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Identification of proteins bound to a thioaptamer probe on a proteomics array

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Abstract

A rapid method to screen and identify unknown bound proteins to specific nucleic acid probes anchored on ProteinChip array surfaces from crude biological samples has been developed in this paper. It was demonstrated with screening specific binding proteins from LPS-stimulated mouse 70Z/3 pre-B cell nuclear extracts by direct coupling of thioaptamer XBY-S2 to the pre-activated ProteinChip array surfaces. With pre-fractionation of crude nuclear extracts by ion exchange method, specific "on-chip" captured proteins have been obtained that were pure enough to do "on-chip" digestion and the subsequent identification of the "on-chip" bound proteins by microsequencing of the trypsin digested peptide fragments through tandem MS. Five mouse heterogeneous nuclear ribonucleoproteins (hnRNPs) A1, A2/B1, A3, A/B, and D0 were identified. To verify those bound hnRNPs, a novel thioaptamer/antibody sandwich assay provides highly sensitive and selective identification of proteins on ProteinChip arrays.

Keywords: Thioaptamer; Aptamer array; SELDI-TOF-MS; Proteomics; hnRNPs, Transcription factor AP-1; Sandwich assay; DNA-binding; Fractionation

Aptamers are nucleic acid ligands that have been selected against their target molecules from combinatorial oligonucleotide libraries by *in vitro* selection. Aptamers, with high affinity and specificity towards their target molecules such as proteins, as well as their chemical diversities, can be made as aptamer arrays for applications as analytical, diagnostic, and therapeutic tools [1–3]. Thioaptamers are aptamers containing randomly selected thiophosphate backbone substitutions which show enhanced affinity, selectivity and nuclease resistance relative to unmodified nucleic acids [3]. In the past several years, we have identified several phosphoromonothioate thioaptamers [4–7] and two duplex 14-mer phosphorodithioate thioaptamers, XBY-S2 and XBY-6 [3], both with six dithioate linkages in the sequences. XBY-S2 has been shown to have broad spectrum antiviral activity (Herzog et al. unpublished), and it binds several proteins in solution including transcription factor AP-1 (Herzog et al. unpublished).

So far, several different detection methods have been attempted on aptamer arrays, such as fluorescence and mass spectrometry detection [8,9]. However, mass spectrometry was previously limited to only detecting the molecular mass of specific binding protein on the array surfaces [10,11]. In this report, we extend the potential of the aptamer array proteomic technology by demonstrating the direct identification of the bound proteins from the proteome of complex biological samples retained on the proteomics array surfaces. When compared to "off-chip" identification of bound proteins [9], this novel "on-chip" analysis method offers much greater speed, accuracy and

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simplicity since one does not have to optimize the elution method and concern regarding stability, degradation and recovery yield of the eluting proteins.

We demonstrate this by screening and identifying proteins from lipopolysaccharide (LPS)-stimulated mouse 70Z/3 pre-B cell nuclear extracts that can bind specifically to a thioaptamer XBY-S2 that has been covalently linked (or affinity attached) to the ProteinChip array surfaces.

ProteinChip arrays, which are the commercialized products for surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry (MS) [12], incorporate the full range of surface properties, extending from classical chromatographic media, such as ion exchange, IMAC, and reverse phase, to more specific biomolecular affinity probes like antibodies, receptors, enzymes, ligands, and nucleotides [13,14]. In short, SELDI-TOF-MS successfully combines chromatographic and affinity captured surfaces with mass spectrometric detection of proteins or other biomolecules retained by those surfaces.

SELDI-TOF-MS provides rapid ''on-chip'' analysis by directly processing crude biological samples with or without pre-fractionation on ProteinChip array surfaces. In addition to rapid ''on-chip'' screening and analysis with minimum management of the crude samples, we present in this paper a novel approach to the subsequent quick identification of those specifically bound proteins—enzymatic digestion of the captured proteins and subsequent microsequencing of the resulting peptides through tandem MS. The identification of the unknown proteins was obtained directly from ''on-chip'' analysis.

In order to verify the identified unknown bound proteins to thioaptamer XBY-S2, we developed a novel thioaptamer/antibody sandwich assay on ProteinChip array surfaces, which provides sensitive and selective identification of both abundant and low-abundant proteins from crude biological samples.

