DNA Nanostructure-Based Universal Microarray Platform for High-Efficiency Multiplex Bioanalysis in Biofluids

Zhenhua Li,[†,‡] Bin Zhao,[†,‡] Dongfang Wang,[†] Yanli Wen,[§] Gang Liu,[§] Haoqing Dong,[∥] Shiping Song,*,[†] and Chunhai Fan[†]

[†]Division of Physical Biology & Bioimaging Center, Shanghai Synchrotron Radiation Facility, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China

[§]Division of Chemistry and Ionizing Radiation Measurement Technology, Shanghai Institute of Measurement and Testing Technology, 1500 Zhangheng Road, Shanghai 201203, China

[∥]Shanghai High School, 989 Baise Road, Shanghai 200231, China

Ⓢ Supporting Information

ABSTRACT: Microarrays of biomolecules have greatly promoted the development of the fields of genomics, proteomics, and clinical assays because of their remarkably parallel and high-throughput assay capability. Immobilization strategies for biomolecules on a solid support surface play a crucial role in the fabrication of high-performance biological microarrays. In this study, rationally designed DNA tetrahedra carrying three amino groups and one single-stranded DNA extension were synthesized by the self-assembly of four oligonucleotides, followed by high-performance liquid chromatography purification. We fabricated DNA tetrahedron-based microarrays by covalently coupling the DNA tetrahedron onto glass substrates. After their biorecognition capability was evaluated, DNA tetrahedron microarrays were utilized for the analysis of different types of bioactive molecules. The gap hybridization strategy, the sandwich configuration, and the engineering aptamer strategy were employed for the assay of miRNA biomarkers, protein cancer biomarkers, and small molecules, respectively. The arrays showed good capability to anchor capture biomolecules for improving biorecognition. Addressable and high-throughput analysis with improved sensitivity and specificity had been achieved. The limit of detection for let-7a miRNA, prostate specific antigen, and cocaine were 10 fM, 40 pg/mL, and 100 nM, respectively. More importantly, we demonstrated that the microarray platform worked well with clinical serum samples and showed good relativity with conventional chemical luminescent immunoassay. We have developed a novel approach for the fabrication of DNA tetrahedron-based microarrays and a universal DNA tetrahedron-based microarray platform for the detection of different types of bioactive molecules. The microarray platform shows great potential for clinical diagnosis.

KEYWORDS: DNA tetrahedron, microarrays, biorecognition, multiplex bioanalysis, biological fluids
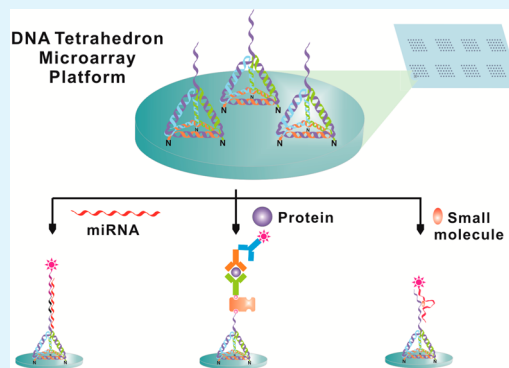
INTRODUCTION

Microarrays of biomolecules have been emerging as powerful tools for genomics, proteomics, and clinical diagnosis because of their remarkably parallel and high-throughput assay capability.[1−7] Typically, selected groups of biomolecules (oligonucleotides or proteins) are immobilized onto a solid support surface with a preset address to fabricate a microarray. Importantly, coating of the surface and immobilization of the biomolecules on the surface are critical for the properties of the microarrays.[8] Many efforts have been devoted to developing various strategies for surface modification and biomolecular immobilization to acquire high-performance microarrays.[9−14] However, several important technical issues still remain challenging, including reducing surface effects such as steric hindrance and electrostatic interaction, controllably arranging the capture biomolecules in an oriented manner, and providing a solution-phase-like environment for biorecognition.[8]

Recently, DNA nanotechnology has attracted tremendous interest because it enables the bottom-up construction of exquisite DNA nanostructures with excellent controllability and high precision.[15−20] Especially, three-dimensional DNA tetrahedron architectures,[21,22] which have mechanical rigidity and structural stability, have proven to be excellent candidates to immobilize biomolecules on surfaces.[23] Taking advantage of the steady structure and consistently favorable orientation of DNA tetrahedron bridges, the capture biomolecules could be indirectly immobilized on the surface with specific orientation

and well-defined spacing. More recent advances have demonstrated that DNA tetrahedron-decorated gold surfaces have great superiority in improving biomolecular recognition and developing bioassay platforms.[24−29] Nevertheless, they still suffer from a complicated fabrication process and the high cost of gold substrates. Especially, strong fluorescence quenching on gold substrates severely hinders the application of DNA tetrahedra in fluorescent microarrays.[27,30]

In order to circumvent the above problems, herein we report a novel approach for the fabrication of DNA tetrahedron-based microarrays on glass substrates placing capture biomolecules in a solution-phase-like environment. A DNA tetrahedron structure probe (TSP) featuring three amine groups and one versatile single-stranded DNA (ssDNA) extension was synthesized through the self-assembly of four ssDNA oligonucleotides. The rationally designed nanostructures were covalently immobilized onto glass surfaces via amine−aldehyde interaction to fabricate microarrays. We then demonstrated the excellent biorecognition capability of the as-fabricated DNA tetrahedron-based microarrays. Finally, we developed a universal multiplex platform for recognizing different types of bioactive molecules including miRNA biomarkers, protein cancer biomarkers, and small molecules.

■ EXPERIMENTAL SECTION

Chemicals and Materials. All of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Aldehyde-functionalized glass slides and a spotting buffer were purchased from CapitalBio Corp. (Beijing, China). Streptavidin was purchased from Sigma-Aldrich (St. Louis, MO). Biotinylated-Cy3 was purchased from Nanocs Inc. (New York). Cocaine was purchased from Shanghai Institute of Control Center (Shanghai, China). Prostate-specific antigen (PSA), α-fetoprotein (AFP), carcinoembryonic antigen (CEA), biotinylated mouse antihuman PSA mAb, and biotinylated mouse antihuman AFP mAb were purchased from United States Biological (Salem, MA). Rabbit antihuman PSA pAb and rabbit antihuman AFP pAb were purchased from Fitzgerald Industries International (Acton, MA). Cy5-labeled goat antirabbit Ab_2 was purchased from Abcam (Cambridge, U.K.). Serum samples were obtained from Renji Hospital, School of Medicine, and Shanghai Jiao Tong University. All oligonucleotides, including miRNA and DNA probes, were synthesized and purified by Invitrogen (Carlsbad, CA). The sequences are shown in Table S1 in the Supporting Information (SI).

Formation of DNA Tetrahedron. DNA tetrahedra were formed based on modified literature protocols.[26] Tetra-A and three amino-modified single strands (tetra-B, tetra-C, and tetra-D) were dissolved in TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.0) with a final concentration of 100 μM. A total of 10 μL of each strand was combined with 60 μL of TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0). The resulting mixture was heated to 95 °C for 10 min and then cooled to 4 °C in 30 s using a Peltier model PTC-200 thermal cycler. The raw self-assembled DNA tetrahedron was isolated and purified by anion-exchange-column high-performance liquid chromatography (HPLC) using a Waters breeze 2 HPLC system. After careful collection and enrichment, the products of HPLC purification were further identified by 8% native polyacrylamide gel electrophoresis (PAGE).

Fabrication of DNA Tetrahedron Microarrays. The fabrication of all DNA tetrahedron microarrays was based on the universal spotting method unless otherwise stated. DNA tetrahedra in a spotting buffer were printed onto aldehyde-functionalized glass slides using a SpotBot 2 microarrayer programmed by *SpotApp* software. The average spot diameter was 100 μm, and the distance between two spots was 500 μm. The arrayed slides were incubated overnight at room temperature in a humidity chamber. After being washed twice with PBST buffer (10 mM phosphate, 140 mM NaCl, 2.7 mM KCl,

0.5% (v/v) Tween20, pH 7.4) and once with PBS buffer (10 mM phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4), the slides were immersed in an aldehyde blocking solution [2.5 mg/mL $NaBH_4$ in PBS buffer with 25% (v/v) ethanol, pH 7.4] at room temperature for 30 min to passivate unreacted aldehyde groups. After being washed twice with PBST buffer and once with PBS buffer, the slides were stored at 4 °C for further use.

