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Journal Name

ARTICLE

Ultralow background substrate for protein microarray technology

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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We herein report an ultralow background substrate for protein microarray. Conventional protein microarray substrates often suffer from non-specific protein adsorption and inhomogeneous spot morphology. Consequently, surface treatment and proper printing solution are required to improve the microarray performance. In the current work, we improved the situation by developing a new microarray substrate based on fluorinated ethylene propylene (FEP) membrane. Polydopamine microspot array was fabricated on the FEP membrane, with proteins conjugated to the FEP surface through polydopamine. Uniform microspots were obtained on FEP without the application of special printing solution. The modified FEP membrane demonstrated ultralow background signal and was applied in protein and peptide microarray analysis.

Introduction

Protein and peptide microarrays are well-developed platforms for biochemical research, drug development, clinical analysis, and etc.[1,2] A typical protein or peptide microarray consists of a substrate, functionalized reaction spots, immobilized proteins or peptides, target protein and labelled reagents for detection.[3,4] In the microarray platform, the substrate is critical to the analysis result in terms of signal consistency and detection sensitivity. An ideal substrate would present zero background signal and uniform signal on the reaction spots with the signal intensity proportional to the target concentration. Nitrocellulose, poly(vinylidene fluoride) (PVDF), and glass are are commonly used substrates in protein microarray.[4,5] Several issues associated with these substrates limit the performance of the microarray. First, the reaction spots on the substrates usually require activation and functionalization with groups such as esters, aldehydes, epoxy, maleimides, hydrazines or with polymers coating such as poly-L-lysine for protein immobilization.[4,6] Most of the methods require special chemical reagents under strictly controlled conditions. Second, these substrates generally suffer from non-specific protein adsorption, which leads to increased background signal and decreased sensitivity and stability of the protein microarray.[7] Therefore, various surface blocking methods have been developed to deactivate the background area, such as grafting polyethylene glycol (PEG)[8] and depositing bovine serum albumin (BSA) onto the substrate,[9] etc.[10,11,12] These methods are usually time-consuming, with still detectable non-specific protein adsorption in most cases.[12] Third, coffee-ring morphology of the spots frequently occurs during microarray preparation, which causes inaccuracy of the quantitative measurement in the microarray-based analysis.[13,14] To eliminate this phenomenon, special printing solution or other substrate treatment methods is required.

To address these issues, and for the purpose of producing a substrate with undetectable, i.e., "ultralow", background and stable target protein signal, we turned our attention to Teflon. Teflon is the brand for a series of synthetic fluoropolymers.[15] Fluoropolymers are known to be both hydrophobic and oleophobic, with extremely low affinity for most materials. Interestingly, the affinity of polydopamine to the Teflon series materials was recently reported and characterized.[16,17] Successful conjugation of amine- and thiol-containing biomolecules onto polydopamine were reported in previous studies.[18,19,20] Among the Teflon series materials, fluorinated ethylene propylene (FEP) is known to be melt-processible and is transparent. Therefore, we decided to evaluate polydopamine modified FEP membrane as a new substrate for protein or peptide microarray analysis.

Materials

Reagents: Siloxane Sylgard® 184 silicone elastomer kit (Dow Corning), dopamine (Sigma-Aldrich), fluorinated ethylene propylene (FEP) (20 μm, Shanghai Yuyisong Plastic Products Co., Ltd.), human IgG, fluorescein-conjugated rabbit anti-human IgG, horseradish peroxidase labelled goat anti-human IgG (Beijing Dingguo Changsheng Biotechnology Co., Ltd.), human cytokine (IL-1β, IL-6, IL-10) cytoset (Life

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Technologies), SuperSignal® ELISA Femto Maximum Sensitivity Substrate (Thermo), anti-Pep-2 (Abcam), anti-Pep-3 (Hangzhou HuaAn Biotechnology), Pep-2, Pep-3 and Pep-N were purchased from GL Biochem (Shanghai).

