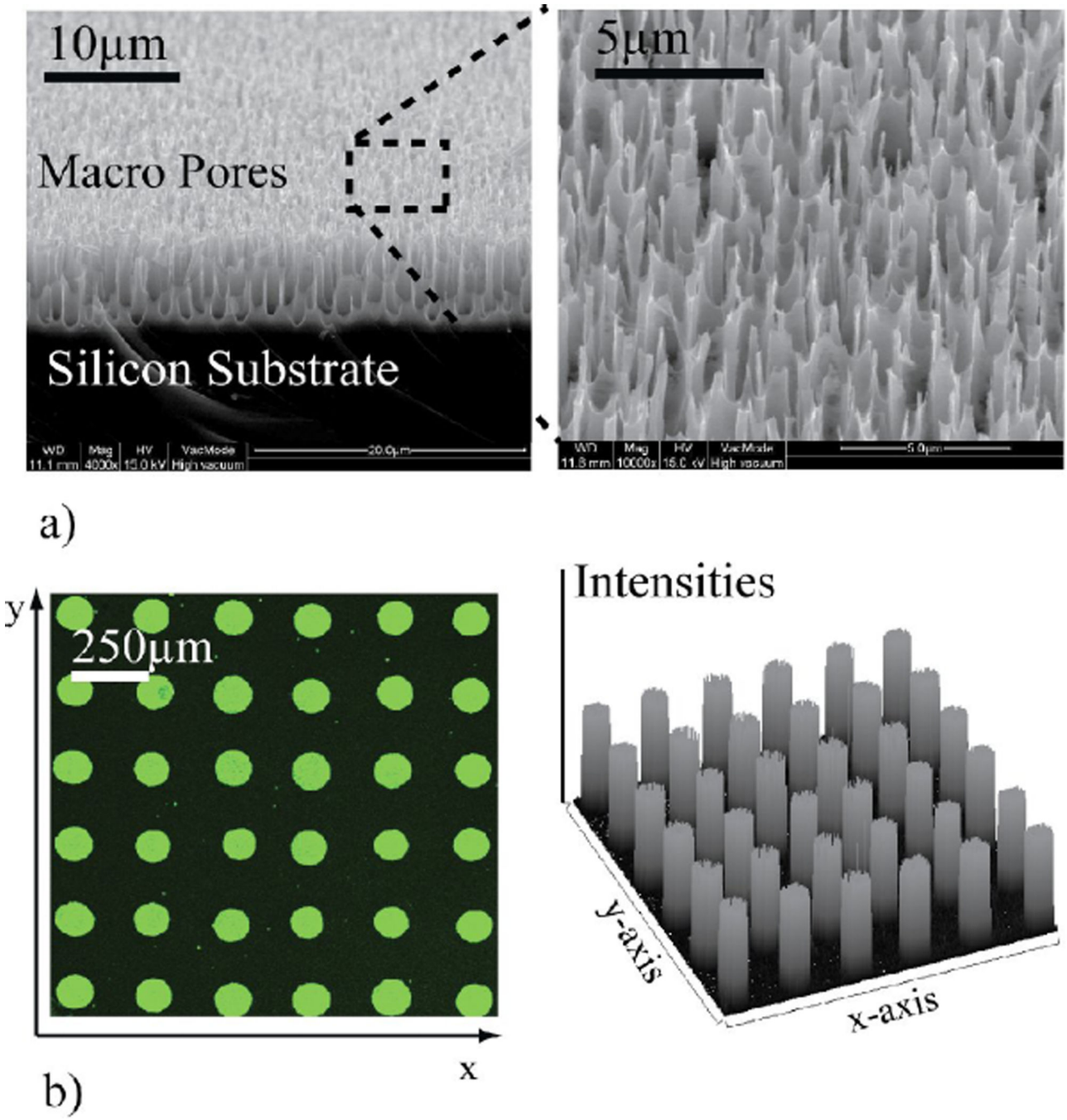

Figure 2.

a) Field emission scanning electron microscope image of the porous silicon surface. Macro pore structure is shown in the zoom insert of the surface. b) Part of spot images and its profile scanning from one of sandwich assay.