Biorecognition Capability of DNA Tetrahedron Microarrays. For DNA hybridization based on DNA tetrahedron microarrays, tetra-A-DNAs (80-nt) were employed for the formation of TSP, named Am-Tet-DNA. ssDNA, unpurified TSP, TSP monomer isolated by HPLC, and TSP polymer containing equimolar capture probes (6.25 μM) were immobilized on aldehyde-functionalized glass slides. Cy3-cDNA (10 nM) in PBS buffer (10 mM phosphate, 1 M NaCl, pH 7.4) was then added to the rectangular fence grids adhering to the slides and incubated at 37 °C for 1 h. After being washed twice with PBST buffer and once with PBS buffer, the N_2-dried slides were scanned by a Genepix 4100A microarray fluorescent scanner. The collected fluorescent signals were analyzed with *GenePix Pro 6.1* software. For DNA tetrahedron microarrays based on different concentrations of TSP, 4 and 12.5 μM, TSP monomer was printed to hybridize with 10 nM Cy5-cDNA. Also, a series of concentrations of Cy5-cDNA (5 nM, 500 pM, 50 pM, 5 pM, and 500 fM) were measured to evaluate the sensitivity of DNA tetrahedron microarrays for direct DNA hybridization. The concentration of DNA tetrahedron (TSP monomer) immobilized on a glass surface was 12.5 μM.

For TSP microarray-based streptavidin−biotin recognition, tetra-A-biotin (80-nt) modified with biotin at the 5′ end and amino-modified single strands (tetra-B, tetra-C, and tetra-D) were annealed to form TSP, named Am-Tet-Biotin. 12.5 μM Am-Tet-Biotin was spotted onto aldehyde-functionalized glass slides to fabricate DNA tetrahedron-based microarrays. 5% bovine serum albumin (BSA) in PBS was used as a blocking solution. 100 μg/mL streptavidin in a spotting buffer was then printed onto DNA tetrahedron-array spots using the micro-arrayer. The slides were incubated at room temperature for 1 h in a humidity chamber. After the slides were washed twice with PBST buffer and once with PBS buffer, biotinylated Cy3 (2.5 μg/mL) was added to the slides and incubated at room temperature for 1 h. After washing twice with PBST buffer and once with PBS buffer, the slides were scanned with a Genepix 4100A microarray fluorescent scanner. For direct immobilization of streptavidin, 100 μg/mL streptavidin in a spotting buffer was directly printed onto aldehyde-functionalized glass slides, followed by the blocking step with 5% BSA in PBS and the binding of biotinylated Cy3 (2.5 μg/mL). The collected fluorescent signals were analyzed with *GenePix Pro 6.1* software.

miRNA Assay. All of the buffers in the microarray-based miRNA assays were prepared with RNase-free water. Four strands—tetra-A-let-7a (75-nt), tetra-B, tetra-C, and tetra-D—were annealed to form DNA tetrahedra, which were used to fabricate microarrays for miRNA assays. The concentration of immobilized DNA tetrahedra was 12.5 μM. The mixture containing let-7a miRNA at variable concentrations (100 pM, 10 pM, 1 pM, 100 fM, and 10 fM), let-7a-hp (50 nM), and let-7a-sp (100 nM) was heated to 80 °C for 5 min and then cooled to room temperature over 10 min. The resulting samples were added to the rectangular fence grids adhering to the slides and incubated at 42 °C for 30 min. After being washed twice with PBST buffer and once with PBS buffer, the fluorescent signals of the slides were collected with a microarray scanner and analyzed with the software.

Protein Assay. Four strands—tetra-A-biotin, tetra-B, tetra-C, and tetra-D—were annealed to form Am-Tet-Biotin. 12.5 μM Am-Tet-Biotin was printed onto aldehyde-functionalized glass slides, followed by a blocking step using 5% BSA in PBS as the blocking solution. 100 μg/mL streptavidin in a spotting buffer was then spotted onto DNA tetrahedron-array spots and incubated at room temperature for 1 h. After being washing twice with PBST buffer and once with PBS buffer, biotinylated anti-PSA mAb (0.5 mg/mL) was spotted onto the microarrays of a DNA tetrahedron streptavidin complex. The slides were incubated at room temperature for 1 h and washed twice with PBST buffer and once with PBS buffer. Subsequently, a series of PSA samples in PBS buffer at variable concentrations were added to the
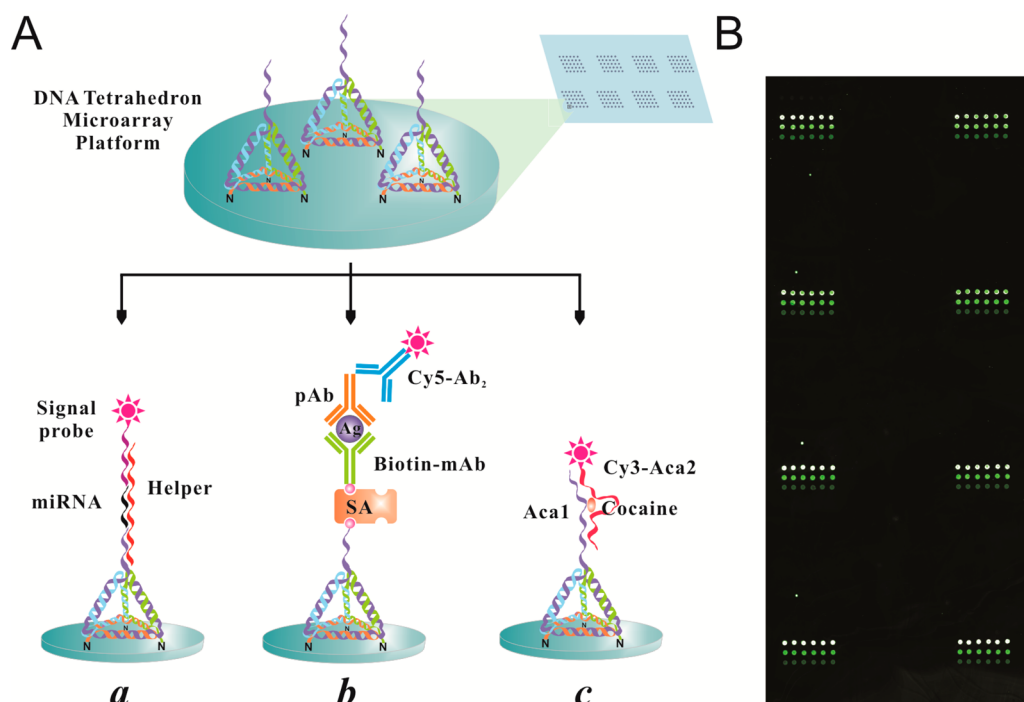
Figure 1. (A) Schematic illustration of the design of a DNA tetrahedron-based microarray platform for multiplex bioanalysis. DNA tetrahedra carrying three amine groups and one ssDNA extension were spotted onto the glass slide to fabricate the microarrays (top). The universal DNA tetrahedron-microarray platform was developed for multiplexing detection of different types of biomolecules (bottom). The gap hybridization strategy (a), the sandwich configuration (b), and the engineering aptamer strategy (c) were employed for the assay of miRNA, PSA, and cocaine, respectively. (B) Microscopic fluorescence images of DNA tetrahedron microarrays (SA, streptavidin; Biotin-mAb, biotinylated monoclonal antibody; Ag, antigen; pAb, polyclonal antibody; Cy5-Ab$_2$, Cy5-labeled secondary antibody; Aca1, anticocaine aptamer 1; Cy3-Aca2, Cy3-labeled anticocaine aptamer 2).

rectangular fence grids adhering to the slides and incubated at 37 °C for 1 h. After washing, anti-PSA pAb (5 μg/mL) was added and incubated at 37 °C for 1 h. The slides were washed twice with PBST buffer and once with PBS buffer, followed by the addition of Cy5-labeled goat antirabbit Ab$_2$ (2.5 μg/mL). After being incubated at 37 °C for 1 h, the slides were washed three times and scanned with a microarray scanner.