Experimental

Fabrication of polydopamine microspots

A two-layered microfluidic chip was fabricated to produce polydopamine microspots array onto the FEP membrane. The upper layer of the chip was a channel layer, and the bottom was a membrane with micropore array. The chip was made of poly(dimethylsiloxane) (PDMS),[21] and was fabricated by photolithography and soft lithography. The bonding of the two layers was achieved through thermo annealing (SI).[22]

To produce the polydopamine microspots on FEP, the microfluidic chip was first bounded to the FEP membrane tightly and degassed in a vacuum desiccator for 30 min (Figure 1a). Due to the gas and water permeable nature of PDMS,[23] the degassing step sufficiently eliminated the unwanted gas bubbles in the following steps.[24] Then fresh dopamine solution (pH=8.5) was continuously injected into the chip for 3 hours. During the period, the solution turned from colourless to dark brown, indicating the formation of polydopamine. After the removal of the microfluidic chip, a microarray of polydopamine spots was formed on the FEP membrane with the pattern defined by the micropore array. In the following text we use "FEP substrate" to refer to the polydopamine modified FEP membrane.

Aqueous solutions can be dispensed onto the microspots through two methods. The first method is by rolling a droplet of the solution on the whole FEP substrate surface. The solution would be pinned by the hydrophilic polydopamine microspots (Figure 1b and Figure 1c). The second method is by using channel dispensing. A PDMS slab engraved with microchannels was fabricated by soft lithography. The channels were aligned with the microspots of the FEP substrate, and solutions were introduced into the channels and in contact with the substrate surface. After the removal of the PDMS slab, solution droplets would be formed on the microspots.

After the droplet formation, the FEP substrate can be immersed into the fluorinated oil (FC3283) to prevent droplet evaporation (Figure 1d).

Protein microarray fabrication

1 mg/ml green fluorescence protein (GFP) and 1 mg/ml red fluorescing protein mCherry solutions were used to test the conjugation efficiency of proteins on the polydopamine spots. The protein solutions were introduced onto the microspots using PDMS microchannels as mentioned in the previous section. Following the formation of protein solution droplet, the whole FEP substrate was stored at 4 ℃ overnight. The substrate was then washed three times by 0.02% Tween buffer and the result was checked by confocal microscopy (Nikon C1si).

To study the non-specific protein adsorption on different surfaces, 1 mg/ml mCherry solution was deposited on a clean pristine FEP membrane, a BSA blocked nitrocellulose membrane, and the FEP substrate. To prepare the surfaces, the 20 μm-thick FEP membrane was cleaned by surfactant, ethanol and DI water with ultra-sonication. The 50 μm-thick nitrocellulose membrane was fixed onto a holder, which was immersed into the 5 mg/ml BSA solution with continuous shaking overnight. The FEP substrate were blocked by the 5 mg/ml BSA solution droplets for 1 hour, and the whole substrate was immersed in FC 3283 oil during the blocking process. To study the non-specific protein adsorption on the three surfaces, mCherry solution was incubated with the three surfaces at 37 □ for 1.5 hours with continuous shaking. Afterwards, the surfaces were washed by 0.02% Tween buffer, and the result was checked by confocal microscopy.

We fabricated a human IgG-based protein microarray on the FEP substrate, and used the microarray to detect the FITC-labelled rabbit anti-human secondary antibody (Figure 4a).

