Supplementary Material (ESI) for Medicinal Chemical Communications

Electronic Supporting Information

Photografted Poly(methyl methacrylate)-based High Performance Protein Microarrays for Hepatitis B Virus Biomarker Detection in Human Serum

Yingshuai Liu, Weihua Hu, Zhisong Lu and Chang Ming Li*

School of Chemical & Biomedical Engineering, Center for Advanced Bionanosystems, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457

* E-mail: ecmli@ntu.edu.sg

Materials and methods

Experimental materials

PMMA sheets (1mm in thickness) were purchased from Ying Kwang Acrylic Trading (Singapore). Goat IgG, Cy3-conjugated anti-goat IgG, hexamethylenediamine, glycidyl methacrylate (GMA), glycerol, triton® X-100 and 0.01 M phosphate buffered saline (PBS, pH7.4) were ordered from Sigma-Aldrich. Alexa Fluor® 546 goat anti-mouse IgG was ordered from Invitrogen. Hepatitis B surface antigen (HBsAg), anti-HBsAg and biotin-labeled anti-HBsAg were purchased from US Biological. Cy3-conjugated streptavidin was obtained from GE healthcare. BlockerTM Casein in TBS was purchased from Pierce. The deionized water used in all experiments was produced by a water purification system, Q-Grad®1, Millipore Corporation.

Preparation of PGMA-PMMA

The PGMA-PMMA slides were prepared step-by-step under mild chemical conditions. The PMMA sheets were tailored to 75 mm×25 mm slides. For thoroughly cleaning, the slides were immersed in 2-propanol for 30min and then cleaned for 5min in an ultrasonic water bath, followed by sequentially rinsing with ethanol and deionized water. The precleaned PMMA slides were

aminated as previously described in [1]. Briefly, the precleaned PMMA slides were incubated in 10% hexamethylenediamine for 2 hours at room temperature, followed by rinsing with deionized water and drying under nitrogen stream. The aminated PMMA slides were soaked in 5% (V/V) GMA monomer solution (prepared in 50% ethanol solution) for 2 hours at room temperature to produce an acrylate-activated PMMA surface. The acrylate groups enable GMA photopolymers to be grafted onto the PMMA surface. For photografting conduction, the acrylate-activated PMMA slides were immersed in GMA monomer solution (5% V/V in 50% ethanol), which contained 5mM sodium persulfate as the photoinitiator and was completely degassed using nitrogen for 30min. Then, the mixture was exposed to UV for 15min with UV flood equipment (shuttered UV system, 400W). After the polymerization, the PMMA slides were washed with ethanol and deionized water to remove unpolymerized monomer and uncoupled oligomer or polymer.

Preparation of GA-PMMA

In order to obtain GA-PMMA, the aminated PMMA slides prepared in 2.2.1 were immersed into 5% glutaraldehyde solution and incubated for 2 hours at room temperature. Then, the GA-PMMA slide was thoroughly rinsed with deionized water and dried under nitrogen flowing.

Preparation of GPTMS-slide

Microscope glass slides were firstly cleaned and hydroxylated in piranha solution (prepared by mixing in a 7/3 volume to volume ratio of concentrated H2SO4 and 30% H2O2) for 2 h in oven at 80℃, followed by three-time washing with deionized water and ethanol respectively. The precleaned slides were immersed in 5% (v/v) (3-Glycidyloxypropyl)trimethoxysilane (GPTMS) in ethanol for 1 h at room temperature to introduce epoxy groups onto the slides surface, then rinsed thoroughly with ethanol and deionized water. The prepared slides were dried

under nitrogen flow and baked in vacuum oven at 110°C for 2h.

AFM imaging

Surface topography of pristine PMMA, aminated PMMA and PGMA-PMMA surfaces were examined with AFM (SPM 3100, Veeco instuments Inc., USA) in tapping mode. The scan area was 20μm×20μm. All images were acquired in open air.

ATR-FTIR analysis

The IR spectra were recorded by using a Nicolet 5700 instrument (Thermo Electron Corporation) equipped with an attenuated total reflection (ATR) accessory. The pristine and modified PMMA slides were placed onto the ATR crystal and pressed tightly to obtain good spectra. The analysises were performed by 16 scans with a resolution of 1 (data spacing is 0.482 cm^{-1}).

