Demonstration and Optimization of Multiple Aptamer-ELISA "ELASA" Assays with Novel DNA Aptamers

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Abstract

While the ELISA method is generally very sensitive with specificity depending on the quality of primary detection antibody, protein-based antibody reagents are not very stable in non-refrigerated (i.e. point-of-care) applications. In contrast, DNA aptamers are extremely stable in both hydrated and lyophilized form and therefore amenable to field-deployed assays. Towards using such reagents, we are demonstrating their suitability in several modified ELISA formats termed enzyme-linked *aptamer* sorbent assays or "ELASA". Using novel aptamers, here we demonstrate both direct and sandwich ELASA approaches. For each of the approaches we determine their apparent limit of detection. In ongoing work, the specificity of our various aptamers are being determined with some of the preliminary data are presented here. Finally, we present a novel application of a reaction to generate sulfonated PVDF membranes. These modified membranes have shown less non-specific binding to negatively charged nucleic acids while retaining their excellent binding capacity for proteins.

Introduction

Aptamers have a number of potential advantages over antibodies [1], especially for point-of-care or "deployed" assays in which antibodies require a cold-chain of custody to prevent degradation. Nevertheless, the extensive use of enzyme linked immunosorbent assays or "ELISAs" has resulted in well-established methods, enzymes, and substrates for sensitive detection of antigens/analytes. Thus, **we are demonstrating aptamers as modular replacements for antibodies in convenient "ELISA-like" formats** familiar to many potential users. Others have rather naturally termed such assays "enzyme linked *aptamer* sorbent assays" or "ELASAs" [2].

Aptamers are single-stranded DNA or RNA oligonucleotides selected to have unique three dimensional folding structure for binding to a variety of targets such as proteins, peptides, and even small molecules with affinity and specificity rivaling that of antibodies. They are typically selected in vitro by a process commonly referred to as "SELEX" as depicted in **Figure 1**.

A key aspect of any SELEX procedure is partitioning of the binding vs. non-binding nucleic acid population. **Figure 2** depicts a sulfonation reaction [3] for modification of standard PVDF membrane. The resulting negative charge results in less non-specific adsorption of nucleic acids while the high capacity of PVDF for adsorbed protein is still retained [3]. This also has obvious implications for the ELASA assays presented here in which minimization of background aptamer binding is desired.

Herein we demonstrate the utility of 3 different ELASA approaches (**Figure 3**) for using aptamers to quantify protein analytes.

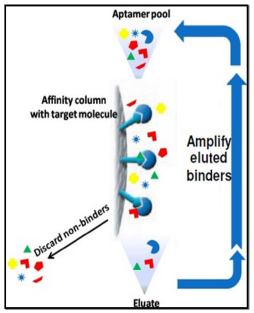

Figure 1: *Schematic representation of conventional single-target DNA aptamer selection*

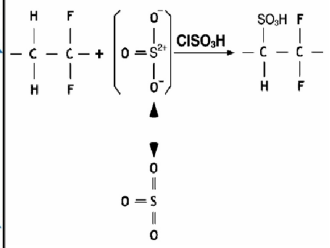

Figure 2: *Reaction involved in sulfonation of Polyvinylidene difluoride*

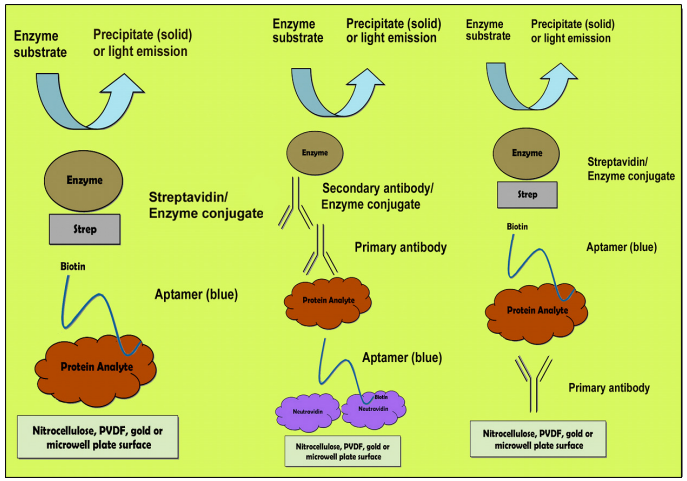

Figure 3: ***Three different approaches to ELISA-like ("ELASA") assays using aptamers.*** ***(3A):*** *ELASA by direct spotting of protein analyte on membrane surface.* ***(3B):*** *Indirect "Method 1" for "sandwich" aptamer/antibody ELISA. In Method 1 an aptamer is used as the primary capture element for the protein analyte.* ***(3C):*** *Indirect "Method 2" for sandwich aptamer/antibody ELISA. In Method 2 an antibody is used as the primary capture element while a novel, biotinylated aptamer is used as the secondary reporter.*