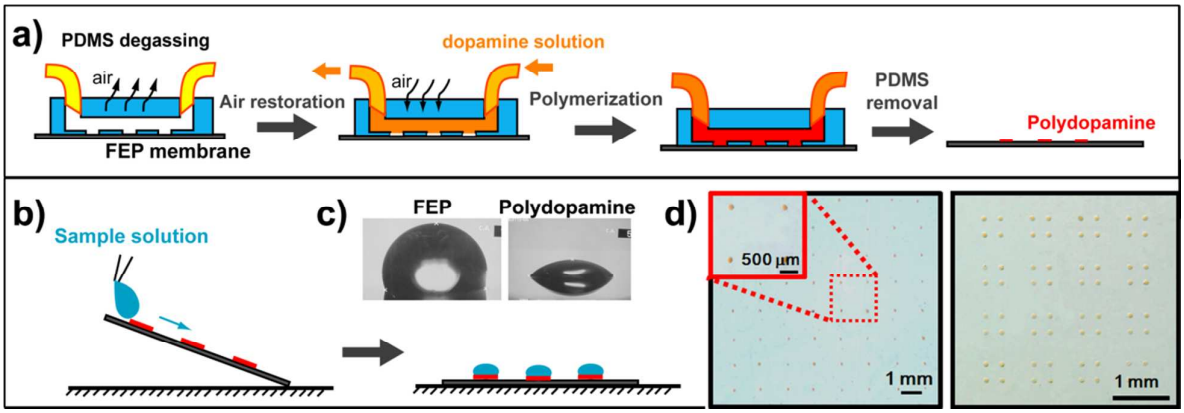

Figure 1 (a) Scheme showing the fabrication method of polydopamine microspots array on the FEP substrate. (b) Scheme illustrating the solution dispensing on the FEP substrate. (c) Images of water droplets sitting on the bare FEP substrate (left) and the polydopamine coated FEP substrate (right). (d) Bright field image showing the red dye solution dispensed on two different types of polydopamine microarray, 8×8, 200 μm in diameter (left) and (4×4)×4, 100 μm in diameter (right).

Briefly, 60 μg/ml human IgG solution was dispensed onto the microspots, and the FEP substrate with IgG solution droplets was incubated at 4 □ overnight. Then 5 mg/ml BSA solution was used to block the polydopamine microspots for 1 hour. The whole substrate was then exposed to the secondary antibody solution, and incubated at room temperature with continues shaking for 3 hours. Different concentrations of the secondary antibody solution were applied, and the fluorescence signals of both the microspots and the background were recorded. Nitrocellulose membrane was used as the control substrate to study the non-specific protein adsorption, and the surface blocking procedure for nitrocellulose membrane was the same as the one for FEP substrate. Following the incubation, the substrates were washed by 0.02% Tween buffer, and the result was observed and analysed by confocal microscopy.

To enhance the detection sensitivity, we used the enzyme-linked signal amplification in the final step. After sampling and surface blocking, the substrate with IgG microarray was incubated with different concentrations of horseradish peroxidase (HRP)-labelled anti-IgG antibody. The incubation was performed for 30 min at either room temperature or 37 ℃ to compare the protein conjugation efficiency. After incubation, the substrate was washed thoroughly with 0.02% Tween buffer. Mixed solution of fresh H_2O_2 and luminol was applied onto a glass slide and the FEP substrate was put onto the glass so that the solution was in contact with the protein microarray. HRP would catalyze the interaction between H_2O_2 and luminol, and induce chemiluminescence. The chemiluminescence signal was detected by GE Imagequant LAS 4000 with 2 min exposure. Each experiment was performed on at least three FEP substrates, and each substrate was fabricated with a $(4\times4)\times4$ array.

Sandwich ELISA for cytokine detection

Sandwich ELISA was used to detect the concentration of cytokines in sample solutions (Figure 6a). Briefly, capture antibody for cytokine was first immobilized on the FEP substrate. The sampling concentration was 200 μg/ml. After the surface blocking by BSA, sample solution was incubated with the substrate for 2 hours to allow the target molecules to be captured. The biotin-labelled detection antibody solution (1 μg/ml) was then added and incubated with the substrate for 1 hour, followed by the incubation with the HRP-labelled streptavidin (1:2000 diluted with buffer) for 30 min. The whole substrate was washed with 0.02% Tween buffer between steps. The signal was detected with GE Imagequant LAS 4000 with 2 min exposure.

Peptide microarray for antibody detection

Different concentrations of the antibodies were incubated with the peptide microarray (Figure 7a). The substrate was then washed thoroughly with 0.02% Tween buffer, followed by the incubation with the HRP-labelled secondary antibody solution. All the incubation steps were performed at 37 ℃ for 30 min. After applying the fresh mixture of H_2O_2 and luminol, we measured the target chemiluminescence signal.

As for the multiplex antibody detection platform, Pep-2, Pep-3 and a non-specific peptide Pep-N were immobilized onto different horizontal rows of the microarray, and PBS buffer with 0.1% glycol was used as the negative control. Anti-Pep-2 (0.5 μg/ml), anti-Pep-3 (1 μg/ml) and the mixture of the two antibodies were added through parallel PDMS microchannels and incubated with the substrate at 37 ℃ for 30 min. The whole substrate was then incubated with the HRP-labelled secondary antibody solution at 37 ℃ for 30 min, followed by the measurement of the chemiluminescence signal.

