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On-Chip Synthesis of RNA Aptamer Microarrays for Multiplexed Protein Biosensing with SPR Imaging Measurements

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Abstract

Microarrays of RNA aptamers are fabricated in a one-step, multiplexed enzymatic synthesis on gold thin films in a microfluidic format and then employed in the detection of protein biomarkers with surface plasmon resonance imaging (SPRI) measurements. Single-stranded RNA (ssRNA) oligonucleotides are transcribed on-chip from double-stranded DNA (dsDNA) templates attached to microarray elements (denoted as generator elements) by the surface transcription reaction of T7 RNA polymerase. As they are synthesized, the ssRNA oligonucleotides diffuse in the microfluidic channel and are quickly captured by hybridization adsorption onto adjacent single-stranded DNA (ssDNA) microarray elements (denoted as detector elements) that contain a sequence complementary to 5'-end of the ssRNA. The RNA aptamers attached to these detector elements are subsequently used in SPRI measurements for the bioaffinity detection of protein biomarkers. The microfluidic generator-detector element format permits the simultaneous fabrication of multiple ssRNA oligonucleotides with different capture sequences that can hybridize simultaneously to distinct detector elements and thus create a multiplexed aptamer microarray. In an initial set of demonstration experiments, SPRI measurements are used to monitor the bioaffinity adsorption of human thrombin (hTh) and vascular endothelial growth factor (VEGF) proteins onto RNA aptamer microarrays fabricated *in situ* with this on-chip RNA polymerase synthesis methodology. Additional SPRI measurements of the hydrolysis and desorption of the surface-bound ssRNA aptamers with a surface RNase H are used to verify the capture of ssRNA with RNA-DNA surface hybridization onto the detector elements. The on-chip RNA synthesis described here is an elegant, one-step multiplexed methodology for the rapid and contamination-free fabrication of RNA aptamer microarrays for protein biosensing with SPRI.

Introduction

The use of nucleic acid aptamer microarrays in conjunction with surface plasmon resonance imaging (SPRI) measurements is emerging as a rapid and robust alternative method to multiplexed antibody screening for the detection and quantitation of protein biomarkers in biological samples^{1–3}. Aptamer microarrays possess several potential advantages over antibody microarrays in terms of their long-term stability, ease of fabrication, and reduced non-specific adsorption^{3–6}. SPRI measurements employing both DNA and RNA aptamer microarrays for protein biosensing have been reported previously^{1, 2, 7, 8}.

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While a number of excellent high fidelity attachment methodologies are currently available for the fabrication of robust single-stranded DNA (ssDNA) microarrays on gold thin films for SPRI measurements^{9–12}, the fabrication of single-stranded RNA (ssRNA) aptamer microarrays to SPRI measurements typically requires more care. Biotinylation or thiol-modification of ssRNA aptamers for surface attachment can often lead to cross-reactions of the oligonucleotides; often an alternative process that uses unmodified ssRNA and an enzymatic reaction to either create a biotin-type tag¹³ or directly attach the ssRNA to 5'-phosphorylated ssDNA microarrays with surface ligation reaction is employed^{14, 15}. Moreover, these procedures for ssRNA aptamer microarrays must take place in a scrupulously clean lab environment because ssRNA is very susceptible to hydrolysis by trace amounts of RNase A. These additional fabrication and handling and requirements have limited the application of ssRNA aptamer microarrays in SPRI biosensing.

In this paper, we demonstrate a simple, on-chip enzymatic synthesis process for the *in-situ* fabrication of ssRNA microarrays in microfluidic format for the detection of protein biomarkers with SPRI. This multiplexed on-chip synthesis employs the surface enzymatic reaction of T7 RNA polymerase with double stranded DNA (dsDNA) templates that encode both an RNA aptamer and a unique capture tag sequence. The surface-transcribed ssRNA are captured by adjacent complementary ssDNA elements to form the RNA aptamer microarray. We have recently employed a similar on-chip RNA synthesis and capture methodology for the amplified SPRI detection of ssDNA at femtomolar concentrations¹⁶. The use of an on-chip RNA transcription methodology greatly reduces the risk of RNA degradation by removing any ex-situ handling steps, and eliminates the need for purification of the synthesized ssRNA product.

