

On-Chip Synthesis of Protein Microarrays from DNA Microarrays via Coupled *In Vitro* Transcription and Translation for Surface Plasmon Resonance Imaging Biosensor Applications

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S Supporting Information

ABSTRACT: Protein microarrays are fabricated from double-stranded DNA (dsDNA) microarrays by a one-step, multiplexed enzymatic synthesis in an on-chip microfluidic format and then employed for antibody biosensing measurements with surface plasmon resonance imaging (SPRI). A microarray of dsDNA elements (denoted as generator elements) that encode either a His-tagged green fluorescent protein (GFP) or a His-tagged luciferase protein is utilized to create multiple copies of mRNA (mRNA) in a surface RNA polymerase reaction; the mRNA transcripts are then translated into proteins by cell-free protein synthesis in a microfluidic format. The His-tagged proteins diffuse to adjacent Cu(II)-NTA microarray elements (denoted as detector elements) and are specifically adsorbed. The net result is the on-chip, cell-free synthesis of a protein microarray that can be used immediately for SPRI protein biosensing. The dual element format greatly reduces any interference from the nonspecific adsorption of enzyme or proteins. SPRI measurements for the detection of the antibodies anti-GFP and antiluciferase were used to verify the formation of the protein microarray. This convenient on-chip protein microarray fabrication method can be implemented for multiplexed SPRI biosensing measurements in both clinical and research applications.

The simultaneous detection of proteins, nucleic acids, lectins, antibodies and other biomolecules in a microarray format is currently revolutionizing the areas of healthcare, biotechnology and biological research.^{1–8} One particularly powerful method for the simultaneous detection of multiple bioaffinity adsorption processes is the use of protein microarrays in conjunction with the optical technique of surface plasmon resonance imaging (SPRI).^{9–17} However, the fabrication of protein microarrays for SPRI can be quite time-consuming: each protein must be spotted from a separately synthesized and purified solution, and then either linked to the surface by a chemical or enzymatic attachment reaction, or modified with a noncovalent bioaffinity adsorption conjugate such as biotin.¹⁸ Moreover, unlike DNA microarrays, protein microarrays have a limited shelf life and can quickly lose functionality if dehydrated or denatured. Recently, a number of new *in vitro* transcription and translation (IVTT) protein synthesis methods have been introduced which can be used to fabricate protein microarrays directly on biochips from template

double-stranded DNA (dsDNA).^{19–22} The conjugation of SPRI and these single-step, cell-free IVTT methods is potentially a very powerful biosensing platform. In this letter, we describe the initial demonstration of a novel dual-element, on-chip protein synthesis/capture methodology to create a protein microarray from a dsDNA microarray in a microfluidic format that can be used immediately for SPRI biosensing measurements. These initial experiments demonstrate that the direct conversion of DNA microarrays to protein microarrays by combining on-chip, picomole protein microarray synthesis on one array element with protein surface capture chemistry on a second adjacent element greatly enhances the capabilities of SPRI protein microarray biosensing.

Our procedure for the on-chip synthesis of a protein microarray directly from a DNA microarray via surface coupled transcription-translation is depicted schematically in Figure 1.

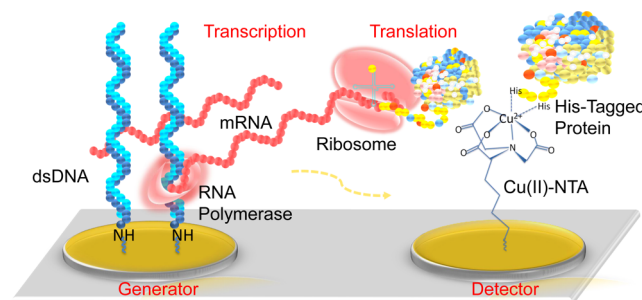


Figure 1. Schematic diagram of the on-chip synthesis of protein microarray from DNA microarray via surface *in vitro* transcription-translation. On the generator elements, the encoding dsDNA was covalently attached to the gold surface and mRNA was transcribed with T7 RNA polymerase. Translated His-tagged protein diffused to the adjacent detector elements and was captured by Cu(II)-NTA surface.