Methods

Membrane preparation: PVDF-coated slides (ArrayIt) were pre-wetted by 100% methanol and allowed to dry for 60 minutes prior to protein spotting. For the "direct" or dot-blot method, 0.5 – 2.0 µl spots of varying concentration were spotted and dried for an hour. Arrays are then blocked with 1 mg/ml non-fat dry milk before aptamer binding. For the indirect methods, neutravidin (Pierce) or analyte-specific antibody, 0.5 – 2.0 µl were similarly spotted and dried for an hour at room temperature. For "Indirect Method 1", 1-2 µl of aptamer was then spotted directly over the neutravidin spot. Arrays thusly prepared were then blocked with non-fat dry milk prior to offering varying concentrations of protein analyte.

Protein:aptamer binding and washing: For the "direct" or dot-blot method, biotinylated aptamer solution was added and allowed to incubate for 120-150 minutes followed by washing with ~500 µl binding buffer in a well created by a "ProPlate™" (GraceBio) slide attachment. Fluorescence was then developed using streptavidin-alkaline phophatase conjugate. In the case of the indirect method 1, suitable primary antibody and secondary antibody conjugate (**Figure 3B**) were allowed to bound for 30 minutes prior to substrate development as described below.

Substrate development and imaging: ELF-97™ (Invitrogen) is a patented alkaline phophatase substrate commonly used for immunohistochemistry. Upon enzymatic conversion, the substrate forms a fluorescent precipitate. Slides were excited by a long wavelength "blacklight blue" source (GE bulb F15T8 BLB 15W) and images were captured using a 14.7 megapixel camera with orange emission filter. The freely available program, NIH **ImageJ** was used to extract fluorescent intensities from the resulting images.

Results

The direct approach (**Figure 4A**, "Method 1") has the advantage of small analyte consumption. Here, the protein (or eventually, serum sample) is spotted in a small (~ 1 µl) volume. Because of this small volume, the ultimate sensitivity is reduced. In contrast, both of the indirect methods ("**Figures 4B** and **4C**") capture protein analyte from a much larger volume (~400 µl). The resulting sensitivity or limit-of-detection is on the order of fmoles/ul as shown below.

Assay Type	Limit of Detection (nM)	Total amount of material detected
Direct Method	259.7	259.7 femtomoles
Indirect (Sandwich) Method 1	5.3	1.6 picomoles
Indirect (Sandwich) Method 2	0.0265	7.95 femtomoles

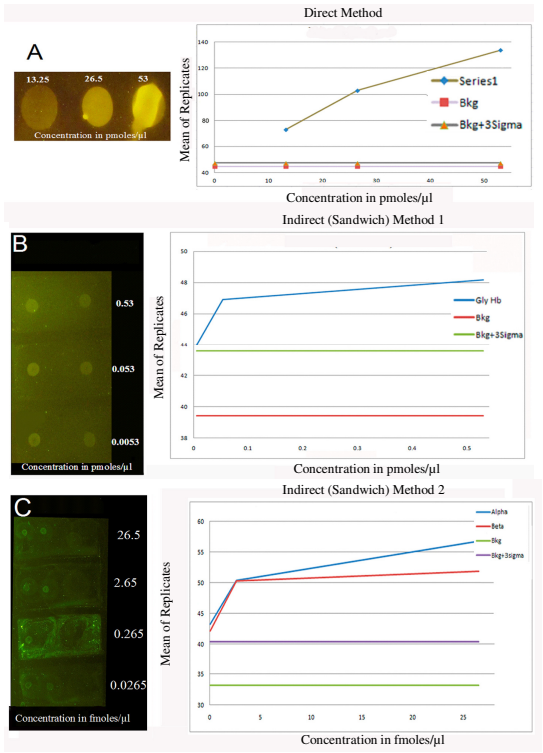

Figure 4A: *Result of direct ELASA sensitive detection of a protein analyte.* ***(4B):*** *Result of " Method 1" indirect or "sandwich" aptamer/antibody ELISA.* ***(4C):*** *Result of "Method 2" indirect or "sandwich" aptamer/antibody ELISA.*

Discussion

The results here demonstrate the feasibility of readily replacing antibodies in many common ELISA assays. Given the lower cost of aptamer materials, their well-defined chemical nature, and their many other potential advantages over protein-based antibodies [1], we find these results especially encouraging for a variety of applications. We are currently investigating complementary aptamers for their ability to bind non-overlapping epitopes and thereby develop "sandwich" assays completely comprising aptamers. Both of the "indirect" methods presented here should be readily translated to standard fluorescent microplate readers.

References

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2. Bruno JG, Carrillo MP, Phillips T. 2007. **Effects of immobilization chemistry on enzyme-linked aptamer assays for *Leishmania* surface antigens.** *J. Clin. Ligand Assay.* **30**: 37–43.
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