Experimental Section

DNA microarray fabrication

SF-10 (Schott Glass) substrates were used to create the SPRI microarrays. First, a hydrophobic layer was formed by spin-coating CYTOP (Asahi Glass) at 5000 rpm on the glass substrate, followed by baking in oven at 70 °C for 50 minutes and then 190 °C for 1 h. The 16 elements (1-mm diameter) were fabricated by physical vapor deposition of chromium (1 nm) and gold (45 nm) utilizing a shadow mask. The details are described elsewhere¹⁷. The slides were then immersed in 1 mM MUAM (11-Amino-1-undecanethiol hydrochloride, Dojindo) overnight, and 2 mg/mL of poly(L-glutamic acid) (pGlu, Sigma) was allowed to react for 1 h. Amino-modified DNA (0.5 μ L, 250 μ M) was immobilized onto the microarray elements using EDC/NHSS coupling reaction as described previously¹⁸.

On-chip transcription

The on-chip transcription process was performed on the SPRI instrument (GWC Technologies) that contains a temperature control system as described previously¹⁶. T7 RNA polymerase (RNAP) was purchased from Promega (RiboMax). First, the template dsDNA duplex on the generator element is formed by applying 1 μ M complimentary sequence (T1 or T2 in 1X PBS; all DNA sequences are listed in Supporting Information) for 10 minutes. 1X PBS contains 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and is adjusted to pH 7.4. A solution of T7 RNAP (1.875 mM rNTP, 200–300 U/mL RNAP, and 1 U/ μ L RNase inhibitor) in the transcription buffer (5X transcription buffer was purchased from Promega and diluted to 1X) was injected into the SPRI flow cell (50 μ L). The temperature was raised to 37 °C for 2 h for the transcription process. After transcription, the SPRI system was cooled down to room temperature and rinsed with buffer.

Protein binding assays

The human thrombin (hTh) and VEGF (165 amino acid form) were purchased from Sigma. Thrombin RNA aptamer was originally selected with affinity to bovine thrombin but can also bind to human thrombin^{19, 20}. In this paper, two nucleotides were added at the end of the hTh aptamer sequence to increase the folding stability. All hTh and VEGF binding assays were performed in 1X PBS.

RNase H surface hydrolysis measurements

After the on-chip transcription process, RNase H (500 μ L of 0.1 U/ μ L; Takara Bio) in Tris buffer (50 mM Tris-HCl, 300 mM KCl, 10 mM MgCl₂, and 10 mM DTT, adjust to pH 7.8) was applied to the chip for 5 min at room temperature²⁰. The real-time SPRI measurement of RNase H surface hydrolysis was shown in Supporting Information (Figure S1).

Results and Discussion

Our method for creating an RNA aptamer microarray via on-chip RNA transcription followed by RNA-DNA hybridization capture is shown pictorially in Figure 1. As in our previous work¹⁶, we employ a generator element-detector element configuration confined in a microfluidic channel (Figure 1a). The generator elements contain the dsDNA template for a ssRNA that consists of two parts, an ssRNA aptamer sequence and a 18-base capture tag on the 5'-end (all DNA and RNA sequences are listed in the Supporting Information). The detector elements contain an ssDNA sequence complementary to a capture tag on the ssRNA. The process of array fabrication is shown in Figure 1b: T7 RNA polymerase and ribonucleotide triphosphates (rNTPs) are introduced into the microfluidic cell. The RNA polymerase adsorbs onto the dsDNA template attached to the generator element and creates multiple copies of the ssRNA sequence. These ssRNA oligonucleotides rapidly diffuse to an adjacent detector element (the distance between elements is ca. 1 mm) where they adsorb by hybridization ("hybridizationadsorption") onto the complementary ssDNA sequence to form an RNA aptamer microarray element where the RNA aptamer is attached to the surface via the DNA-RNA heteroduplex. Each RNA aptamer can be encoded with a different capture sequence to attach to a different detector element and thus in one step the surface RNA polymerase reaction can form a multiplexed RNA aptamer microarray. This microarray can be used immediately for SPRI protein biosensing by rinsing the microfluidic cell with a buffer solution and then introducing a sample solution with the target proteins (Figure 1c). Since entire process - *in-vitro* transcription, array fabrication and SPRI protein adsorption detection - occurs in a single, low-volume microfluidic flow cell, the opportunity for contamination is greatly reduced, and the implementation of RNA aptamer microarrays is greatly simplified.