Cocaine Assay. In cocaine assay, we engineered the anticocaine aptamer (Aca) by splitting it into two fragments: Aca1 and Aca2. Tetra-A-cocaine containing Aca1 sequence (78-nt), tetra-B, tetra-C, and tetra-D were annealed to form DNA tetrahedra. 12.5 μM DNA tetrahedron carrying Aca1 was spotted onto aldehyde-functionalized glass slides to fabricate DNA tetrahedron microarrays. Samples in phosphate buffer (PB) including cocaine at variable concentrations (1 mM, 100 μM, 10 μM, 1 μM, and 100 nM) and Cy3-labeled Aca2 (Cy3-Aca2; 100 nM) were heated to 80 °C for 5 min and cooled to room temperature over 10 min. The samples were then added to the rectangular fence grids adhering to the slides and incubated at room temperature for 30 min. After washing twice with PBST buffer and once with PBS buffer, the fluorescent signals of the slides were collected with a microarray scanner and analyzed with the software.

Multiplex Detection of Cancer Protein Markers in Human Serum. Several addressable DNA tetrahedron microarrays were first fabricated. Cy3-linked DNA tetrahedra were prepared by annealing tetra-A-Cy3, tetra-B, tetra-C, and tetra-D, termed m-Tet-Cy3. Am-Tet-Cy3, Am-Tet-DNA, and Am-Tet-Biotin (12.5 μM each) were immobilized on aldehyde-functionalized glass slides followed by a blocking step using 5% BSA in PBS. Am-Tet-Cy3 (row 1) and Am-Tet-DNA (row 2) acted as positive and negative controls, respectively. Am-Tet-Biotin (rows 3 and 4) was used to immobilize streptavidin for the detection of cancer markers. 100 μg/mL streptavidin in a spotting buffer was then printed onto DNA tetrahedron-array spots (rows 2−4) using a microarrayer. The slides were incubated at room temperature for 1 h in a humidity chamber. After washing, biotinylated anti-AFP

mAb (0.5 mg/mL) was spotted onto the dots of rows 2 and 3. Biotinylated anti-PSA mAb (0.5 mg/mL) was spotted onto the dots of row 4. The slides were incubated at room temperature for 1 h and washed twice with PBST buffer and once with PBS buffer. Subsequently, serum sample 1 only containing PSA (2.68 ng/mL), serum sample 2 only containing AFP (5.9 ng/mL), mixed serum sample 3 containing PSA (1.84 ng/mL) and AFP (2.95 ng/mL), and serum sample 4 (blank control) were added to the rectangular fence grids adhering to the slides. The slides were incubated at 37 °C for 1 h and washed three times. Then the mixture of anti-PSA pAb (5 μg/mL) and anti-AFP pAb (5 μg/mL) was added and incubated at 37 °C for 1 h. After being washed three times, Cy5-labeled goat antirabbit Ab$_2$ (2.5 μg/mL) was added and incubated at 37 °C for 1 h. The slides were washed three times and scanned with a microarray scanner.

■ RESULTS AND DISCUSSION

Fabrication of DNA Tetrahedron Microarrays on Glass Substrates. A DNA tetrahedron was formed by annealing one DNA fragment (tetra-A, 75−80 nucleotides, nt) and three amine-modified DNA fragments (tetra-B, tetra C, and tetra D, 55-nt). Once assembled, the DNA tetrahedron contained one ssDNA extension at one vertex and three amine groups at the other three vertices for its immobilization on the glass surface. This tetrahedron was termed Am-Tet-DNA to reflect its composition of amine groups and ssDNA extension. It was worth noting that the tetrahedron assembly process was extremely fast (within 2 min) and simple with a high yield of over 85%.[28] The raw self-assembled DNA tetrahedra were isolated and purified by HPLC. The main contents isolated from HPLC were further identified as TSP monomers and polymers by using native PAGE. Having established DNA tetrahedra with amine groups, we covalently immobilized DNA
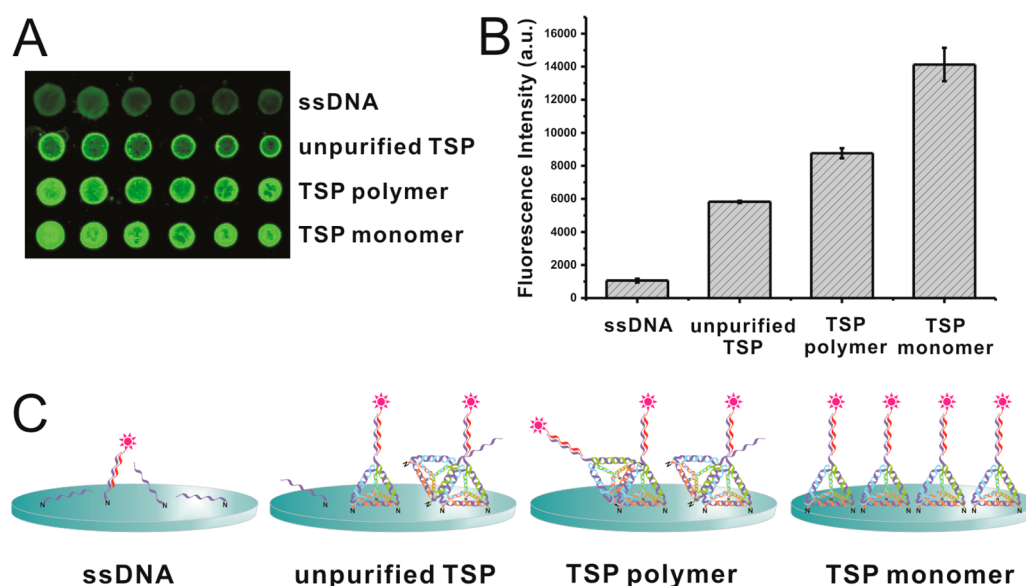
17946

dx.doi.org/10.1021/am5047735 | ACS Appl. Mater. Interfaces 2014, 6, 17944−17953

Figure 2. (A) Microscopic fluorescence image of four microarrays, using ssDNA, unpurified TSP, TSP polymer, and TSP monomer for DNA hybridization. All four probes contained an equimolar capture probe (6.25 μM). TSP polymer and monomer were obtained via HPLC purification. The complementary ssDNA labeled with Cy3 (10 nM) acted as a fluorescent reporter. (B) Quantitative comparison of the DNA hybridization ability of microarrays based on ssDNA, unpurified TSP, TSP polymer, and TSP monomer. (C) Schematic illustration of possible arrangements for ssDNA, unpurified TSP, TSP polymer, and TSP monomer on microarrays.

tetrahedra onto glass slides in an array manner to fabricate DNA tetrahedron-based microarrays (Figure 1A). Three amine groups acted as anchors to react covalently with the aldehyde groups on glass slides, leading to strong and rigid fixing of DNA tetrahehra. We validated the immobilization model by using a fluorophore-contained DNA tetrahedron, named Am-Tet-Cy3, where a fluorophore was premodified at the end of the ssDNA extension. Homogeneous and reproducible fluorescent signals (Figure 1B) were observed with a microarray fluorescent scanner when we spotted different concentrations of Am-Tet-Cy3 on the glass slides. The fluorescent intensity decreased gradually as the Am-Tet-Cy3 concentration decreased (12.5, 4, and 1 μM).