We employ a generator-detector microarray element format in which single stranded mRNA molecules (mRNA) are transcribed from surface-bound dsDNA on one microarray element (the “generator element”), and translated protein molecules are captured by a second adjacent element (the “detector element”) for immediate use in SPRI bioaffinity biosensing. The three coupled processes that comprise our methodology for on-chip protein microarray fabrication are: (i) an on-chip

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RNA polymerase transcription reaction from the surface-bound dsDNA templates to create multiple copies of mRNA, (ii) the immediate translation of the mRNA transcripts into a His-tagged protein in the microfluidic channel using a cell free protein expression mixture, and (iii) the specific adsorption of the expressed His-tagged protein onto an adjacent detector element that has an Cu(II)-NTA modified gold surface. As our first example, we fabricated a 16 element, dsDNA/protein microarray that contains two protein microarray elements: green fluorescent protein (GFP) and luciferase. After fabrication, this microarray was used directly in SPRI measurements to monitor the specific adsorption of anti-GFP and antiluciferase onto the biosensor chip. The use of the generator-detector format greatly reduces interferences from nonspecific enzyme and protein adsorption in the subsequent SPRI measurements.

The first step in our on-chip synthesis of protein microarrays was a surface reaction of RNA polymerase with adsorbed dsDNA to create multiple mRNA transcripts in solution. We have used surface RNA polymerase reactions previously in conjunction with SPRI for both the amplified ultrasensitive detection of ssDNA and the on-chip fabrication of ssRNA aptamer microarrays for protein biosensing.^{23,24} In comparison with our previous application of surface RNA polymerase reactions to create RNA microarrays, the on-chip protein synthesis methods additionally require the ribosomal translation of the transcribed mRNAs to proteins. A 16-element microfluidic chip that contained eight “generator” elements was used to immobilize DNA templates and create the mRNA transcripts. The gold thin film generator elements were first modified with a monolayer of 11-mercapto-undecamine (MUAM), followed by the electrostatic adsorption of a monolayer of the biocompatible polyelectrolyte poly-L-glutamic acid (pGlu). The dsDNA was then covalently attached to the pGlu monolayer via an NHSS-EDC coupling chemistry that also covalently attached the pGlu monolayer to the MUAM as described in a previous paper.^{25,26} The dsDNA was PCR amplified from a protein expression plasmid (for GFP, we employed a plasmid based on the pIVEX2.3d vector containing a gene encoding wild-type GFP (pIVEX-GFP, Roche); for luciferase, the plasmid we used was based on a pET vector containing a gene encoding luciferase). The PCR products consisted of the following components: a T7 promoter site, a ribosomal binding site, a gene encoding GFP or luciferase, a hexahistidine tag and a T7 polymerase terminator sequence. The forward primer used in the PCR experiments included a PEG based spacer to facilitate the surface enzymatic reaction and was 5'-amino-modified for the immobilization chemistry (see the Supporting Information for the primer sequences used in this paper).

In addition to the generator elements, four thin gold film “detector” elements in the microarray were functionalized with a Cu(II)-nitriloacetic acid (NTA) monolayer for the capture of the synthesized His-tagged protein. The NTA monolayers were created by linking amino-NTA (*N*-(5-amino-1-carboxypentyl)-iminodiacetic acid, Dojindo) to a MUAM/pGlu monolayer via NHSS-EDC coupling chemistry.²⁵ The detector elements were then exposed to 1 mM Cu(II) solution to create the Cu(II)-NTA modified surfaces. Cu(II) was used in these experiments because surfaces modified with Cu(II)-NTA exhibit stronger binding affinity to His-tagged proteins as compared to Ni(II)-NTA surfaces.^{27,28} Four additional thin gold film “control”

elements were functionalized with a metal-free NTA monolayer, but not exposed to Cu(II).

After the fabrication of the dsDNA/NTA microarray, a cell free protein expression solution (RTS 100 *E. coli* HY protein expression kit, Roche) based on an enhanced *E. coli* cell lysate with all the necessary components including T7 RNA polymerase, rNTPs, tRNAs, amino acids and ribosome was introduced into the 25 μ L microfluidic channel at 32 °C. The RNA polymerase adsorbed onto the dsDNA template T7 promoter site on the generator element surface and commenced the transcription of multiple copies of mRNA. Simultaneously, the ribosomal units nearby in solution started to translate the nascent mRNA into protein. This simultaneous, coupled IVTT system has been shown to be highly productive as compared to the use of sequential mRNA transcription and translation reactions.^{29,30} The translated His-tagged protein then diffused to the neighboring detector element and was captured by the Cu(II)-NTA surface to form the protein microarray. This protein microarray was then used directly for SPRI biosensing after simply rinsing with detergent buffer solution to wash off any adsorbed enzymes or other reaction mixture components.