A first application of these transcription-based microarrays to SPRI thrombin biosensing is shown in Figure 2. Human thrombin (hTh) is a 37-kDa serine protease that catalyzes many coagulation-related reactions and has been used as a biomarker for various coagulation-related and cardiovascular diseases^{21, 22}. A number of both DNA and RNA aptamers have been identified for hTh; in these experiments we employ the 31-base RNA aptamer that binds to the fibrinogen binding site^{19, 20}. A three-component, 16-element ssDNA microarray is fabricated in a 50 μ L microfluidic SPRI cell as shown in Figure 2a. The DNA microarray elements are created by attaching amino-modified DNA to an electrostatically adsorbed poly-L-glutamic acid monolayer via an NHSS-EDC coupling chemistry described elsewhere¹⁸. Three different types of array elements -- generator elements (sequence G1), detector elements (sequence D1), and control elements (sequence C1) -- are fabricated in the spatial pattern shown in the Figure 2a. The D1 and C1 sequences are ssDNA oligonucleotides, whereas the G1 and T1 sequence form a dsDNA 81-mer that contains both

the RNA aptamer sequence and a capture sequence complementary to D1. On-chip *in-vitro* transcription is initiated by exposing the microarray to a solution containing T7 RNA polymerase and rNTPs for 2 hours at 37 °C. The transcribed ssRNA aptamers diffuse from the generator elements and are captured by the adjacent detector elements as described in Figure 1. After microarray fabrication, the SPRI microfluidic chip is cooled to room temperature, flushed clear with a HEPES buffer solution and then a solution containing 25 nM hTh (total volume of 1 mL) is flowed through the SPRI chip. Figure 2b shows a SPRI difference image obtained after exposure of chip for 120 sec; the four detector elements for hTh are clearly visible in the Figure 2b. A line profile obtained from the image (red line) is also shown in Figure 2b, indicating that the detector elements exhibit an increased signal of $\Delta\%R=1.07\pm0.09\%$. This value is comparable to that reported previously²⁰ in other SPRI measurements. The real-time microarray image data can also be processed to create real-time binding curves for the D1 and C1 elements as shown in Figure 2c. The solid lines are fits of the SPRI data to adsorption/desorption kinetic equations²³ from which we determine the rate constants k_{on} and k_{off} . The ratio of these two constants yields a Langmuir adsorption coefficient K_{ads} of $1.5\times10^8\text{ M}^{-1}$. An adsorption kinetics curve is observed for hTh adsorption onto element D1 with the limiting value of 1.0%. The kinetics data also shows negligible adsorption onto the control element C1, indicating the suppression of non-specific protein adsorption.

As an additional demonstration of the presence of an RNA aptamer microarray in this experiment, the microfluidic cell was flushed with 500 μL of an RNase H solution and the SPR image in Figure 2d was obtained. As seen in previously experiments^{14, 24}, RNase H will very efficiently hydrolyze any DNA-RNA heteroduplexes on the microarray element, yielding a loss in the differential reflectivity SPR image. As seen in the Figure 2d, $\Delta\%R$ for the detector elements decreased by the significant amount of $-3.2 \pm 0.2\%$, whereas very little loss was observed on the control elements. A little bit of reflectivity loss was observed due to removal of some RNA on the generator elements, but this did not interfere with the quantitation of the SPRI data (indeed, this is the value of the generator-detector dual element strategy). The loss of SPRI signal from the detector elements was larger than the signal increase during the previous hTh adsorption step, indicating that both the RNA and the hTh have been removed from the surface. From the amount of loss in the SPRI signal and previous SPRI measurements²⁴, we can estimate that the amount of RNA on each detector element was approximately 10 femtomoles. Since the RNase H enzyme will not hydrolyze DNA (neither ssDNA nor dsDNA), the RNase H hydrolysis reaction has restored the generator and detector elements on the SPRI biochip to their original state. After the RNase H process, the biochip in principle is able to create new RNA aptamer microarrays by re-applying a new solution of T7 RNA polymerase and rNTPs onto the surface²⁵.