Protein binding assay

The protein binding assay was conducted using fluorescence based method. The Alexa Fluor 546 conjugated goat anti-mouse IgG at 1 mg mL^{-1} was printed onto PGMA-PMMA, GA-PMMA and GPTMS-glass with VersArray chip writer. The printed slides were put in dark box and incubated for overnight at room temperature to immobilize the protein onto different substrates. After the incubation, all slides were thoroughly washed in TBS with 0.05% Tween 20 and deionized water. Then, the dried slides were scanned with microarrays scanner.

Protein microarray fabrication

Protein probes were prepared in the printing buffer and then transferred to 384-well microtiter plate, which was pre-blocked with Blocker^{TM} casein in TBS solution for 1 hour to prevent protein probe adsorption during printing. For optimization of printing buffer, 0.01M PBS containing 2.5%-40% glycerol and 0.001%-0.012% triton X-100 were applied as printing buffers. For other

experiments, the optimal buffer was employed as the printing buffer. 20μL of the protein solution was dispensed to each well. The contact printing process was performed by using VersArray chipwriter^{TM} (BIO RAD) with telechem printhead and stealth microspotting pins. At 60% humidity, ~ 0.3nL of sample per spot was delivered to different substrates for microarray fabrication.

The protein coupling reaction was allowed to proceed in humid chamber for 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 hours respectively for immobilization kinetics investigation, and 8 for other experiments. The arrayed slides were then rinsed 3 times for 2 min each with TBS containing 0.05% Tween 20 to remove unbound proteins. After washing steps, all slides were immersed in Blocker^{TM} casein in TBS solution for 1 hour not only to quench the unreacted functional groups on the substrate surface, but to form a molecular layer of casein that could reduce the nonspecific binding of other proteins in subsequent steps.

Imaging and data analysis

The fluorescence images were obtained with ScanArray GX Microarray Scanner (PerkinElmer, USA). And then the acquired images were quantitatively analyzed with ScanArray® Express analysis software. The fluorescent intensities are local background corrected means, which are used for downstream statistical analysis. The acquired data were inputted to Origin 7.0 for further analysis and plots.

Experimental results

Low intrinsic fluorescence of PMMA

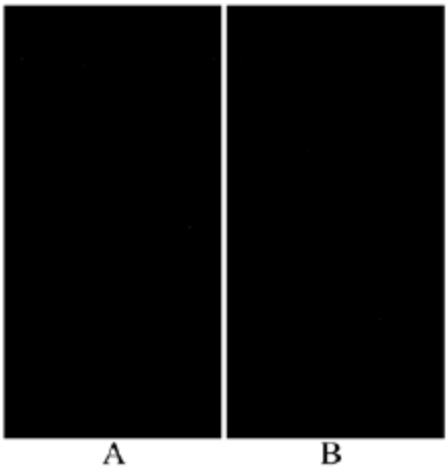

Fig. S1: Intrinsic fluorescence of PMMA under 543 nm and 633 nm, which are two most common excitations in microarray scanner.

Optimization of printing buffer

Printing buffer plays a very important role in fabricating protein microarrays by affecting the quality of microspot, the stability of immobilized protein and the protein binding capability of substrate. To obtain the best performance of protein microarrays on PGMA-PMMA, the printing buffer was optimized by spotting goat IgG in 0.01M PBS, which is suitable to preserve the bioactivity of printed proteins due to its pH is close to physiological value, with different concentrations of glycerol and triton X-100 as additives. The quality and signal intensity of the microspot were examined by fluorescent scanner after applying of Cy3-labeled anti-goat IgG.