Several efforts have been made recently to fix DNA tetrahedra on solid substrates for biorecognition and biosensing applications, which are dependent on the principle of the Au−S bond linkage.[23−28] However, they still suffer from some tough problems such as the high cost of gold substrates and a complicated fabrication process. In particular, gold substrates greatly restrict their practical use in the field of fluorescent microarrays, as a result of the intrinsic property of gold as the superquencher of fluorophores.[30] More recently, patterned and biofunctionalized surfaces have been created by combining the bottom-up fabrication of DNA tetrahedra with the top-down fabrication of patterned gold surfaces.[27] Although nanoscale DNA tetrahedra on patterned gold surfaces showed improved biorecognition capability, more than 90% of the fluorescent signals were quenched by gold. Because of distortion resulting from the distance-dependent quenching between fluorophores and the gold surface, the surface fluorescent densities had to be determined via the cumbersome desorption of fluorophores and additional quantitation procedures. Immobilization of DNA tetrahedra on microarrays in our work was achieved via the covalent coupling of amine groups to aldehyde groups on glass slides. The glass substrates enabled direct microscopic imaging of fluorescent signals. Thus, the biorecognition events on tetrahedron microarrays could be directly evaluated by

quantifying their microscopic fluorescent signals. In addition, such glass slides are low-cost and commercially available. We estimated that the glass slides can reduce the cost by 90% compared with the same-sized gold substrates.

Biorecognition Capability of DNA Tetrahedron Microarrays. Having established DNA tetrahedron-anchored microarrays on glass substrates, we evaluated their performance for immobilizing capture biomolecules to improve biorecognition. First, we investigated the ability of DNA tetrahedron-based microarrays to anchor capture ssDNA probes for DNA hybridization. We fabricated four kinds of microarrays, using ssDNAs, unpurified TSP, TSP polymers, and TSP monomers. The ssDNA extensions at one vertex of these TSPs acted as inherent capture ssDNA probes to recognize and hybridize with their Cy3 fluorophore-labeled complementary ssDNA (Cy3-cDNA) at the same concentration. The microscopic fluorescence image is shown in Figure 2A. We found that the TSP-monomer-based microarray showed the strongest fluorescent signals. The quantitative results suggested that its signals were approximately 14 times as strong as that of the ssDNA probe microarray (Figure 2B; 14131 ± 1008 versus 1059 ± 116 fluorescence counts). This means that the extension probe tethered at the vertex of the DNA tetrahedron could specifically capture approximately 14 times the amount of its DNA targets than the ssDNA probe. Also, the fluorescent signal of the TSP-monomer-based microarray was much stronger than that of other TSP-based microarrays.

Such strong signals of the TSP-monomer-based microarray indicated its high performance for DNA hybridization. On the basis of the comparison of the signals, we think that the ssDNA capture probes on different microarrays may have different arrangements (Figure 2C). TSP monomers can render the ssDNA probe with a solution-phase-like environment because of its highly ordered arrangement on the glass surfaces. The pyramidal structure of DNA tetrahedra can provide the ssDNA probe with upright consistent orientation and well-defined spacing.[23,24] However, linear ssDNA probes may be inclined to
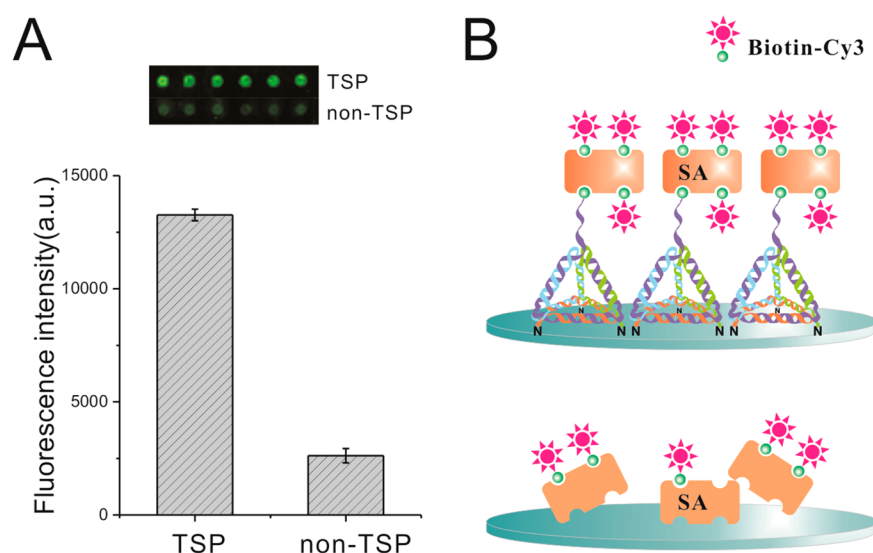
Figure 3. (A) Quantitative comparison of DNA tetrahedron-based streptavidin microarrays (TSP) and microarrays with streptavidin only (non-TSP) for biorecognition. Inset: Microscopic fluorescence images. In the DNA tetrahedron-based streptavidin microarrays, DNA tetrahedra carrying biotin moieties were employed to immobilize the streptavidin, followed by the binding of biotinylated Cy3 (Biotin-Cy3). For the microarrays with streptavidin only, streptavidin was spotted onto the glass slide directly. The concentration of streptavidin in both strategies was 100 μg/mL. (B) Scheme of possible biorecognition models for DNA tetrahedron-based streptavidin microarrays (top) and microarrays with streptavidin only (bottom).

lie flat on the glass surfaces, leading to much lower hybridization efficiency.[31] The aggregation of TSPs may result in a reduction of the hybridization efficiency because of steric hindrance and electrostatic interaction.

Different concentrations of Am-Tet-DNAs (4 μM and 12.5 μM) were tested to hybridize with a defined concentration of Cy5 fluorophore-labeled complementary ssDNA (Cy5-cDNA; 10 nM). We found that 12.5 μM Am-Tet-DNA showed stronger microscopic fluorescent signals than 4 μM Am-Tet-DNA, indicating that the former captured approximately 2.3-fold more Cy5-cDNA than the latter (Figure S1A in the SI; 9040 ± 261 vs 3879 ± 121 fluorescence counts). This result demonstrated that the DNA hybridization capability of DNA tetrahedron microarrays could be regulated by changing the concentration of the DNA tetrahedron receptors. Also, we tested a series of concentrations of complementary ssDNA (cDNA) targets (5 nM to 500 fM) to evaluate the sensitivity of DNA tetrahedron microarrays for direct DNA hybridization (Figure S1B in the SI). The fluorescent signals decreased gradually with the concentration of the cDNA targets. Quantitative analysis revealed a good response of the fluorescence intensities to the concentrations of the cDNA targets. The DNA tetrahedron microarrays could recognize as low as 500 fM cDNA. The improved hybridization efficiency should be due to the highly ordered arrangement of purified DNA tetrahedron monomers on the glass surfaces and the elimination of fluorescence quenching.

Streptavidin and biotin, a typical ligand−receptor pair, were also chosen to examine the ability of DNA tetrahedron-anchored microarrays for enhanced biorecognition. The DNA tetrahedron containing a biotin tethered by an ssDNA extension, named Am-Tet-Biotin, was exploited to bind to streptavidin, followed by the binding of biotinylated Cy3. We found that DNA tetrahedron-based microarrays featured much stronger fluorescence signals than microarrays with streptavidin only (Figure 3A). The quantitative comparison suggested that the former could capture biotinylated Cy3 5-fold more than the

latter (13264 ± 255 vs 2620 ± 321 fluorescence counts). Previous studies had demonstrated that highly ordered DNA tetrahedra could provide biotin moieties with well-defined nanoscale spacing to accommodate streptavidin molecules.[23] Therefore, all individual biotin moieties tethered on the DNA tetrahedra would bind to streptavidin molecules so that streptavidin could be orderly immobilized away from the glass surfaces. Thus, its remaining binding sites toward biotin were fully exposed to the ambient, leading to high-efficiency capture of biotinylated Cy3 (Figure 3B). In contrast, streptavidin directly immobilized in a random fashion on the glass surfaces may undergo complications and steric hindrance, resulting in the loss of binding capability (Figure 3B).[32] Importantly, the DNA tetrahedron could serve as engineered bridges that effectively eliminate the chemical damage and avoid the loss of the binding activity due to the directly covalent coupling of streptavidin with glass surface.[9] Furthermore, while antibodies are linked to the streptavidin layer, the DNA tetrahedron bridges can provide them with a specific orientation away from the surfaces. Thus, the antigen binding efficiency can also be greatly improved.