In a second experiment, a two-component RNA aptamer microarray was transcribed and utilized in SPRI protein biosensing measurements in order to demonstrate the multiplexing capabilities of this fabrication methodology. This experiment employed a four-component, sixteen element microarray (as depicted in Figure 3a) that consisted of generator elements that contained a mixture of two dsDNA sequences (50% of G1-T1 duplex and 50% of G2-T2 duplex), two different detector elements (with sequences D1 and D2 attached) and one control element (sequence C2). The sequence G1 is the same as that used in the first experiment and encodes the hTh aptamer; the sequence G2 encodes a second RNA aptamer for vascular endothelial growth factor (VEGF). VEGF is a 46 kDa cell signaling protein whose over-expression is used a serum cancer biomarker, and the 29-mer RNA aptamer has been selected by previous researchers²⁶. As in the first experiment, T7 RNA polymerase and rNTPs were introduced into the microfluidic cell for 2 hrs at 37 °C. The hTh and VEGF RNA aptamers were simultaneously transcribed, diffused, and then specifically attached by hybridization adsorption onto two different detector spots labeled D1 and D2 respectively.

The SPRI difference images shown in Figure 3a and 3b were obtained after the sequential introduction of 40 nM of VEGF and 25 nM of hTh to the microfluidic cell (see Supporting Information for details), and clearly indicate that the microarray can be used to identify and quantitate these two proteins simultaneously. Figure 3d plots in two line profiles obtained from the images; both RNA aptamer array elements showed an increase in differential reflectivity of approximately 1% when exposed to the corresponding protein target. A small amount of non-specific adsorption (<0.2%) was also observed on the control elements of this microarray.

In summary, we have demonstrated an elegant one-step, on-chip synthesis methodology for the fabrication of RNA aptamer microarrays from DNA microarrays that uses a multiplexed surface-based RNA transcription reaction followed by hybridization adsorption capture in a microfluidic format. This rapid on-chip methodology has a number of significant advantages: (i) the integrated on-chip approach provides a simple method for generating multiple RNA aptamers simultaneously without the need for further purification or modification, and the microfluidic format greatly reduces the risks of RNase A contamination from the external environmental sources; (ii) only a very small amount of each RNA aptamer is needed (on the order of 10 femtomoles), but it is sufficient in the microfluidic format for biochip fabrication; (iii) the use of different capture sequences for the different RNA aptamers means that the aptamer microarray is formed from a single solution without the need for separate reaction compartments or spotting procedures, (iv) since each dsDNA on the generator element can transcribe multiple copies of ssRNA, the number of generator elements does not need to scale with the number of distinct aptamer sequences. In the future, in addition to SPRI detection, we will combine our new SPR phase imaging^{27, 28} techniques with this fabrication methodology for ultrasensitive protein biosensing with RNA aptamer microarrays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Iliuk AB, Hu L, Tao WA. Aptamer in bioanalytical applications. *Anal. Chem.* 2011; 83(12):4440–4452. [PubMed: 21524128]
2. Tombelli S, Minunni M, Luzi E, Mascini M. Aptamer-based biosensors for the detection of HIV-1 Tat protein. *Bioelectrochemistry.* 2005; 67(2):135–141. [PubMed: 16027048]
3. Bunka DHJ, Stockley PG. Aptamers come of age - at last. *Nat. Rev. Microbiol.* 2006; 4(8):588–596. [PubMed: 16845429]
4. Rowe W, Platt M, Day PJR. Advances and perspectives in aptamer arrays. *Integr. Biol.* 2009; 1(1): 53–58.
5. Nimjee SM, Rusconi CP, Sullenger BA. Aptamers: an emerging class of therapeutics. *Annu. Rev. Med.* 2005; 56:555–583. [PubMed: 15660527]
6. Liu J, Cao Z, Lu Y. Functional nucleic acid sensors. *Chem. Rev.* 2009; 109(5):1948–1998. [PubMed: 19301873]
7. Potty ASR, Kourentzi K, Fang H, Jackson GW, Zhang X, Legge GB, Willson RC. Biophysical characterization of DNA aptamer interactions with vascular endothelial growth factor. *Biopolymers.* 2009; 91(2):145–156. [PubMed: 19025993]

