

A modular platform to display multiple hemagglutinin subtypes on a single immunogen

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eLife Assessment

This **valuable** manuscript describes the immunogenicity of a bead-on-a-string immunogen that allows the inclusion of multiple HA subtypes. The evidence to support the claims is **convincing**, and more importantly, this approach could be adapted to other vaccine platforms.

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Abstract

Next-generation influenza vaccines aim to elicit cross-reactive humoral responses to multiple influenza subtypes. Such increased breadth would not only improve seasonal vaccines but may afford ‘universal’ protection against influenza subtypes including those with pandemic potential. Here, we describe a “beads-on-a-string” (BOAS) immunogen, that tandemly links up to eight distinct hemagglutinin (HA) head domains from circulating and non-circulating influenzas. These BOAS are immunogenic in the murine model and elicit comparable serum responses to each individual component. Notably, we also find that BOAS elicit cross-reactive responses to influenza subtypes not included in the immunizing immunogen. Furthermore, BOAS conjugation to protein-based ferritin nanoparticles does not significantly augment serum responses suggesting that our BOAS platform is sufficient for eliciting cross-reactive responses without off-target effects induced by the nanoparticle scaffold. Finally, vaccination with a mixture of the same HA head domains is not sufficient to elicit the same neutralization profile as the BOAS immunogens or nanoparticles. This mix-and-match immunogen design strategy is a robust platform for eliciting responses to multiple influenza subtypes via a single immunogen, and a potential platform for other viral glycoproteins.

Introduction

Influenza viruses, despite continuous surveillance and annual vaccination, are a significant public health problem resulting in ~500,000 deaths per year¹. This is largely a consequence of viral evolution and antigenic drift as it circulates seasonally within humans and ultimately impacts vaccine effectiveness. Additionally, the chance for spillover events from animal reservoirs (e.g., avian, swine) is increasing as population and connectivity also increase. To combat this, global surveillance is necessary to inform re-formulation of influenza vaccines to match circulating strains. However, long lead times for vaccine preparation in eggs and mutations acquired during that process² can lead to vaccine mismatch and subsequent ineffectiveness. The SARS-CoV-2 pandemic has additionally underscored the need for pandemic preparedness as humans increasingly come into contact with animal reservoirs. Indeed, several avian influenzas, such as H5N1, H7N9 and H9N2, are currently being monitored for their pandemic potential. Thus, there is an urgent need to develop so-called “universal” influenza vaccines that provide protection against both currently circulating (e.g., H1N1s, H3N2s, and Bs) and potentially pandemic viruses.

Several approaches have been taken to improve upon current influenza vaccines, largely focused on the influenza surface glycoprotein, hemagglutinin (HA). A major goal of immunogen design efforts is to elicit antibodies toward conserved epitopes on HA, including the receptor binding site (RBS)^{3–9}, trimer interface^{10–14}, and stem^{15–20}; antibodies targeting such epitopes are often broadly protective. Indeed, RBS-directed antibodies often are potently neutralizing, as they directly inhibit HA engagement with host cell sialic acid, while TI- and stem-directed antibodies generally protect via Fc-dependent mechanisms (e.g., ADCC, ADCP). Several rational immunogen design approaches²¹ such as epitope removal^{22,23}, domain chimeras²⁴, computational sequence optimization (COBRAs)^{25–29}, epitope resurfacing³⁰ and hyperglycosylation^{14,31,32} attempt to focus to these epitopes.

An additional strategy to enrich for cross-reactive antibodies includes increasing antigen valency by displaying multiple antigens on a scaffold, such as a nanoparticle^{33–35}. Such multivalency is thought to enhance immunogenicity and antigenicity by spacing antigens near one another in order to effectively cross-link B cell receptors (BCRs)³⁶. Ferritin, which can display 24 antigens at its 8 three-fold axes, has been used for influenza, Epstein-Barr virus, coronavirus, and HIV glycoproteins^{37–43}. For influenza, following immunization with a mixture of homotypic full-length seasonal H1, H3, and influenza B HA trimers displayed on ferritin nanoparticles, mice elicited responses with enhanced potency and breadth relative to the trivalent inactivated influenza vaccine (TIV) standard³⁸. Based on this success, they then designed “mosaic” ferritin nanoparticles that displayed up to eight antigenically distinct H1 subtype HA receptor binding domains (RBDs) on a single particle, which outperformed admixtures of homotypic nanoparticles and elicited cross-reactive H1 broadly neutralizing antibodies⁴¹. Further iterations of nanoparticle immunogens have co-displayed multiple full-length HA antigens on virus-like particles (VLPs)⁴⁴ and *de novo* designed particles⁴⁵, and the latter is currently in clinical trials^{45,46,47}.