The simultaneous on-chip synthesis and adsorption of a GFP microarray was monitored with real-time SPRI measurements, and Figure 2 plots the SPRI synthesis/adsorption kinetics

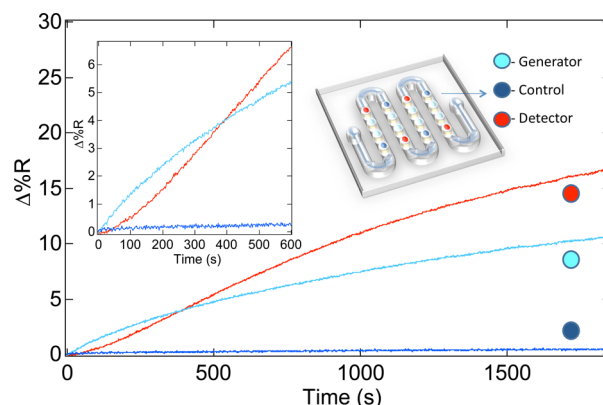


Figure 2. Real-time SPRI synthesis/adsorption kinetics measurements of newly synthesized GFP His-tagged protein onto detector, generator and control elements. The inset plot shows the initial SPRI Reflectivity changes at times less than 600 s.

curves from those experiments. As seen in the diagram in Figure 2, the microarray elements are coded as G, D and C for generator, detector and control elements respectively, and the figure plots the changes in SPRI Reflectivity, ΔR , for these three elements in real time following introduction of 25 μ L of the cell free protein expression mixture at 32 °C into the microfluidic channel. The SPRI adsorption kinetics curve for the detector elements exhibits a large increase in SPRI Reflectivity ($\Delta R = 16.4 \pm 0.5\%$ in 1800 s) that we attribute to the specific adsorption of synthesized protein onto the microarray elements; the ΔR value suggests the formation of a full GFP monolayer (ca. 10^{12} molecules/ cm^2).^{25,31} In contrast, only a negligible ΔR was observed for the control elements, indicating a lack of nonspecific adsorption of protein, polymerase or ribosome onto the microarray.

Figure 2 also plots the SPRI adsorption kinetics curves for the generator elements. As seen in the inset plot in Figure 2, at the beginning of the synthesis/adsorption kinetics curves, the

$\Delta\%R$ curves for the generator element increases before the detector element, as expected. A significant final $\Delta\%R$ was also observed for the generator elements; this SPRI signal is attributed to a combination of adsorption of both RNA polymerase and ribosome onto the generator elements. This coupling of the simultaneous transcription and translation processes has been observed previously in IVTT reactions.²⁹ There may also be some nonspecific adsorption of GFP onto the generator elements (even though none is observed on the control elements), but it must be stressed that any nonspecific adsorption of polymerase, ribosome, or GFP onto the generator elements will not interfere with any subsequent SPRI measurements of protein adsorption onto the detector elements.

Finally, to demonstrate the application of protein microarrays fabricated with this on-chip synthesis methodology to SPRI biosensing measurements, a GFP-luciferase protein microarray was translated and then utilized to detect the adsorption of the antibodies anti-GFP and antiluciferase (Figure 3). In this

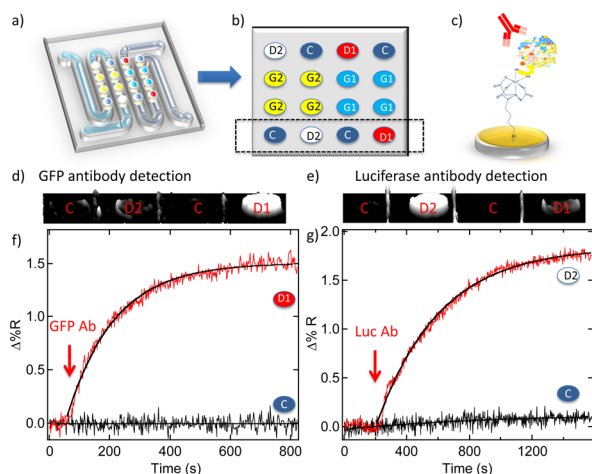


Figure 3. (a) Five-component, 16-element GFP-luciferase DNA microarray in a dual-channel microfluidic cell. (b) Spatial diagram of the five components on the DNA microarray. (c) Schematic diagram of antibody binding onto synthesized protein microarray. (d) SPRI difference images taken before and after the exposure to anti-GFP solutions showing the antibody binding onto corresponding element D1. (e) SPRI difference images taken before and after the exposure to antiluciferase solutions showing the antibody binding onto corresponding element D2. (f) Real-time SPRI adsorption kinetics measurements of anti-GFP specific binding onto D1. (g) Real-time SPRI adsorption kinetics measurements of antiluciferase specific binding onto D2.