8. de-los-Santos-Álvarez N, Lobo-Castanon MJ, Miranda-Ordieres AJ, Tunon-Blanco P. SPR sensing of small molecules with modified RNA aptamers: Detection of neomycin B. *Biosens. Bioelectron.* 2009; 24(8):2547–2553. [PubMed: 19217276]
9. Thiel AJ, Frutos AG, Jordan CE, Corn RM, Smith LM. In situ surface plasmon resonance imaging detection of DNA hybridization to oligonucleotide arrays on gold surfaces. *Anal. Chem.* 1997; 69(24):4948–4956.
10. Lee SJ, Youn B-S, Park JW, Niazi JH, Kim YS, Gu MB. sDNA aptamer-based surface plasmon resonance biosensor for the detection of retinol binding protein 4 for the early diagnosis of type 2 diabetes. *Anal. Chem.* 2008; 80(8):2867–2873. [PubMed: 18324839]
11. Phillips KS, Wilkop T, Wu J-J, Al-Kaysi RO, Cheng Q. Surface plasmon resonance imaging analysis of protein-receptor binding in supported membrane arrays on gold substrates with calcinated silicate films. *J. Am. Chem. Soc.* 2006; 128(30):9590–9591. [PubMed: 16866487]
12. Wolf LK, Fullenkamp DE, Georgiadis RM. Quantitative angle-resolved SPR Imaging of DNA-DNA and DNA-drug kinetics. *J. Am. Chem. Soc.* 2005; 127(49):17453–17459. [PubMed: 16332097]
13. Srisawat C, Engelke DR. RNA affinity tags for purification of RNAs and ribonucleoprotein complexes. *Methods.* 2002; 26(2):156–161. [PubMed: 12054892]
14. Li Y, Lee HJ, Corn RM. Fabrication and characterization of RNA aptamer microarrays for the study of protein-aptamer interactions with SPR imaging. *Nucleic Acids Res.* 2006; 34(22):6416–6424. [PubMed: 17130155]
15. Li YA, Wark AW, Lee HJ, Corn RM. Single-nucleotide polymorphism genotyping by nanoparticle-enhanced surface plasmon resonance imaging measurements of surface ligation reactions. *Anal. Chem.* 2006; 78(9):3158–3164. [PubMed: 16643008]
16. Sendroiu IE, Gifford LK, Luptak A, Corn RM. Ultrasensitive DNA microarray biosensing via in situ RNA transcription-based amplification and nanoparticle-enhanced SPR imaging. *J. Am. Chem. Soc.* 2011; 133(12):4271–4273. [PubMed: 21391582]
17. Goodrich TT, Wark AW, Corn RM, Lee HJ. Surface plasmon resonance imaging measurements of protein interactions with biopolymer microarrays. 2006; Vol. 328:113–130. In.
18. Chen YL, Nguyen A, Niu LF, Corn RM. Fabrication of DNA microarrays with poly(L-glutamic acid) monolayers on gold substrates for SPR imaging measurements. *Langmuir.* 2009; 25(9):5054–5060. [PubMed: 19253965]
19. Liu X, Cao G, Ding H, Zhang D, Yang G, Liu N, Fan M, Shen B, Shao N. Screening of functional antidotes of RNA aptamers against bovine thrombin. *FEBS Letters.* 2004; 562(1,Äi3):125–128. [PubMed: 15044012]
20. Li Y, Lee HJ, Corn RM. Detection of protein biomarkers using RNA aptamer microarrays and enzymatically amplified surface plasmon resonance imaging. *Anal. Chem.* 2007; 79(3):1082–1088. [PubMed: 17263339]
21. Yang XH, Wang L, Wang KM, Tan WH, Tang HX, Meng XX, Guo QP. Novel protein detection method based on proximity-dependent polymerase reaction and aptamers. *Chinese Science Bulletin.* 2008; 53(2):204–208.
22. Huang YC, Ge BX, Sen D, Yu HZ. Immobilized DNA switches as electronic sensors for picomolar detection of plasma proteins. *J. Am. Chem. Soc.* 2008; 130(25):8023–8029. [PubMed: 18517197]
23. Jensen KK, Ørum H, Nielsen PE, Nordén B. Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. *Biochemistry.* 1997; 36(16):5072–5077. [PubMed: 9125529]
24. Seefeld TH, Zhou W-J, Corn RM. Rapid microarray detection of DNA and proteins in microliter volumes with surface plasmon resonance imaging measurements. *Langmuir.* 2011; 27(10):6534–6540. [PubMed: 21488682]
25. Lee HJ, Wark AW, Li Y, Corn RM. Fabricating RNA microarrays with RNADNA surface ligation chemistry. *Anal. Chem.* 2005; 77(23):7832–7837. [PubMed: 16316195]
26. Jellinek D, Green LS, Bell C, Janjic N. Inhibition of receptor binding by highaffinity RNA ligands to vascular endothelial growth factor. *Biochemistry.* 1994; 33(34):10450–10456. [PubMed: 7520755]