Universal DNA Tetrahedron-Microarray Platform. Having demonstrated that the DNA tetrahedron microarrays can immobilize nucleic acids and proteins for improved biorecognition, we developed a universal tetrahedron-microarray platform for multiplex detection of different types of bioactive analytes including miRNAs, proteins, and small molecules. let-7a miRNA (a promising cancer biomarker), PSA (an important protein biomarker of prostate cancer), and cocaine (a recreational drug) were chosen as target molecules.[33−37] Figure 1A schematically illustrates the design of universal DNA tetrahedron-based microarrays for these target analytes. We designed a specific detection strategy for each type of analyte to get the best assay performance. These strategies were integrated onto a piece of glass slide and performed independently by using rectangular fence grids. Thus, addressable and high-throughput analysis had been
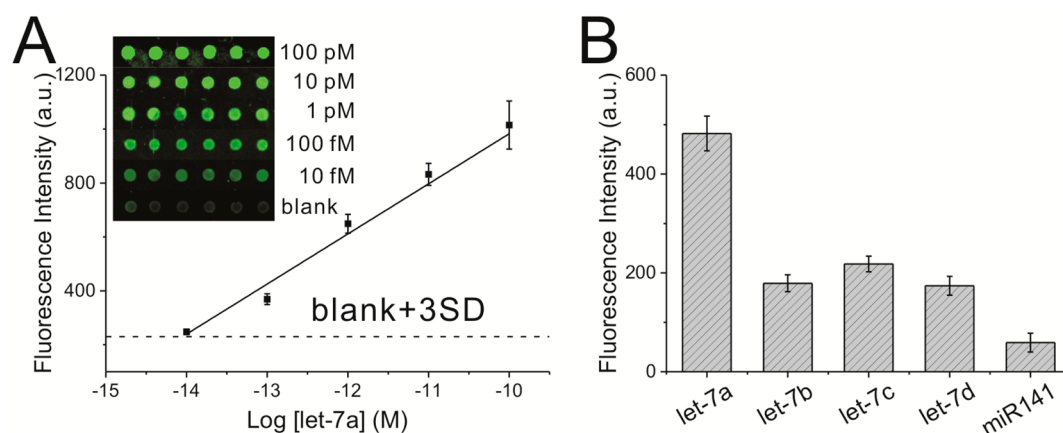
Figure 4. (A) DNA tetrahedron-based microarrays for the let-7a assay. The concentration of let-7a ranged from 10 fM to 100 pM. The linear fitting equation was $y = 185.80x + 2840.73 \pm 134.35$, and R^2 was 0.961. The dashed lines stand for the threshold (blank + 3SD). Inset: Microscopic fluorescence images of different concentrations of let-7a samples. (B) Specificity of DNA tetrahedron-based microarrays for the let-7a assay. The concentration of all of the miRNAs was 1 pM. The error bars represented the SD of 18 spots from three independent measurements.
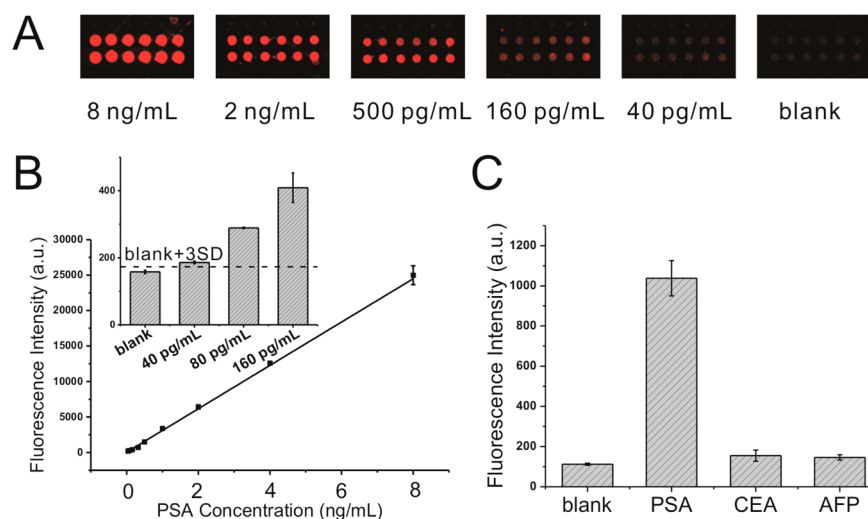

Figure 5. (A) Microscopic fluorescence images of DNA tetrahedron-based microarrays for PSA assay. (B) Linear calibration curve of PSA assay with DNA tetrahedron-based microarrays. The linear fitting equation was $3.06x + 47.01 \pm 2.47$, and R^2 was 0.989. Inset: Histogram showing the LOD of PSA assay. The dashed lines stand for the threshold (blank + 3SD). (C) Selectivity tests of DNA tetrahedron-based microarrays for PSA (500 pg/mL). Negative controls were CEA and AFP (1 μg/mL each).

achieved for the simultaneous detection of three different types of analytes.

For the miRNA assay, a gap hybridization strategy was employed because of its simplicity and high sensitivity (Figure 1A-a).[38] In this design, the tetrahedron containing one capture probe is immobilized onto the glass surface. In the presence of miRNA targets, miRNA binds to a gap built of a capture probe, a helper probe, and a signal probe. The complementary binding favors hybridization of all four of these components and brings the fluorophore to the vicinity of the surface, showing fluorescence signals. In the absence of miRNA targets, the gap between the capture and signal probes is not filled. The missing base-stacking energy destabilizes the DNA hybridization complex, showing no signals.[38]

let-7a miRNA, an important serologic cancer biomarker, was chosen as the target.[33] As shown in Figure 4A, the fluorescence intensity increased gradually as the miRNA concentration increased from 10 fM to 100 pM, while the blank control only showed low background signals. It is notable that the DNA tetrahedron-microarray assay for let-7a miRNA had a broad

dynamic range (4 decades) and a good log−linear range from 10 fM to 100 pM. The limit of detection (LOD) was determined as the lowest detected concentration whose signal was higher than the threshold equal to the blank control signal plus three standard deviations (3SD). The LOD of the assay for let-7a was 10 fM, which was 200 times more sensitive than the ssDNA-based gap hybridization assay reported previously.[38] This improvement was due to the consistently favorable orientation of DNA tetrahedra on glass surfaces, which could avoid possible steric crowding and electrostatic interaction often encountered by soft ssDNA probes.[24] Of note, this LOD is even lower than several reported methods with significant signal amplification techniques.[39−41] We ascribe the high sensitivity to inherently strong base stacking of the gap hybridization assay, leading to a high capture efficiency of miRNA on microarrays, as well as the use of a DNA tetrahedron, providing capture probe a solution-phase-like environment to enhance the biorecognition ability. The selectivity of the assay for detection of let-7a was tested using a family of human let-7 sequences and a noncognate sequence

(miRNA-141) as negative controls (Figure 4B). We found that let-7a could be distinguished clearly from other negative controls even though there was only one or two base mismatches. This high mismatch discrimination ability demonstrated that the DNA tetrahedron-microarray assay for miRNA has high specificity.

We combined a typical sandwich configuration with the DNA tetrahedron microarray for the detection of PSA (Figure 1A-b). Am-Tet-biotin was covalently coupled onto glass slides, followed by the binding of streptavidin. Biotinylated anti-PSA monoclonal antibody (mAb) then was specifically bound to streptavidin for the capture of PSA. After anti-PSA polyclonal antibody (pAb) was bound to PSA, a fluorophore-labeled secondary antibody (Ab$_2$) was involved to generate fluorescence signals.

The results of the DNA tetrahedron-microarray platform for the highly sensitive and selective PSA assay are shown in Figure 5. We challenged the microarrays with a series of diluted samples (40 pg/mL to 8 ng/mL PSA). AFP and CEA were selected as negative controls. Figure 5A presented the fluorescence images of the subarrays. The fluorescent intensities decreased concomitantly with decreasing PSA concentration, while the blank control showed negligible background signals. The intensities were analyzed to generate a dose−response curve (Figure 5B). A good linear response was achieved in the concentration range. The regression equation was $y = 3.06x + 47.01 \pm 2.47$, and R^2 was 0.989. The LOD of the PSA assay was 40 pg/mL (Figure 5B, inset), which was 25 times lower than that of conventional streptavidin-based assay (1 ng/mL; Figure S2 in the SI). We ascribed this improvement to the high-efficiency, intact, and orienting immobilization of the biotinylated antibody, which benefited from Am-Tet-biotin. We also investigated the specificity of the DNA tetrahedron-microarray assay for PSA (Figure 5C). Control experiments revealed that 500 pg/mL PSA could be detected very clearly, while 1 μg/mL negative samples showed no significant difference from the blank control, indicating that the DNA tetrahedron-microarray assay for PSA has good specificity.