Results

Patterning FEP with polydopamine

We tried both microfluidic devices and micro-contact printing[25] for patterning the FEP membranes with polydopamine. For the microspots with diameters 500 μm or 200 μm, a uniform polydopamine microarray by micro-contact printing could be obtained. However, micro-contact printing failed to make microspots with diameter less than 100 μm. Liquid droplets could not be trapped on the projected microspot positions, which may be due to the limited amount of polydopamine transferred from the PDMS stamp. By contrast, the two-layer microfluidic chip was able to reliably fabricate the microspots with all the tested diameters (Figure 1d, Figure S1). The deposition of polydopamine onto the FEP substrate was characterized by AFM and XPS (Figure S4 and Figure S5). The thickness of the polydopamine was 10.5 ± 1.5 nm.

Solution dispensing on the patterned FEP

Solution could be dispensed onto the microspots by directly flowing over the substrate surface (Figure 1b). Due to the different hydrophilicity between polydopamine and FEP, solution would be pinned only on the polydopamine microspots, while no solution would stick to the hydrophobic background area (Figure 1c, d, Figure 2a). Different sample solutions could be introduced onto the specified microspots in this way to form a multi-functionalized array (Figure 2a). As shown from the result, solutions were evenly distributed on each polydopamine microspots which facilitated consistency of parallel experiments.

Proteins would form covalent bonding through the amine and thiol groups of the protein with the quinone or catechol groups on polydopamine, and therefore be immobilized on the FEP membrane.[20] XPS result demonstrated the successful conjugation of human IgG onto the FEP substrate (Figure S5). Green fluorescence protein (GFP) and red fluorescing protein mCherry were successfully conjugated to the polydopamine spots without extra steps of functional modification or agent linkage (Figure 2b). In addition, the fluorescence intensity on the microspot was uniform with no “coffee ring” or “donut” pattern,[26] indicating the even distribution of the proteins on polydopamine microspots. The fluorescence intensity from

different microspots containing the same protein was also consistent. The uniform distribution of the proteins on polydopamine is likely a result of the hybrid hydrophobic/hydrophilic nature of the substrate.[27] The spot uniformity did not require special printing solution and facilitated quantitative measurement using the protein microarray.

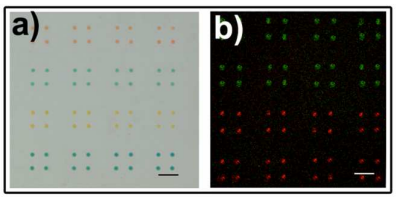

Figure 2 (a) Bright field image showing the dispensing of different dye solutions onto the polydopamine microarray. (b) Confocal image showing the conjugation of different fluorescence proteins onto the polydopamine microarray, GFP (upper two rows), mCherry (bottom two rows). Scale bar: 500 μm.

Fluorescence based protein microarray analysis

We incubated the substrates with 1 mg/ml mCherry solution to test the non-specific protein adsorption (Figure 3). Non-specific protein adsorption on both the pristine FEP membrane and the BSA-blocked polydopamine microspots was minimal. On the contrary, nitrocellulose suffered from severe non-specific protein adsorption even after the optimization of the surface blocking procedure.

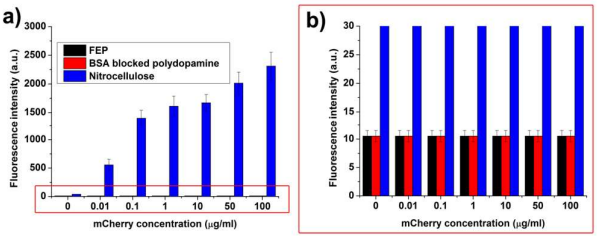

Figure 3 Measurement of the non-specific protein adsorption on different substrates with the red fluorescence protein mCherry. (a) Fluorescence signal intensity change when the substrates were incubated with different concentrations of mCherry. (b) The blow-up of the red rectangular in (a), showing the signal from the FEP membrane and the BSA blocked polydopamine microspots.

In the IgG microarray experiment, the target FITC signal from both the polydopamine microspots and nitrocellulose increased proportionally with the anti-IgG antibody concentration. The nitrocellulose membrane suffered from much more severe non-specific protein adsorption than the FEP substrate in every anti-IgG antibody concentration (Figure 4c). The signal-to-background ratio demonstrated the better performance of FEP substrate (Figure 4b). However, the target fluorescence intensity from nitrocellulose was actually much stronger than the intensity from polydopamine (Figure S2). The difference in the fluorescence signal originates from the structure of the materials: nitrocellulose is a three-dimensional substrate with the thickness at micrometer scale, while the thickness of polydopamine is within nanometers.[28] Consequently, the polydopamine microspots were easily saturated with the capture protein and presented lower signal intensity.