27. Halpern AR, Chen Y, Corn RM, Kim D. Surface plasmon resonance phase imaging measurements of patterned monolayers and DNA adsorption onto microarrays. *Anal. Chem.* 2011; 83(7):2801–2806. [PubMed: 21355546]
28. Zhou W-J, Halpern AR, Seefeld TH, Corn RM. Near infrared surface plasmon resonance phase imaging and nanoparticle-enhanced surface plasmon resonance phase imaging for ultrasensitive protein and DNA biosensing with oligonucleotide and aptamer microarrays. *Anal. Chem.* 2012; 84(1):440–445. [PubMed: 22126812]

Figure 1.

Schematic diagram of on-chip synthesis of RNA aptamer microarrays. (a) On the generator elements, surface promoter DNA was covalently attached to the gold surface and then hybridized with a template DNA. (b) The mixture solution of T7 RNA polymerase and rNTPs was injected into the microfluidic channel. RNA was transcribed with T7 RNA polymerase on the generator spot. Transcribed RNA was diffused to the adjacent detector elements and captured by bound ssDNA. (c) Injected protein was detected by RNA aptamer on the detector elements.

Figure 2.

(a) Schematic of chip patterning on the microarray. generator, detector and control elements are labeled G1, D1 and C1, respectively. (b) A SPRI difference image after injecting 25 nM human thrombin (hTh) and corresponding line profile taken from the array image containing G1, D1 and C1 elements. (c) Normalized real-time SPRI kinetic curves for the detection of 25 nM hTh on D1 and C1 elements. (d) A SPRI difference image by injecting 500 μL , 0.1 U/ μL RNase H solution and corresponding line profile taken from the difference image. RNA at the DNA-RNA duplex was degraded by RNase H and protein-RNA aptamer complex was removed from the detector element.

Figure 3. SPRI difference images after sequential injection of (a) 40 nM VEGF and (b) 25 nM hTh for ten minutes, respectively. (c) Schematic of surface patterning of the microarray for multiplexed protein detection. Generator, detector element for VEGF and hTh, and Control elements are represented as G (a mixture of sequences G1 and G2), D1, D2, and C2. (d) Line profiles of VEGF (red) and hTh (black) obtained by the corresponding difference images shown in (a) and (b). Line profiles for VEGF contains D2 and C2, and that for hTh contains G and D1, confirming very little non-specific binding.