The detection of cocaine was achieved by using its engineering aptamers combined with the tetrahedron-microarray platform (Figure 1A-c).[42,43] We engineered the single-stranded Aca by splitting it into two fragments: Aca1 and Aca2. Aca1 was appended to the DNA tetrahedron as the capture probe, while Aca2 was labeled with a fluorophore to produce signals. After immobilization of the DNA tetrahedron, Aca1 and Aca2 could be glued to form a sandwich complex in the presence of cocaine.

The sensitivity and specificity of the DNA tetrahedron microarray for cocaine are shown in Figure 6A,B, respectively. The quantitative fluorescence signals increased proportionally with the increasing concentration of cocaine (100 nM to 1 mM). The log−linear fitting equation was $y = 38.67x + 336.13 \pm 18.88$, and R^2 was 0.982. The lowest concentration of cocaine detected was 100 nM. This high sensitivity is comparable to that of the DNA tetrahedron-based electrochemical cocaine sensor we previously reported.[26] In the control experiments, benzoylecgonine (BE) and methylecgoine (ME), two cocaine analogues, were selected to test the specificity of the microarray assay. As shown in Figure 6B, as low as 100 μM cocaine could be clearly distinguished with 1 mM BE, 1 mM ME, and the blank control, demonstrating that the split aptamer remained selective of the original aptamers.
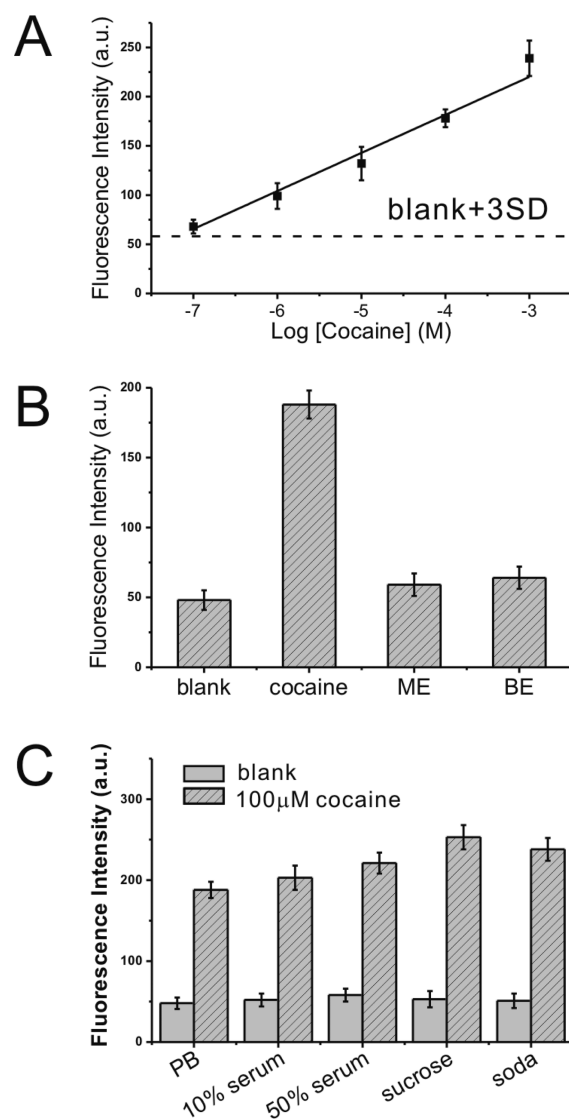

Figure 6. (A) Detection of cocaine using DNA tetrahedron-based microarrays. Samples containing cocaine varying from 100 nM to 1 mM were tested. The log−linear fitting equation was $y = 38.67x + 336.13 \pm 18.88$, and R^2 was 0.982. The dashed lines stand for the threshold (blank + 3SD). (B) Specificity tests of tetrahedron-based microarrays, using cocaine (100 μM) and two analogues (ME and BE, 1 mM each). (C) Tetrahedron-based microarrays for cocaine assay in several media. From left to right: 100 μM cocaine in PB, 100 μM cocaine adulterated in 10% and 50% serum, and 100 μM cocaine (1:1 m/m) "masked" with sucrose and soda.

Multiplex Detection in Human Serum. Having demonstrated the feasibility and versatility of the DNA tetrahedron-microarray platform for the assay of various analytes, we further evaluated its feasibility and capability for the detection of targets in human serum and its multiplexing ability. First of all, the DNA tetrahedron microarrays were challenged with various cocaine-containing media that were critical to their practical application (Figure 6C). Considering that the DNA tetrahedron-decorated surface was resistant to protein adsorption, the microarray assay was performed for the detection of 100 μM cocaine in 10% serum. We found that the background signal in serum was only slightly larger than that in pure PB. Moreover, the DNA tetrahedron microarrays could work well with 50% serum. Similarly, the microarrays could be effectively used for
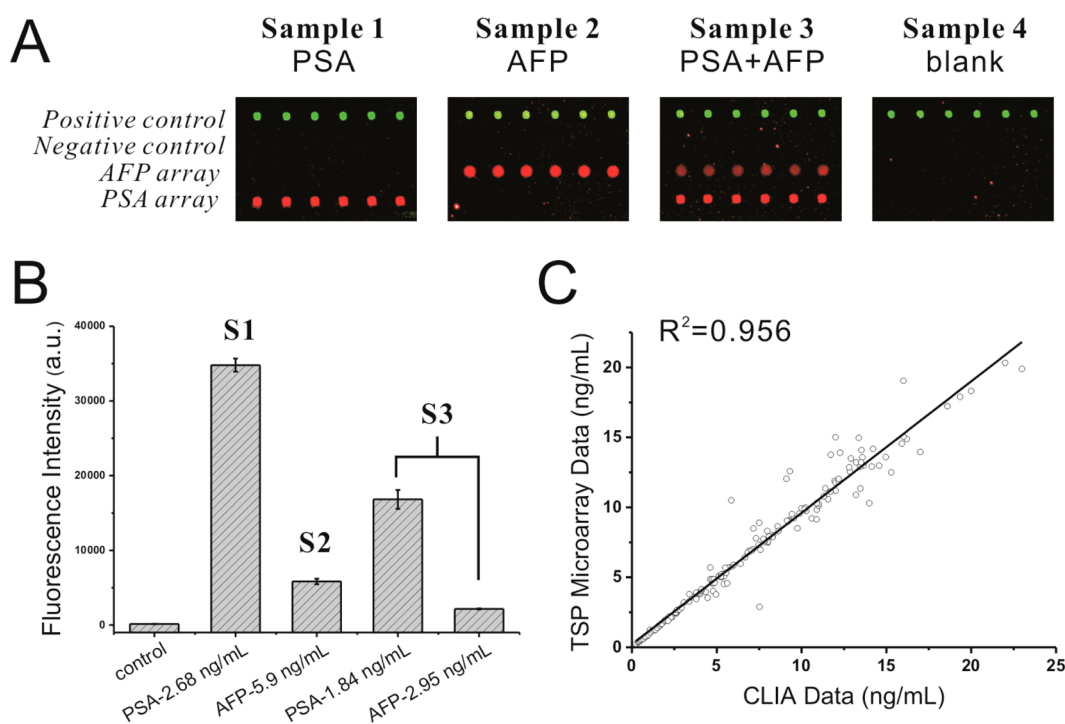
Figure 7. (A) Microscopic fluorescence images of DNA tetrahedron-based addressable microarrays for multiplex detection of PSA and AFP in serum samples. Am-Tet-Cy3 and Am-Tet-DNA were employed to act as positive and negative controls, respectively. AFP and PSA arrays were obtained by sequentially spotting Am-Tet-biotin, streptavidin, and biotinylated mAb onto the glass surface. Sample 1 (S1) containing PSA, sample 2 (S2) containing AFP, sample 3 (S3) containing PSA and AFP, and sample 4 from a healthy individual were tested. (B) Quantitative results of all three positive samples and all negative controls. (C) Relativity analysis of DNA tetrahedron-based microarrays data compared with CLIA data. A total of 159 serum samples from patients were tested. The relativity equation was $y = 1.02x + 0.10$, and R^2 was 0.956.

the detection of cocaine in tainted samples (with sucrose or soda) with minimal perturbation of its performance.

