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Replacing antibodies with modified DNA aptamers in vaccine potency assays

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ABSTRACT

Vaccine *in vitro* potency assays are vital regulatory tests that are used to confirm the presence and concentration of an antigen of interest in a form that directly or indirectly relates to protective activity in patients. Current assays come in many forms, but they almost exclusively use antibody reagents for selective detection of the target antigen. Antibodies provide specific recognition of vaccine antigens but also exhibit drawbacks such as stability limitations, cost, and lot-to-lot variation, which can make it challenging to maintain the reagent throughout the lifetime of the vaccine.

We explored replacing antibodies with aptamers. Aptamers are macromolecules, such as nucleic acids, which can bind to their targets with high specificity and affinity, similar to that of antibodies. Some of the advantages of using aptamers over antibodies is that aptamers can be more stable, smaller, less expensive to produce, synthesized *in vitro*, and logistically easier to supply throughout the multi-decade lifespan of a commercial vaccine. We created modified DNA aptamers against the common vaccine carrier protein, CRM_{197}. Several aptamers were discovered and one was chosen for further characterization. The binding kinetics of the aptamer revealed an off-rate 16-fold slower than anti-CRM_{197} antibodies used for comparison. The aptamers were more sensitive than available antibodies in some assay formats and comparable in others. The aptamer epitope was mapped to the receptor-binding domain of CRM_{197}, a site adjacent to a known antibody binding site. These data address some key aspects for a path forward in replacing antibodies with aptamers for use as critical reagents in vaccine assays. We further highlight the possibility of using nucleic acid reagents to develop next generation potency assays.

1. Introduction

Vaccine potency assays are critically important in maintaining the quality, safety and efficacy of a vaccine throughout its lifetime. These assays are used for a variety of purposes, including confirming the presence and concentration of the vaccine antigen in a form that directly relates to protective activity. The assays may be used for characterization, product release or stability [1]. Historically, the potency of live virus or subunit vaccines was determined using either plaque assays or animal immunogenicity assays. Many modern recombinant vaccines [2] have taken advantage of *in vitro* potency assays such as ELISA (enzyme-linked immunosorbent assay), nephelometry, or *in vitro* neutralization assays.

Within immuno-based potency assays, antibodies are used as critical reagents. Antibodies offer high affinity and specificity to the target, potential clinical relevance, and well-established production methods. However, significant drawbacks of both monoclonal and polyclonal antibodies independent of the target antigen include (a) potentially complex long-term storage and limited shipping stability; (b) high discovery and supply costs; (c) long discovery timelines; (d) limited control of the immunization discovery process; (e) lot-to-lot inconsistencies between polyclonal antibody (pAb) immunizations as well as between monoclonal antibody (mAb) contract manufacturers; (f) the use of immunized animals; and (g) the maintenance of cell-lines, complex expression systems, or immunized animals throughout the multi-decade lifetime of a vaccine.

A possible alternative, which can address many of the disadvantages of antibodies, are aptamers. Aptamers are affinity reagents, such as nucleic acids, that specifically bind to a target molecule [3,4]. In many cases, aptamers are comparable to antibodies in both affinity and specificity with dissociation rates in the low nanomolar to mid-picomolar range [3,5,6]. In some cases, aptamers can even discriminate proteins that differ by a single amino acid [7] or small molecules that differ by a single atom

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[8]. Aptamers are generated using an *in vitro* process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) that employs iterative rounds of partitioning and amplification of a random nucleic acid library [9,10].

Aptamers offer significant advantages over monoclonal antibodies (mAbs) as reagents for *in vitro* potency assays. Aptamers are developed *in vitro*, allowing for greater control over the resulting reagent attributes such as specificity [11] and binding kinetics [12]. Once generated, an aptamer can simply be synthesized to deliver a pure and consistent supply throughout the lifetime of a vaccine. In addition, aptamer functionalization with biotin or fluorophores is straight forward. The smaller size of aptamers (8–25 kDa [13]) compared to antibodies (IgG ~150 kDa) potentially allows binding to sterically challenged epitopes. DNA aptamers can be very stable, facilitating global distribution for assays used to support testing upon importation. Finally, aptamers can be less expensive than antibodies resulting in significant cost savings over the vaccine life cycle.

Although aptamers have the potential to improve potency assays, many questions still need to be addressed. Because an antibody is generated by an immune system, they often bind to clinically relevant epitopes. Aptamers are selected *in vitro* and could bind similar or very different epitopes on the vaccine. Therefore, the potential clinical relevance of aptamer epitopes needs to be explored.

To explore the feasibility of using aptamers in a vaccine potency assay, we chose a polysaccharide vaccine conjugated to the protein carrier CRM_{197}. CRM_{197} is a non-toxic form of diphtheria toxin used in many vaccines to improve the immunogenicity of a conjugated antigen such as a polysaccharide [14,15]. CRM_{197} is currently used in approved conjugate vaccines against meningitis, pneumococcus and Haemophilus influenza B [16,17] to improve immunogenicity. Several CRM_{197} epitopes have been mapped that might be relevant for the carrier's function in a vaccine [18,19]. As aptamers against polysaccharide target in our experience have not been feasible yet, we targeted the protein carrier as a proof of principle to incorporate aptamers in a vaccine-related potency assay.