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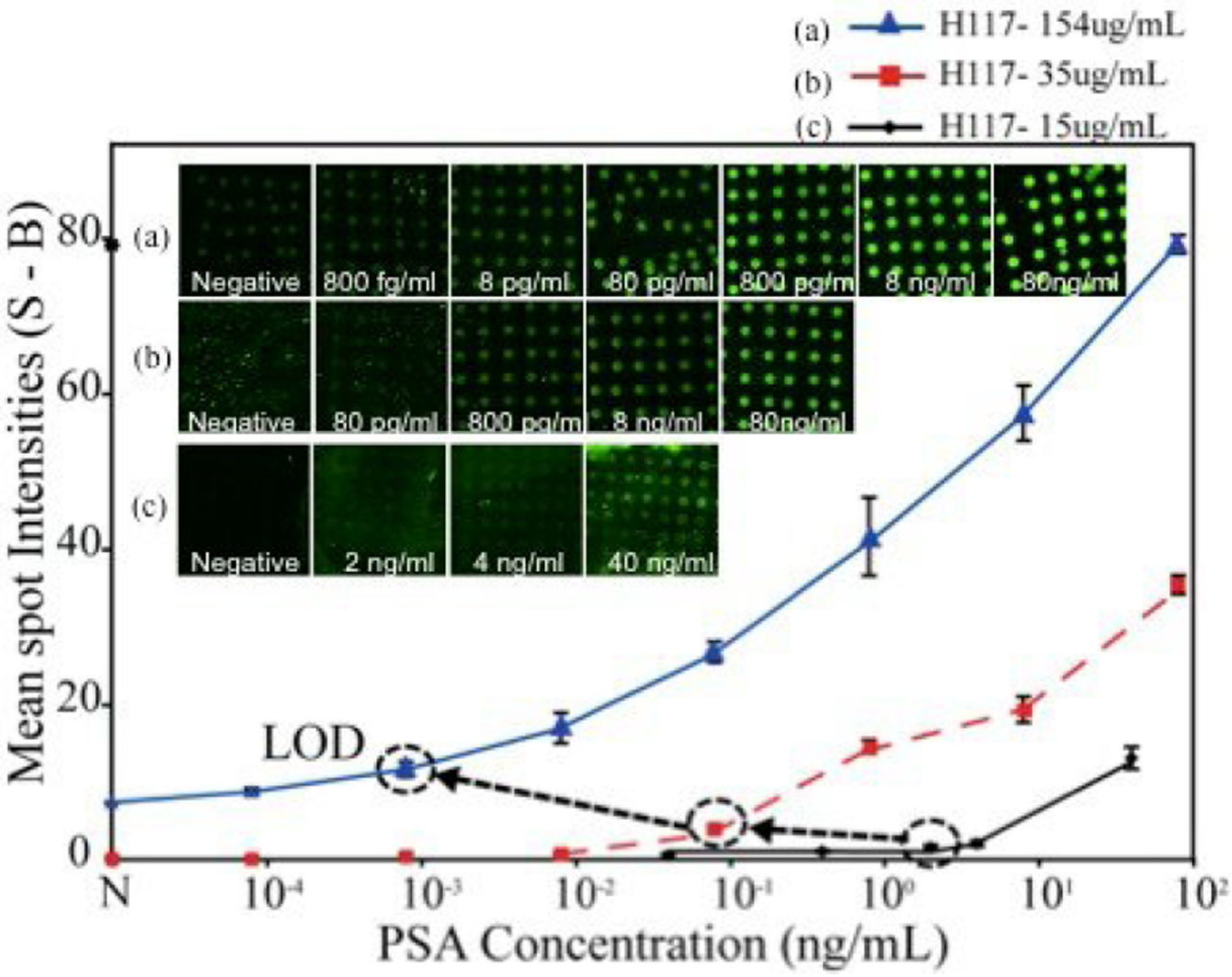

Figure 3.

Titration series of PSA in buffer (PBS) solution at three different concentrations of the capturing antibody H117 (15 μgmL^{-1}, μgmL^{-1} and 154 μgmL^{-1}). The LOD was found to be 2.5 ngmL^{-1} when the capturing antibody was 15 μgmL^{-1} and was reduced to 80 pgmL^{-1} and 800 fgmL^{-1} when the capturing antibody concentrations were 35 μgmL^{-1} and 154 μgmL^{-1}, respectively. Signal of negative control (N) increases significantly at the higher concentration of H117 (154μgmL^{-1}).

(a)

PSA conc.	H117 (35μg/mL) mean spot intensities	CV(%) n=18	array image	H117 (77μg/mL) mean spot intensities	CV(%) n=18	array image	H117 (154μg/mL) mean spot intensities	CV(%) n=18	array image
N	0.45	88		1.97	20		6.73	11.1	
800pg/mL	1.84	40		4.02	27		12.28	10	
8 ng/mL	0.98	8.1		8.17	18		14.61	10	
80 ng/mL	19.77	19.8		19.77	20		43.02	4	

(b)

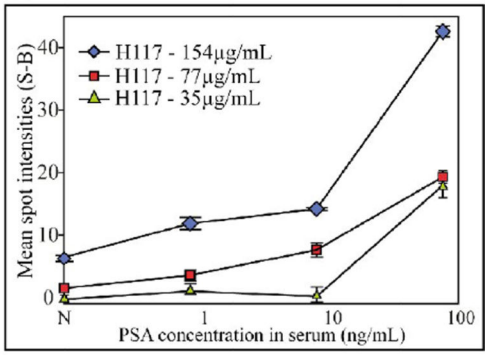

Figure 4.