Subsequently, PSA and AFP in serum samples were simultaneously tested by using a typical sandwich configuration and DNA tetrahedron microarrays. To test the specific binding between Am-Tet-Biotin and streptavidin, we employed Am-Tet-DNA to act as the negative control. Am-Tet-Cy3 was used as the positive control.

DNA tetrahedron, streptavidin, and biotinylated mAb were spotted onto the glass substrates to fabricate addressable microarrays (Figure 7A). Biotinylated anti-AFP mAb and biotinylated anti-PSA mAb were employed for AFP and PSA arrays, respectively. The microarrays were first challenged with the serum sample only containing PSA (sample 1, 2.86 ng/mL). As expected, only the PSA array showed strong microscopic fluorescence signals of Cy5, while both the AFP array and negative control showed no signals. Similarly, when the serum sample only contained AFP (sample 2, 5.9 ng/mL), we could only observe the Cy5 fluorescence signals from the AFP array rather than the PSA array and other negative controls. The negative controls of Am-Tet-DNA did not generate any signals, indicating the excellent binding specificity between streptavidin and Am-Tet-Biotin and a low background signal of the tetrahedron microarrays. For the detection of PSA or AFP, the microarrays exhibited good sensitivity and high specificity.

Importantly, the microarrays were challenged with the serum sample containing PSA and AFP even at lower concentrations (sample 3). 1.84 ng/mL PSA and 2.95 ng/mL AFP in serum could be clearly detected. The serum sample from a healthy individual served as the blank control and showed no

fluorescence signals, suggesting that the DNA tetrahedron microarrays have satisfactory sensitivity and high selectivity for multiplex proteins analysis in real-life samples. The microarrays herein showed a high signal-to-noise ratio owing to the good resistance of the DNA tetrahedron-decorated glass surface to interferential proteins in serum. The quantitative results (Figure 7B) indicated that the fluorescence signals of all three positive samples were much stronger than those of all negative controls.

To evaluate the relativity of the DNA tetrahedron microarrays, we compared them with conventional chemical luminescent immunoassay (CLIA) by testing serum the PSA levels of a number of patients. As shown in Figure 7C, 159 serum samples from patients were analyzed using DNA tetrahedron-based microarrays and CLIA. There was a good correlation between the DNA tetrahedron-microarray assays and CLIA with a R^2 value of 0.956, demonstrating that the microarray platform holds great promise for clinical diagnosis.

■ CONCLUSIONS

In conclusion, we have developed a novel approach for the fabrication of microarrays by covalently coupling rationally designed DNA tetrahedra onto glass surfaces. We demonstrated its excellent capability of biorecognition for DNA hybridization and streptavidin—biotin binding and further developed a universal and multiplex platform for the highly sensitive and selective detection of different types of bioactive molecules including miRNAs, proteins, and small molecules. The DNA tetrahedron-microarray platform has several advantages. First, to our knowledge, this is the first example for immobilizing DNA tetrahedra on the glass surfaces to fabricate microarrays. The DNA tetrahedron bridges on microarrays could effectively

arrange the capture probes with specific orientation and well-defined spacing and provide them with a solution-phase-like environment, improving the biorecognition capabilities of the microarrays for various bioactive molecules. Furthermore, the microarray platform is expected to be used for the detection of many other targets because of its universality and versatility. Second, the glass-surface-based DNA tetrahedron microarrays enable the direct use of fluorescent probes, greatly expanding their scope of application compared with gold surfaces. Third, the DNA tetrahedron-microarray platform is competent for the assays in biological fluids because of its high protein-resistance ability. In addition, it is low-cost and is expected to be commercialized in the future. Thus, we believe that the DNA tetrahedron-microarray platform holds great promise for life science and biomedicine.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Comparison of different concentrations of DNA tetrahedron-immobilized microarrays for DNA hybridization, detection of PSA with conventional streptavidin-based microarrays, and the sequences of oligonucleotides involved in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: spsong@sinap.ac.cn. Tel.: +86 21 39194065. Fax: +86 21 39194702.

Author Contributions
‡Z.L. and B.Z. contributed equally.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Schena, M.; Shalon, D.; Davis, R. W.; Brown, P. O. Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray. *Science* **1995**, *270*, 467−470.
(2) Kononen, J.; Bubendorf, L.; Kallionimeni, A.; Barlund, M.; Schraml, P.; Leighton, S.; Torhorst, J.; Mihatsch, M. J.; Sauter, G.; Kallionimeni, O.-P. Tissue Microarrays for High-throughput Molecular Profiling of Tumor Specimens. *Nat. Med.* **1998**, *4*, 844−847.
(3) MacBeath, G.; Schreiber, S. L. Printing Proteins as Microarrays for High-Throughput Function Determination. *Science* **2000**, *289*, 1760−1763.
(4) MacBeath, G. Protein Microarrays and Proteomics. *Nat. Genet.* **2002**, *32*, 526−532.
(5) Heller, M. J. DNA Microarray Technology: Devices, Systems, and Applications. *Annu. Rev. Biomed. Eng.* **2002**, *4*, 129−153.
(6) Sassolas, A.; Leca-Bouvier, B. D.; Blum, L. J. DNA Biosensors and Microarrays. *Chem. Rev.* **2007**, *108*, 109−139.
(7) Nie, S.; Benito-Peña, E.; Zhang, H.; Wu, Y.; Walt, D. R. Multiplexed Salivary Protein Profiling for Patients with Respiratory Diseases Using Fiber-Optic Bundles and Fluorescent Antibody-Based Microarrays. *Anal. Chem.* **2013**, *85*, 9272−9280.