experiment, a five-component, 16-element DNA microarray chip was fabricated in a 25 μL dual-channel microfluidic cell as shown in Figure 3a. The five components are shown in the spatial diagram in Figure 3b: (i) four GFP generator elements (G1, light blue) fabricated with dsDNA encoding His-tagged GFP; (ii) four luciferase generator elements (G2, yellow) fabricated with dsDNA encoding His-tagged luciferase; (iii) two GFP detector elements (D1, red), (iv) two luciferase detector elements (D2, white), and (v) four control elements (C, dark blue). Upon completion of the on-chip self-assembly of the protein microarray in cell free protein expression solution after incubation at 32 $^{\circ}\text{C}$, the protein microarray was washed with 3% BSA, PBST and 1 $\times$ PBS buffer solutions. The microarray was then used directly for SPRI antibody biosensing measure-

ments as shown schematically in Figure 3c; alternatively, the microarray could be kept stable at 4 $^{\circ}\text{C}$ for up to 3 days if necessary. SPRI difference images and kinetics curves for the specific adsorption of GFP and luciferase antibodies (Abcam, rabbit polyclonal antibodies, 50 and 100 nM, respectively) onto their respective microarray elements are shown in Figure 3d–g. The SPRI difference images taken before and after the exposure to anti-GFP and antiluciferase solutions are shown in Figure 3d and e. The specific adsorption of the antibodies onto the correct protein microarray elements are clearly observed in these figures, with negligible nonspecific adsorption onto the control elements of the microarray. Quantitative real-time SPRI adsorption kinetics curves were also obtained for the specific adsorption of anti-GFP and antiluciferase and are shown in Figure 3f and g, respectively. A $\Delta\%R$ of $1.4 \pm 0.2\%$ was observed after 800 s for the GFP detector elements upon exposure to anti-GFP, and a $\Delta\%R$ of $1.7 \pm 0.3\%$ was observed after 1400 s for the luciferase detector elements upon exposure to antiluciferase. These experiments clearly verify that the multiplexed on-chip synthesis method can be used to rapidly create protein microarrays for SPRI bioaffinity adsorption experiments directly from dsDNA microarrays.

This integrated on-chip synthesis process (RNA surface transcription, protein translation, protein immobilization, and protein purification) creates a multielement, ready-to-use protein microarray in a single step from a dsDNA microarray in just a few hours. There are several significant advantages for using this on-chip protein microarray fabrication method: (i) it greatly reduces the risk of RNA and protein degradation by removing any ex situ handling steps and creating proteins just in-time, (ii) it eliminates the need for the off-chip purification and storage of separate proteins, (iii) only a very small amount (10^{-14} – 10^{-15} moles) of dsDNA template is needed to generate a small amplified amount (10^{-11} – 10^{-12} moles) of each protein,²³ (iv) this cell-free methodology can be used to express proteins that cannot be cloned in *E. coli* due to toxicity issues,³² (v) the encoding dsDNA microarray can be easily transported, stored and then converted to protein microarray in a single step in just a few hours, (vi) even though only a small amount of protein is synthesized, the bioaffinity adsorption of the His-tag to the Cu(II)-NTA surface occurs rapidly since the local concentration is high in the small volume microfluidic cell (we estimate it to be 10^{-5} – 10^{-6} M), (vii) the use of the His-tag capture mechanism creates an oriented protein microarray for better bioaffinity measurements as compared to a lysine-based attachment chemistry,³³ and (viii) the chips can be recycled after washing and recharging with Cu(II), although for reusable chips, it is better to use Ni(II) rather than Cu(II) for the His-tag adsorption of the synthesized protein. A current limitation of this method is that we have only implemented one type of surface attachment chemistry on the detector elements (His-tagged protein capture with Cu(II)-NTA), and therefore the different proteins must be transcribed in separate wells or channels. We hope to eliminate this problem by implementing multiple surface attachment chemistries in the future. Even with this limitation, the convenient on-chip synthesis method described here should be easily integrated into SPRI applications for both research and point-of-care applications.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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