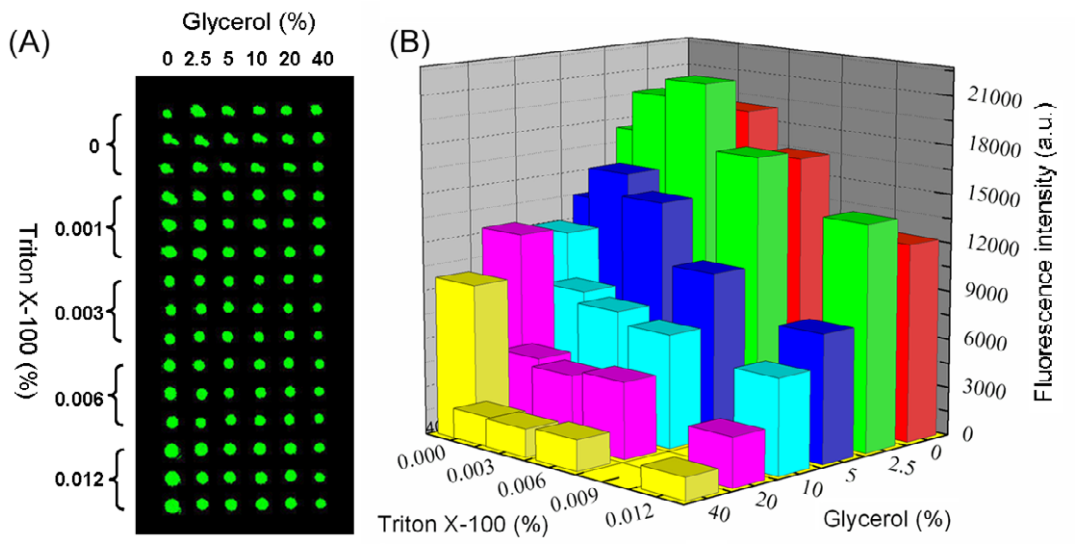

Fig. S2 Affect of glycerol and triton X-100 as printing buffer additives on microspot morphology **(A)** and signal intensity **(B)**.

During the protein microarrays fabrication, subnano- or nanoliter volume of protein solution is delivered onto substrate. To prevent the dehydration of printing solution, glycerol was used as supplements of the protein printing buffer due to its hygroscopic nature result from the three hydrophilic alcoholic hydroxyl groups. Furthermore, glycerol can affect the size and morphology of printed protein microspot. As shown in Fig. S2A, the spot size decreases with the increasing of glycerol in the printing buffer, and the spot morphology is normalized by adding high percent of glycerol [2]. However, high concentration of glycerol significantly reduces the signal intensity (Fig. S2B), because the high viscousity retard the protein diffusion in printing buffer [3]. Therefore, the optimal amount of glycerol is determined to 2.5%, which can efficiently prevent printing buffer dehydration and give the highest signal intensity.

Nonuniform or irregular such as ring-like and smearing spot profile can result in poor reproducibility and reliability of protein microarrays. Recently, ring-like formation in protein microarrays was eliminated by adding competitive surfactant triton X-100 to printing buffer [4]. In

this study, the typical smearing spot occurs on PGMA-PMMA (Fig. S2A , rows 1-6) when there is no or a low level of triton X-100 in printing buffer. It is demonstrated that the smearing profile can be completely removed by applying higher amount of triton X-100 (Fig. S2A, rows 7-15). However, too much triton X-100 results in significantly reduction of signal intensity (Fig. S2B). It is because that triton X-100, as a non-ionic detergent, can block protein adsorption on the substrate and damage the bioactivity of protein [5-7]. Fig. S2B demonstrates that the signal intensity enhances first and then decreases with the increasing of the concentrations of triton X-100. The highest fluorescence intensity is obtained when 0.003% triton X-100 is applied. Combined the effect of glycerol, the optimal printing buffer is 0.01M PBS with 2.5% glycerol and 0.003% triton X-100 as additives. Therefore, the optimal printing buffer was applied in following experiments.

Protein immobilization kinetics

Protein immobilization kinetics on the PGMA-PMMA surface was investigated to determine efficient immobilization time, which is essential parameter in protein microarrays fabrication. On PGMA-PMMA, protein probes were immobilized covalently via the reaction between epoxy groups on the substrate surface and the amino groups on the protein molecules. Therefore, the immobilization progress can be simplified as a chemical reaction:

$$P + A \underset{k_d}{\overset{k_a}{\rightleftharpoons}} PA \qquad (1)$$

Where P is the probe protein in the printing spot solution, A is the available anchor site on the substrate surface, and *PA* is the complex of immobilized probe and anchor sites. k_a is the association rate constant, and k_d is the dissociation rate constant.