(8) Oh, S. J.; Hong, B. J.; Choi, K. Y.; Park, J. W. Surface Modification for DNA and Protein Microarrays. *OMICS* **2006**, *10*, 327−343.
(9) Peluso, P.; Wilson, D. S.; Do, D.; Tran, H.; Venkatasubbaiah, M.; Quincy, D.; Heidecker, B.; Poindexter, K.; Tolani, N.; Phelan, M.; Witte, K.; Jung, L. S.; Wagner, P.; Nock, S. Optimizing Antibody Immobilization Strategies for the Construction of Protein Microarrays. *Anal. Biochem.* **2003**, *312*, 113−124.
(10) Chen, S.; Liu, L.; Zhou, J.; Jiang, S. Controlling Antibody Orientation on Charged Self-Assembled Monolayers. *Langmuir* **2003**, *19*, 2859−2864.
(11) Hong, B. J.; Oh, S. J.; Youn, T. O.; Kwon, S. H.; Park, J. W. Nanoscale-Controlled Spacing Provides DNA Microarrays with the SNP Discrimination Efficiency in Solution Phase. *Langmuir* **2005**, *21*, 4257−4261.
(12) Hong, B. J.; Sunkara, V.; Park, J. W. DNA Microarrays on Nanoscale-Controlled Surface. *Nucleic Acids Res.* **2005**, *33*, e106.
(13) Kausaite-Minkstimiene, A.; Ramanaviciene, A.; Kirlyte, J.; Ramanavicius, A. Comparative Study of Random and Oriented Antibody Immobilization Techniques on the Binding Capacity of Immunosensor. *Anal. Chem.* **2010**, *82*, 6401−6408.
(14) Trilling, A. K.; Beekwilder, J.; Zuilhof, H. Antibody Orientation on Biosensor Surfaces: A Minireview. *Analyst* **2013**, *138*, 1619−1627.
(15) Wei, B.; Dai, M.; Yin, P. Complex Shapes Self-Assembled from Single-Stranded DNA Tiles. *Nature* **2012**, *485*, 623−626.
(16) Nykypanchuk, D.; Maye, M. M.; van der Lelie, D.; Gang, O. DNA-Guided Crystallization of Colloidal Nanoparticles. *Nature* **2008**, *451*, 549−552.
(17) Auyeung, E.; Li, T. I. N. G.; Senesi, A. J.; Schmucker, A. L.; Pals, B. C.; de la Cruz, M. O.; Mirkin, C. A. DNA-Mediated Nanoparticle Crystallization into Wulff Polyhedra. *Nature* **2014**, *505*, 73−77.
(18) Zhang, Z.; Wang, Y.; Fan, C.; Li, C.; Li, Y.; Qian, L.; Fu, Y.; Shi, Y.; Hu, J.; He, L. Asymmetric DNA Origami for Spatially Addressable and Index-Free Solution-Phase DNA Chips. *Adv. Mater.* **2010**, *22*, 2672−2675.
(19) Rothemund, P. W. K. Folding DNA to Create Nanoscale Shapes and Patterns. *Nature* **2006**, *440*, 297−302.
(20) Seeman, N. C. DNA in a Material World. *Nature* **2003**, *421*, 427−431.
(21) Goodman, R. P.; Berry, R. M.; Turberfield, A. J. The Single-Step Synthesis of a DNA Tetrahedron. *Chem. Commun.* **2004**, 1372−1373.
(22) Goodman, R. P.; Schaap, I. A. T.; Tardin, C. F.; Erben, C. M.; Berry, R. M.; Schmidt, C. F.; Turberfield, A. J. Rapid Chiral Assembly of Rigid DNA Building Blocks for Molecular Nanofabrication. *Science* **2005**, *310*, 1661−1665.
(23) Mitchell, N.; Schlapak, R.; Kastner, M.; Armitage, D.; Chrzanowski, W.; Riener, J.; Hinterdorfer, P.; Ebner, A.; Howorka, S. A DNA Nanostructure for the Functional Assembly of Chemical Groups with Tunable Stoichiometry and Defined Nanoscale Geometry. *Angew. Chem., Int. Ed.* **2009**, *48*, 525−527.
(24) Pei, H.; Lu, N.; Wen, Y. L.; Song, S. P.; Liu, Y.; Yan, H.; Fan, C. H. A DNA Nanostructure-Based Biomolecular Probe Carrier Platform for Electrochemical Biosensing. *Adv. Mater.* **2010**, *22*, 4754−4758.
(25) Wen, Y. L.; Pei, H.; Shen, Y.; Xi, J. J.; Lin, M. H.; Lu, N.; Shen, X. Z.; Li, J.; Fan, C. H. DNA Nanostructure-Based Interfacial Engineering for PCR-Free Ultrasensitive Electrochemical Analysis of MicroRNA. *Sci. Rep.* **2013**, *2*, 867.
(26) Wen, Y. L.; Pei, H.; Wan, Y.; Su, Y.; Huang, Q.; Song, S. P.; Fan, C. H. DNA Nanostructure-Decorated Surfaces for Enhanced Aptamer-Target Binding and Electrochemical Cocaine Sensors. *Anal. Chem.* **2011**, *83*, 7418−7423.
(27) Schlapak, R.; Danzberger, J.; Armitage, D.; Morgan, D.; Ebner, A.; Hinterdorfer, P.; Pollheimer, P.; Gruber, H. J.; Schäffler, F.; Howorka, S. Nanoscale DNA Tetrahedra Improve Biomolecular Recognition on Patterned Surfaces. *Small* **2012**, *8*, 89−97.
(28) Pei, H.; Wan, Y.; Li, J.; Hu, H. Y.; Su, Y.; Huang, Q.; Fan, C. H. Regenerable Electrochemical Immunological Sensing at DNA Nanostructure-Decorated Gold Surfaces. *Chem. Commun.* **2011**, *47*, 6254−6256.

17952

dx.doi.org/10.1021/am5047735 | *ACS Appl. Mater. Interfaces* 2014, 6, 17944−17953

(29) Ge, Z.; Lin, M.; Wang, P.; Pei, H.; Yan, J.; Shi, J.; Huang, Q.; He, D.; Fan, C.; Zuo, X. Hybridization Chain Reaction Amplification of MicroRNA Detection with a Tetrahedral DNA Nanostructure-Based Electrochemical Biosensor. *Anal. Chem.* **2014**, *86*, 2124−2130.

(30) Dulkeith, E.; Morteani, A. C.; Niedereichholz, T.; Klar, T. A.; Feldmann, J.; Levi, S. A.; van Veggel, F. C. J. M.; Reinhoudt, D. N.; Möller, M.; Gittins, D. I. Fluorescence Quenching of Dye Molecules near Gold Nanoparticles: Radiative and Nonradiative Effects. *Phys. Rev. Lett.* **2002**, *89*, 203002.

(31) Levicky, R.; Herne, T. M.; Tarlov, M. J.; Satija, S. K. Using Self-Assembly to Control the Structure of DNA Monolayers on Gold: A Neutron Reflectivity Study. *J. Am. Chem. Soc.* **1998**, *120*, 9787−9792.

(32) Wacker, R.; Schröder, H.; Niemeyer, C. M. Performance of Antibody Microarrays Fabricated by either DNA-Directed Immobilization, Direct Spotting, or Streptavidin−Biotin Attachment: A Comparative Study. *Anal. Biochem.* **2004**, *330*, 281−287.

(33) Roush, S.; Slack, F. J. The Let-7 Family of MicroRNAs. *Trends Cell Biol.* **2008**, *18*, 505−516.

(34) Oesterling, J. E. Prostate Specific Antigen: A Critical Assessment of the Most Useful Tumor Marker for Adenocarcinoma of the Prostate. *J. Urol.* **1991**, *145*, 907−923.

(35) Mendelson, J. H.; Mello, N. K. Management of Cocaine Abuse and Dependence. *N. Engl. J. Med.* **1996**, *334*, 965−972.

(36) Yan, X.; Cao, Z.; Lau, C.; Lu, J. DNA Aptamer Folding on Magnetic Beads for Sequential Detection of Adenosine and Cocaine by Substrate-Resolved Chemiluminescence Technology. *Analyst* **2010**, *135*, 2400−2407.

(37) Sheng, Q.; Liu, R.; Zhang, S.; Zheng, J. Ultrasensitive Electrochemical Cocaine Biosensor Based on Reversible DNA Nanostructure. *Biosens. Bioelectron.* **2014**, *51*, 191−194.

(38) Pöhlmann, C.; Sprinzl, M. Electrochemical Detection of MicroRNAs via Gap Hybridization Assay. *Anal. Chem.* **2010**, *82*, 4434−4440.

(39) Harcourt, E. M.; Kool, E. T. Amplified MicroRNA Detection by Templated Chemistry. *Nucleic Acids Res.* **2012**, *40*, e65.

(40) Yang, L.; Liu, C.; Ren, W.; Li, Z. Graphene Surface-Anchored Fluorescence Sensor for Sensitive Detection of MicroRNA Coupled with Enzyme-Free Signal Amplification of Hybridization Chain Reaction. *ACS Appl. Mater. Interfaces* **2012**, *4*, 6450−6453.

(41) Yin, B.-C.; Liu, Y.-Q.; Ye, B.-C. Sensitive Detection of MicroRNA in Complex Biological Samples via Enzymatic Signal Amplification Using DNA Polymerase Coupled with Nicking Endonuclease. *Anal. Chem.* **2013**, *85*, 11487−11493.

(42) Zhang, J.; Wang, L. H.; Pan, D.; Song, S. P.; Boey, F. Y. C.; Zhang, H.; Fan, C. H. Visual Cocaine Detection with Gold Nanoparticles and Rationally Engineered Aptamer Structures. *Small* **2008**, *4*, 1196−1200.

(43) Li, F.; Zhang, J.; Cao, X. N.; Wang, L. H.; Li, D.; Song, S. P.; Ye, B. C.; Fan, C. H. Adenosine Detection by Using Gold Nanoparticles and Designed Aptamer Sequences. *Analyst* **2009**, *134*, 1355−1360.