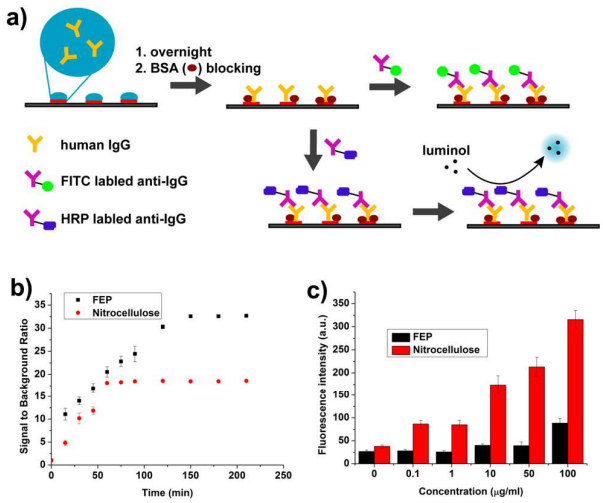

Figure 4 (a) Scheme illustrating the principle of the establishing of IgG microarray and the fluorescence based and enzyme linked immune chemiluminescence assay for the detection of anti-IgG. (b) The signal-to-background ratio change through time from different substrates. The anti-IgG concentration was 50 μg/ml. (c) Signals from the background protein adsorption of different chips in the FITC-labeled anti-IgG detection experiment.

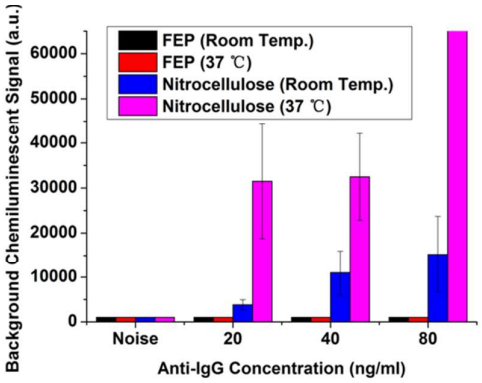

Figure 5 Signals from non-specific protein adsorption of different substrates under different incubation conditions.

Enzyme linked immune chemiluminescence assay based on the FEP substrate

To evaluate the assay sensitivity of the FEP substrate based protein microarray, we used the human IgG microarray to detect the HRP-labelled goat anti-human IgG antibody (Figure 4a).

The assay used HRP-induced chemiluminescence to amplify the target signal and thus enhances the detection sensitivity. The nitrocellulose membrane showed much more severe non-specific protein adsorption after incubation with the secondary antibody at 37 ℃. Although the non-specific protein adsorption on the nitrocellulose membrane was significantly reduced when the incubation was performed at room temperature, the background signal was still significantly higher than the undetectable background signal from the FEP substrate (Figure 5).

Multiplex immune assay platform for cytokine detection

We first used sandwich ELISA to obtain the standard curves of three kinds of cytokines, IL-1β, IL-6 and IL-10 using the FEP substrate based simplex protein microarray (Figure 6b, c, d). The dynamic range of the simplex protein microarray for the three cytokines was from a few pg/ml to ng/ml, and the detection limit was 8.91 pg/ml for IL-1β, 1.33 pg/ml for IL-6, 6.12 pg/ml for IL-10, respectively. For the simplex platform, 16 spot replicates were used in each microchip, and three microchips were used to determine the mean values and standard deviations for each target protein. The Z-factor for each data point was listed in Table S1, indicating the reliability of the assay.