2. Materials and methods

2.1. Aptamer generation and monoclonal antibody information

Aptamers were generated through SELEX by SomaLogic® through their SOMAmer discovery service. The target of the selection was CRM197 conjugated to a polysaccharide. The protein, CRM_{197}, was biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin labeling kit (Thermo, 21327) following the manufactures instructions. A molar ratio of 5 Sulfo-NHS-LC-Biotin for every protein was used. The SELEX protocol has been published previously [12,20]. Briefly, a pool of random modified DNA sequences were incubated with the CRM_{197} conjugate. Sequences that bound were enriched using streptavidin magnetic beads. All rounds but round 1 were significantly diluted in buffer (20–400-fold) prior to separation to select reagents with slow dissociations. Later rounds also included 10 mM dextran sulfate. Sequences were eluted using sodium perchlorate and amplified into natural DNA. The modified sequences were then generated using a primer extension step and introduced into the next round of selection. A total of 16 aptamers or SOMAmers (slow-off rate modified aptamers) were chosen for synthesis based on their phylogenetic separation. All aptamers contain a hydrophobic amino acid-like modification off of every uridine base in the sequence [12,21].

All anti-CRM_{197} antibodies are monoclonal mouse IgG1 with the exception of mAb-01 which is IgG2b. They were generated by immunization with CRM_{197} or CRM_{197} conjugated to a polysaccharide.

2.2. Electrophoretic mobility shift assay (EMSA)

Aptamers at 500 nM were incubated with 0 μM and with 5 μM CRM_{197} in the manufacturer's recommended buffer (40 mM Hepes pH 7.5, 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA) supplemented with 1X Type III DNA loading dye. After a 30 min incubation on ice, 7 μL were added to a 10% native TBE gel (Invitrogen). The gel was run for 45 min at 120 V, stained with GelRed for 30 min, imaged on an AlphaImager system (ProteinSimple), and quantified for the percent shift in ImageJ (NIH).

2.3. Surface plasmon resonance (SPR)

Data were generated on a Biacore™ 3000 (GE Healthcare) using a running buffer of 10 mM Hepes pH 7.5, 150 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.05% Tween 20. For aptamer experiments, the surface of a CM4 chip was activated with a 1:1 mixture of 0.1 M EDC/0.1 M NHS followed by covalent binding of 10 μg/mL streptavidin (Thermo Fisher, 21122) in 10 mM sodium acetate at pH 5.0 to give 200 reference units (RU) on both the control and sample flow cells. The reaction was quenched using 1 M ethanolamine pH 8.0. The control flow cell was saturated with 1 μM biotinylated aptamer of similar chemical composition as the aptamer of interest but evolved to a different target. The sample cell was saturated with 1 μM of the biotinylated aptamer of interest. Both surfaces were blocked with 1 mM biotin. Kinetic data were collected by injecting CRM_{197} over the surface at 30 μL/min for 5 min, and the dissociation was observed by flowing buffer over the surface for 30 min. Five different concentrations (not including 2 buffer injections) were injected in a randomized order over both the control and the sample flow cells with the middle concentration point run in duplicate. The surface was regenerated with 0.5% SDS at 30 μL/min for 30 s.

For antibody experiments, the surface was labeled similarly but with 600 RUs of an anti-mouse antibody (Jackson ImmunoResearch, 115-005-068). The anti-CRM_{197} mAb was bound with 5 μL/min at a concentration of 10 μg/mL for 5 min. The chip was regenerated with 10 mM Glycine pH 2.0 at 30 μL/min for 60 s. Since this stripped off the anti-CRM_{197} antibody, the mAb was bound again before each injection of CRM_{197}.

Data were processed with the BIAevaluation 3.2 software (GE Healthcare) using the double-referenced subtraction method and fit to a 1:1 Langmuir model. All data presented were averaged from three independent injection series with the error representing the standard deviation.

2.4. ELISA

All ELISAs were carried out with 100 μL volumes in 96-well black maxisorp plates (NUNC 437111) at ambient temperature using assay buffer with 10 mM Tris-HCl pH 7.7, 150 mM NaCl, 0.05% Tween-20, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mg/mL BSA, and 0.1 mg/mL Salmon Sperm DNA. After each step, plates were washed three times using 1X TTBS (10 mM Tris-HCl pH 7.7, 150 mM NaCl, 0.05% Tween-20) and six times prior to substrate addition.

In a direct assay, CRM_{197} in TBS was absorbed to the plate (2 h) starting at 1 μg/mL followed by 2-fold dilutions across the plate. Plates were blocked with 200 μL assay buffer (1 h) followed by 10 nM anti-CRM_{197} aptamer or antibody (2 h). Subsequently, streptavidin conjugated to alkaline phosphatase (Thermo Fisher, 21324) or anti-mouse mAb-alkaline phosphatase conjugated antibody

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