According to the proposed reaction in equation (1), the immobilization rate can be expressed as follows:

$$\frac{d[PA]}{dt} = k_a[P][A]_t - k_d[PA] \qquad (2)$$

The concentration of *P* in printing solution is assumed to be constant with time for simplifying the reaction. The fluorescence intensity is proportional to the concentration of *PA* on the substrate surface. Therefore, the fluorescence intensity as a function of immobilization time is according to the following equation [8]:

$$F_t = E^*(1-e^{-k_s t}) + F_0 \qquad (3)$$

With

$$E^* = \frac{k_a C F_{\max}}{(k_a C + k_d)},$$

$$k_s = k_a C + k_d .$$

Where *F* is the fluorescence intensity, $F_{\max}$ is the maximal fluorescence intensity at a certain concentration, F_0 is the fluorescence intensity at $t = 0$, *C* is the concentration of protein in printed solution.

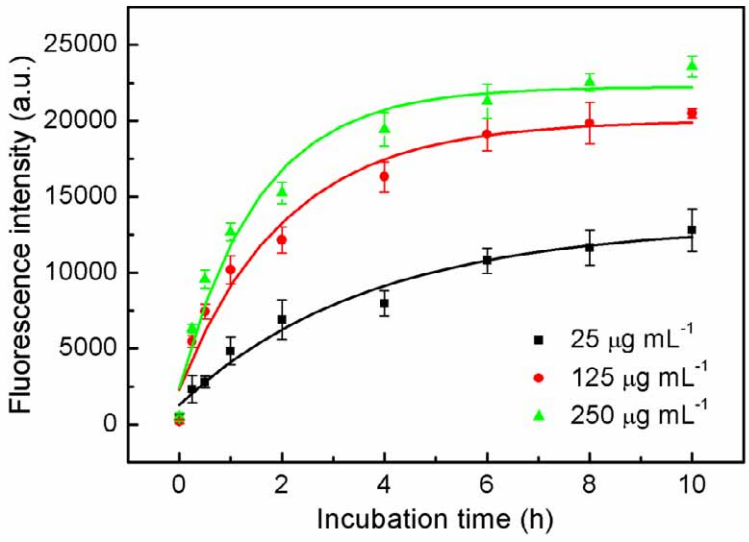

Fig. S3 Protein binding curves (scatter points) and fitting curves (solid lines) with different printing concentrations of Alexa 546-labeled IgG on PGMA-PMMA

Fig. S3 shows the binding curves of Alexa Fluor 546-labeled goat anti-mouse IgG at 25 μg mL^{-1}, 125 μg mL^{-1} and 250 μg mL^{-1}. The fitting curves (solid lines) based on equation (3) are in reasonably agreement with the experimental data (scatter points). It suggests that protein immobilization kinetics on PGMA-PET surface follows the proposed reaction. The parameters E^*, k_s and F_0 were obtained by the fitting, and then k_a and k_s were further determined to be 88.5 $M^{-1}S^{-1}$ and 6.03×10^{-5} S^{-1}, respectively. Therefore, k_a/k_s defined as the affinity coefficient (K) between P and A is equal to 1.47×10^{-6} M^{-1}.

According to both the experimental results and the theoretical simulation, the protein binding is dependent on the concentration of spotted proteins. For 250 μg mL^{-1}, the amount of immobilized protein reaches more than 94% of the theoretical maximum after 6-8 hours incubation. The results can guide protein microarrays construction on PGMA-PMMA surface with efficient incubation time. For further microarrays fabrication, 8 hours was employed for optimal protein immobilization.

Optimization of capture antibody concentration

The amount of immobilized protein can directly affect the sensitivity and detection limitation of protein microarrays. Thus, a maximum immobilization of protein has to be achieved on given substrate to obtain the best performance of protein microarrays. As protein probes (mostly antibodies or antigens) are very expensive and the available concentration is limited. For both performance and economy, the immobilization of protein on PGMA-PMMA was characterized to find efficient protein concentration. To conduct this experiment, the PGMA-PMMA was printed with a series of double-diluted Alexa Fluor 546-conjugated goat anti-mouse IgG in range from 1 mg mL^{-1} to 7.8 μg mL^{-1}. Fluorescence intensity as a function of printed protein concentration is

shown in Fig. S4.