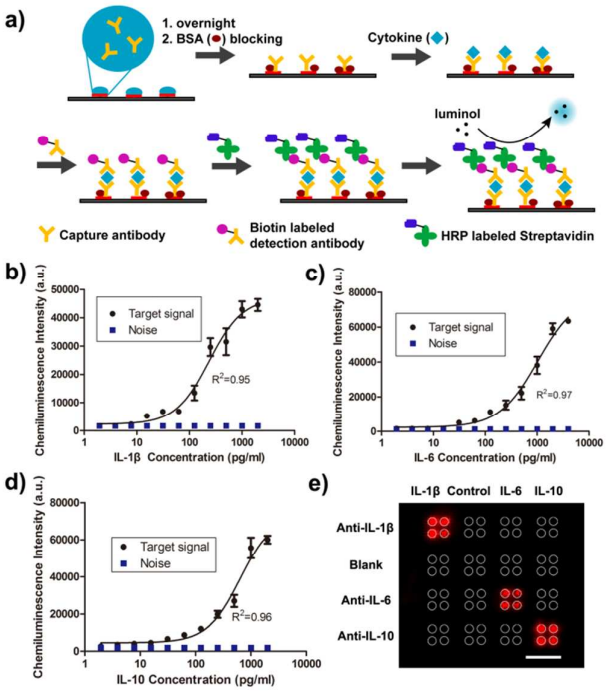

Figure 6 (a) Scheme showing the fabrication and experimental process for cytokine detection. (b), (c), (d) Standard curves for IL-1β, IL-6, IL-10 detection using the FEP substrate. "Noise" is averaged from both the microarray spots and the FEP substrate. The fitting was by using the four-parameter logistic model in Graphpad. (e) Image of the multiplex cytokine detection platform. The concentration for the cytokines here is 31.2 pg/ml. Scale bar: 500 μm.

We then performed the multiplex assay for cytokine detection. As shown in Figure 6e, the capture antibodies for IL-1β, IL-6 and IL-10 were sequentially immobilized onto different rows of the microspots, and the blank microspots were treated only with buffer. The sample solutions spiked with the cytokines were added to each column of the microspots in the sequence of IL-1β, PBS buffer, IL-6 and IL-10. The three biotin-labelled detection antibodies solutions were mixed and incubated with the whole FEP substrate, followed by the deposition of HRP-labelled streptavidin solution. No cross interaction among the three cytokines was observed, and the background signal was undetectable. The blank microspots also showed undetectable signal, indicating the non-specific protein adsorption was minimal (Figure 6e).

We also performed a quantitative cytokine detection experiment based on the multiplex cytokine assay platform. We prepared a cytokine antibody microarray with increasing concentration, and compared the detected target signal from the multiplex platform with the simplex platform. For the multiplex platform, 4 spot replicates were used in each microchip, and three microchips were used to determine the mean values and standard deviations. The results showed no obvious difference between the multiplex and the simplex microarrays (Figure S3).

Peptide microarray analysis

The FEP substrate was further applied to the peptide microarray-based analysis. First we used a FITC-labelled peptide with the sequence KKKKRGD ("Pep-1", Figure 7b) to test the peptide conjugation to the polydopamine microspots. Increasing concentrations of the peptide solution were dispensed onto the polydopamine microspots and the FEP substrate with peptide droplets was incubated overnight at 4 □. From the confocal microscope image, the conjugation of the peptide onto the microspots was successful, with saturated peptide conjugation when the concentration of the peptide was 200 μg/ml (Figure 7b).

To perform the peptide microarray analysis, peptides Pep-2, Pep-3 and a non-specific peptide Pep-N were immobilized onto the polydopamine microspots. Antibodies anti-Pep-2 and anti-Pep-3 were the target proteins. The target signal increased exponentially as the concentration increased. The detection limits for anti-Pep-2 anti-Pep-3 were found to be 30.8 ng/ml and 38.1 ng/ml, respectively (Figure 7c). The Z-factor for each data point was listed in Table S2, indicating the reliability of the assay. The background signal for the peptide experiments was undetectable, and the target signal from the microspots was consistent in the parallel experiments (Figure 7c).

Finally multiplex peptide microarray based analysis was performed. Assays for the detection for the antibodies at different concentrations with the orthogonal platform were