Materials and methods

Oligonucleotides (ODNs) synthesis, purification, and characterization

Oligonucleotides (ODNs) were synthesized on a 1 μmol scale on an Expedite 8909 DNA synthesizer (Applied Biosystems). These samples were prepared without dimethoxytrityl (DMT) groups at their 5′ termini and purified as previously described [15]. The samples were further purified on a Hamilton PRP-1 (Polymeric Reverse Phase) high-performance liquid chromatography (HPLC) column (305 × 7.0 mm) with a flow rate of 2.0 ml/min using a linear gradient of two buffers. Buffer A was 100 mM ammonium acetate at pH 7.2. Buffer B was acetonitrile. Buffer B was increased linearly from 0 to 25% over a 55 min interval. Then the ODNs were lyophilized and re-dissolved in distilled water and the concentrations were determined by measuring the UV absorbance at 260. The ODNs were characterized by MALDI-TOF mass spectrometer (ABI Voyage DE MALDI-TOF) for molecular mass and ^{31}P-NMR for purity, respectively.

Double-stranded oligonucleotide probes

Equal moles of the oligonucleotide and its complementary strand was mixed together containing 100 mM NaCl at pH 7.2. After heating to 80 °C, the solution was gradually cooled to room temperature. The extra salt and excess of single strand were removed from the duplex by ultrafiltration using Centricon-3 (Amicon). It was assumed that 1 OD of duplex corresponds to ca. 50 μg at 260 nm.

Cell culture

70Z/3 cells (kind gift of Dr. Carol Sibley, University of Washington) were maintained between 1×10^5 and 1×10^6 cells/ml in RPMI 1640 (Biofluids) supplemented with 5% fetal calf serum (Hyclone), 2 mM glutamine, 55 mM β-mercaptoethanol, 20 mM Hepes, 100 U/ml penicillin G, and 100 mg/ml streptomycin. The cells were kept in a humidified 37 °C incubator with a 7% CO_2/93% air mixture. For induction, cells were split to 3–3.5 × 10^5/ml in the evening prior to stimulation. The next morning, cells were stimulated with 10 mg/ml LPS (*Salmonella typhosa*, Difco). Prior and 2 h after the induction, the cells were harvested and nuclear and cytoplasmic extracts were prepared [16].

Nuclear extract preparation

Nuclear extracts were prepared following standard procedures [16]. Pelleted cells were resuspended in buffer I with sucrose (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris (pH 8.0), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). Nuclei were pelleted, washed in buffer I without sucrose, and repelleted. Nuclei were then resuspended in a low-salt buffer (20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, and 0.5 mM PMSF), an equal volume of high-salt buffer (20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.8 M KCl, 0.2 mM EDTA,and 0.5 mM PMSF) was added, and the nuclei were incubated on ice for 20 min. They were then diluted by the addition of 2.5 volumes of dilution buffer (25 mM Hepes (pH 7.6), 25% glycerol, 0.1 mM EDTA, and 0.5 mM PMSF), and debris was pelleted. The supernatant was removed as the nuclear lysate and stored at −80 °C with aliquots.

Screening bound proteins by thioaptamer XBY-S2 from stimulated 70Z/3 nuclear extracts

One microliter of each 5′-amino linked double-stranded oligonucleotide (25 μM) (see Table 1) in 4 μl of 50 mM sodium bicarbonate (pH 9) was added to each spot on the PU ProteinChip array containing epoxy functional group (Ciphergen, under development), which interacts with primary amines to form a covalent bond. The reaction proceeded at 4 °C overnight in a humidified chamber. Excess oligonucleotides were removed and the array was blocked with the buffer containing 5 mg/ml BSA and 0.1% Triton X-100 for 2 h. The array was sequentially washed with PBS containing 0.1% Triton X-100, PBS, and 1× binding buffer (50 mM Hepes, 50 mM KCl, and 1 mM $MgCl_2$, pH 7.4). The array was then ready to capture proteins from crude nuclear extracts. Five microliters of reaction mixture was added to each spot including 4 μg nuclear extracts, 0.75 μg poly(dI:dC) buffered with 1× binding buffer, 0.5 mM DTT, and 0.1% Nonidet P-40. The array was incubated at 4 °C for 2 h with gentle shaking and high humidity. The excess reaction mixture was removed and the array was washed separately with 1× binding buffer containing 0.1 M KCl and 0.3% Triton X-100 and with 1× binding buffer. After washing, the array was quickly rinsed with 5 mM Tris (pH 7.5), followed by the

Table 1
Double-stranded oligonucleotide probes (C_{12} represents 12 methylene flexible linker)

Name	Sequences
XBY-S2	5′-CC AGT$_{S2}$G ACT$_{S2}$C AGT$_{S2}$G-3′
	3′-GG$_{S2}$TCA C$_{S2}$TGA G$_{S2}$TCA C-5′
Scrambled-XBY-S2	5′-CC AGG AG AT$_{S2}$T$_{S2}$CC AC-3′
(XBY-6)	3′- GG$_{S2}$TCC$_{S2}$TC$_{S2}$TA A GG$_{S2}$TG-5′
Poly(IC)$_7$	5′-ICICICICICIC-3′
	3′-CICICICICICI-5′