PSA-spiked human female serum analyzed with the sandwich microarray at three different capturing antibody concentrations (H117: 35 μgmL^{-1}, 77 μgmL^{-1} and 154 μgmL^{-1}). Increased assay sensitivity was observed with the elevated concentration of the capturing antibody (H117). The negative signal also increased at the higher concentrations of H117.

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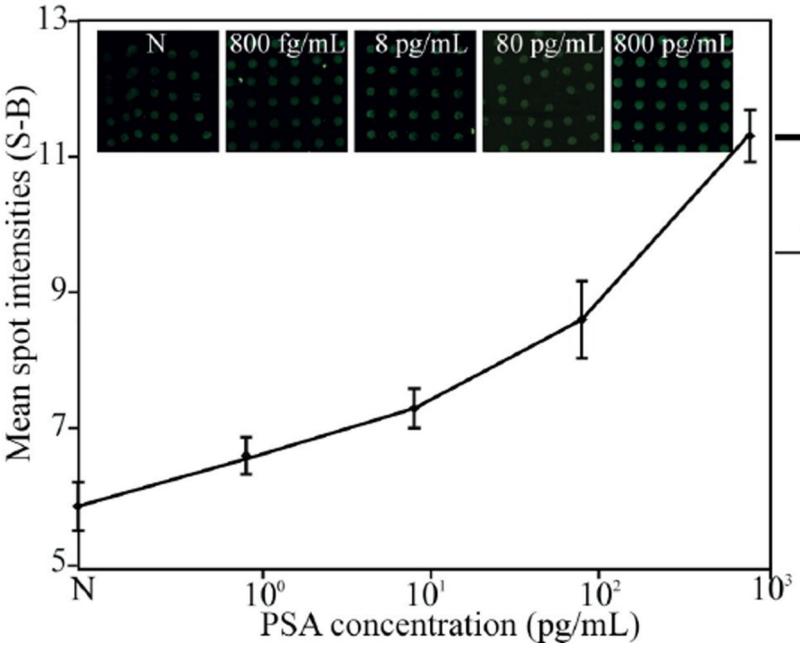

PSA conc.	Mean spot intensities	CV(%) n=18
N	5.87	12.8
800fg/mL	6.61	13.3
8pg/mL	7.31	13.9
80pg/mL	8.62	21.8
800pg/mL	11.33	8.20

Figure 5.

Titration of PSA spiked in female serum as monitored with the sandwich antibody microarray. The assay was performed using a capturing antibody concentration of 77μgmL^{-1}. Mean spot intensities and standard deviations were calculated from the spots by imaging via a 10x lens. The size of each spot was around 120 μm and spot to spot distance about 250 μm. Each mean spot intensity (y-axis) was calculated from 12 microarray spots (S) subtracted by background signal (B). The LOD was defined as the lowest detectable signal compared to the false positive (N) and was found to be 800 fgmL^{-1}.

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Table 1

Mean spot intensities and its coefficient of variance (CV) of antibody microarrays

	PSA concenentration	Mean spot intensities	CV(%) n=18
H117 Conc. (15μgmL)	Negative	0.41	80
	2 $ngmL^{-1}$	1.45	20
	4 $ngmL^{-1}$	1.92	19
	20 $ngmL^{-1}$	13.0	10
H117 Conc. (35μgmL)	Negative	0.34	85
	8 $pgmL^{-1}$	0.41	75
	80 $pgmL^{-1}$	3.85	6
	800 $pgmL^{-1}$	14.56	5
	8 $ngmL^{-1}$	19.23	8
	80 $ngmL^{-1}$	35.28	3
H117 Conc. (154μgmL)	Negative	7.22	10
	80 $fgmL^{-1}$	8.63	5
	800 $fgmL^{-1}$	11.5	8.3
	8 $pgmL^{-1}$	19.42	6.8
	80 $pgmL^{-1}$	26.65	5.1
	800 $pgmL^{-1}$	41.48	3.0
	8 $ngmL^{-1}$	57.39	6.1
	80 $ngmL^{-1}$	79.10	2