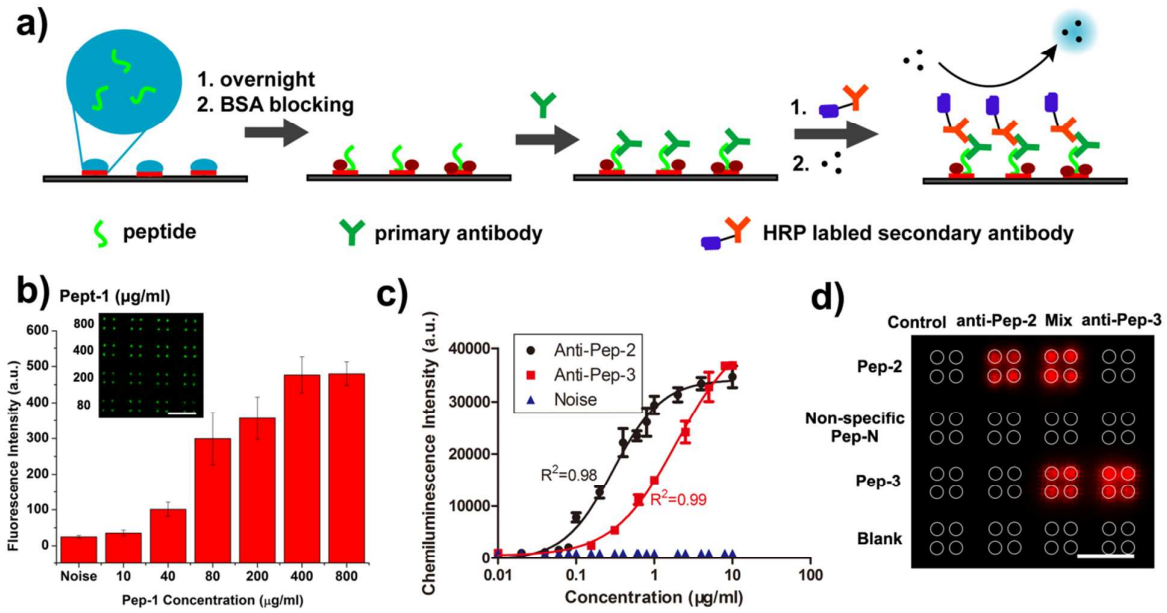

Figure 7 (a) Scheme showing the fabrication of the peptide microarray and the peptide microarray for antibody detection. (b) Optimization of the sampling concentration of Pep-1 onto the polydopamine microspots. Insertion is the confocal image of the peptide microarray with different Pep-1 concentrations. Scale bar: 500 μm. (c) Standard curves for anti-Pep-2 and anti-Pep-3 antibody detection based on the peptide microarray. The fitting was by the four-parameter logistic model in Graphpad. (d) Fluorescence image of the multiplex antibody detection platform based on the peptide microarray. Scale bar: 500 μm.

illustrated in Figure 7d. No obvious difference was found between the detection results of the simplex and multiplex platforms. The background signals from both the FEP substrate and the polydopamine microspots were undetectable, with no cross-interaction between non-target antibodies and peptides. Additionally, the two antibodies showed undetectable non-specific adsorption on both Pep-N and the blank microspots (Figure 7d).

Conclusion

The current work reports the first microfluidic fabrication of the polydopamine microspot array with the spot diameter smaller than 500 μm. By coupling with a microfluidic system, the size, shape and geometry of the microspot array can be easily controlled. The microarray substrate in this work is an FEP membrane modified with polydopamine microspot array. The hydrophilicity difference between polydopamine and FEP allows fast solution dispensing and on-chip droplet-based reaction.

The protein microarray based analysis for IgG and cytokines demonstrated ultralow background signal of the FEP substrate with no need of FEP surface blocking. The detection limit of the FEP substrate based protein microarray analysis is much lower than the conventional nitrocellulose membrane and comparable with other state-of-art techniques, which is in the range of 1-10 pg/ml.[29,30,31,32]

In conclusion, we have successfully fabricated the protein and peptide microarrays on the FEP substrate for protein analysis. Simple dispensing method was used to deposit capture proteins or peptides onto the polydopamine microspots. No complicated methods were needed to activate the microspots or to deactivate the background area of the FEP substrate. Capture proteins or peptides formed covalent bonding with polydopamine and were conjugated onto the microspots. Uniform microspots were resulted without the need of special printing solutions. The experiments of both protein and peptide microarrays have demonstrated ultralow non-specific protein adsorption on the background area of the FEP substrate. The signals were consistent from different batches of FEP substrates, which is critical for quantitative analysis. In future, we will explore methods to optimize the conjugation density and orientation of the capture protein or peptide on the FEP substrate. We believe the FEP substrate is a promising tool for high sensitivity microarray-based analysis.

Acknowledgements

We thank the financial support from CUHK (TBF13SC1019, 4053061) and the Hong Kong Ph.D. Fellowship (H. F.) from RGC.
We thank Prof. Jiang Xia, Dr. Jianpeng Wang, Feng Huang, and Sze Nga Lai for the gift of GFP and mCherry and support to this work.

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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