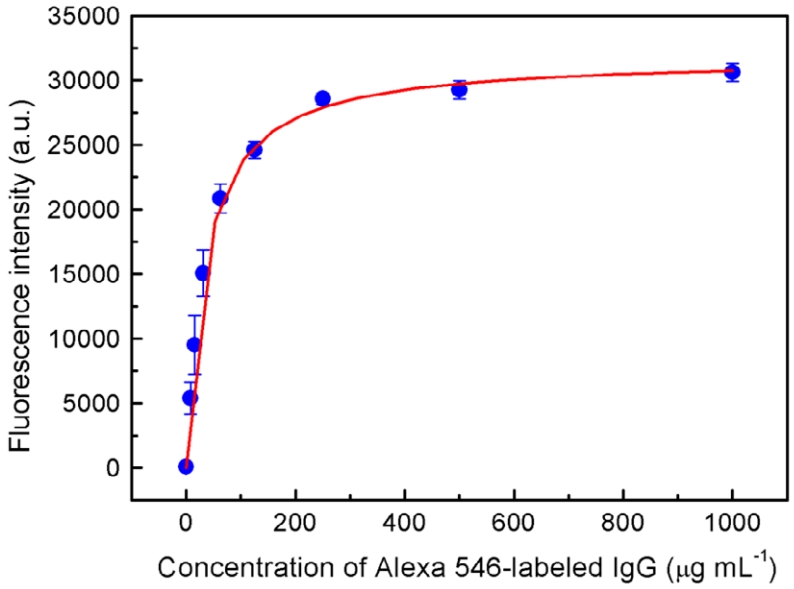

Fig. S4 Fluorescence intensity as a function of concentrations of printed Alexa 546-labeled anti-mouse IgG (0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 μg mL^{-1} in optimal printing buffer) on PGMA-PMMA.

Based on immobilization kinetics described in equation (2), the fluorescence intensity dependent on printed protein concentration could be given by the Langmuir adsorption isotherm at equilibrium state:

$$F_{eq} = \frac{K[P]F_0}{1+K[P]} \qquad (4)$$

Where K is the affinity coefficient, defined as $K = k_a / k_d$, F_{eq} is the equilibrium signal intensity in response to a certain concentration and F_0 is the fluorescence intensity for a complete coverage.

The experimental scatter points were fitted based on equation (4). A typical Langmuir plot is shown in Fig. S4 with an affinity coefficient K (k_a/k_s) of 4.29×10^6 M^{-1} and maximum fluorescence intensity of 32000 a.u.. By printing 250 μg mL^{-1} Alexa Fluor labeled IgG, the fluorescence

intensity reaches 90% of the maximal value, i.e. the protein immobilization on the PGMA-PET substrate is nearly saturated at this concentration. For both efficiency and economy considerations, this concentration was used in the microarrays fabrication. In addition, the value of affinity coefficient K is reasonably consistent with that (1.47×10^{6} M^{-1}) calculated from immobilization kinetics in part 3.5. The results both from kinetics and thermodynamics indicate that protein binding on the PGMA-PMMA surface follows our proposed reaction.

Reference

1. **F. Fixe, M. Dufva, P. Telleman and C. B. V. Christensen, *Nucleic Acids Res.*, 2004, 32.**
2. **E. W. Olle, J. Messamore, M. P. Deogracias, S. D. McClintock, T. D. Anderson and K. J. Johnson, *Exp. Mol. Pathol.*, 2005, 79, 206.**
3. **Y. S. Liu, C. M. Li, L. Yu and P. Chen, *Front. Biosci.*, 2007, 12, 3768.**
4. **Y. Deng, X. Y. Zhu, T. Kienlen and A. Guo, *J. Am. Chem. Soc.*, 2006, 128, 2768.**
5. **K. L. Brogan, J. H. Shin and M. H. Schoenfisch, *Langmuir*, 2004, 20, 9729.**
6. **J. S. Wang, J. X. Shan, Q. Xu, X. Ruan, Y. D. Gong, T. Y. Kuang and N. M. Zhao, *Journal of Photochemistry and Photobiology B-Biology*, 2000, 58, 136.**
7. **S. Zampieri, A. Ghirardello, A. Doria, M. Tonello, R. Bendo, K. Rossini and P. F. Gambari, *J. Immunol. Methods*, 2000, 239, 1.**
8. **W. H. Hu, C. M. Li, X. Q. Cui, H. Dong and Q. Zhou, *Langmuir*, 2007, 23, 2761.**