An alternative approach for multivalent display involves tandemly linking antigens into a single polypeptide. This method may alleviate manufacturing challenges related to preparing multiple individual components for mosaic nanoparticles while still increasing size and valency of antigens. For example, using domain III from the dengue envelope surface glycoprotein, a bivalent⁴⁶ or quadrivalent⁴⁷ tandemly linked immunogen elicited neutralizing antibody responses to all four dengue serotypes and provided protection upon challenge. An analogous approach tandemly linked four different coronavirus RBDs into a single immunogen⁴⁸. These “quartets” elicited responses to each individual components similar to a “mosaic-4” nanoparticle that displayed monomeric RBDs on the surface of a virus like particle (VLP) nanoparticle^{49,50}.

these approaches were then combined and quartets displaying four or eight unique RBDs were multivalently displayed on VLP nanoparticles. This combinatorial approach augmented both neutralization titers and cross-reactive responses.

Here, we tandemly linked multiple HA subtypes into a single immunogen via short, flexible linkers in a “plug and play” platform we call “beads-on-a-string” or BOAS. These recombinantly produced, protein-based immunogens have between three and eight unique HA heads including circulating and non-circulating A and B influenzas. Mice immunized with either 3, 4, 5, 6, 7, or 8mer BOAS elicited responses to matched and mismatched HA components and could neutralize subsequent replication-restricted reporter (R3) viruses⁵¹. To further enhance valency, we generated two 4mer BOAS that included 8 different HA heads, conjugated both to ferritin nanoparticles using SpyTag-SpyCatcher^{52,53}, and showed that the serum responses elicited similar responses to the 8mer. Additionally, we showed that a mixture of the same HA head components was not sufficient to recapitulate the neutralizing responses elicited by the BOAS or BOAS NP. Collectively, these BOAS immunogens could serve as a means to elicit responses to multiple HA subtypes as a single immunogen and represents a general approach for multivalent display of other viral antigens.

Results

Design and characterization of beads-on-a-string (BOAS)

Monomeric hemagglutinin (HA) heads representing antigenically distinct subtypes were tandemly linked together as a single linear construct, separated by a short, flexible glycine-serine-serine (GSS) linker, which we termed beads-on-a-string (BOAS) immunogens. Eight unique HA subtypes spanning group 1 and 2 influenza A viruses, as well as an influenza B virus, from seasonal, circulating, and non-circulating influenzas were selected to understand the permissibility of the platform to express different HAs (**Figure 1A**). We found that monomeric HA head domains varied in expression levels depending on the boundaries selected: H1, H5, and H7 HA monomers spanned residues 57-261 (H3 numbering), whereas H2, H3, H4, H9, and B HA monomers spanned residues 37-316 (H3 numbering) (**Figure 1B**). We then asked whether we could tandemly link multiple HA heads together as a single construct. Using linkers with either two, three, or four GSS repeats, we tandemly linked representative H1, H3, and B HAs (Supplemental Figure 1A) together for recombinant expression in mammalian cells. Each construct, regardless of linker length expressed to similar levels (Supplemental Figure 1B) and each individual HA component could be detected with a conformation specific and subtype specific monoclonal antibody (mAb) (Supplemental Figure 1C). We next assessed whether the order of the HA heads adversely affected expression or accessibility to the conformation-specific mAb. Using H5, H7, and H9 HA heads we created two constructs 1) H5-H9-H7 and 2) H9-H5-H7 each with a four GSS linker. Each construct had comparable levels of expression and reactivity to the respective mAbs (Supplemental Figure 2).

Based on these initial data, we next designed six constructs with three to eight individual HA head components from representative H1, H2, H3, H4, H5, H7, H9, and B influenzas; to maximize spacing between each HA component we used the 4x GSS linker. Each BOAS was cloned into a single polypeptide chain with a protease-cleavable C-terminal purification tag (**Figure 1C**). We synthesized six different BOAS, and define each construct as 3mer, 4mer, etc., referencing the number of HA components (**Figure 1D**). Each BOAS construct could be purified to homogeneity as assayed by SDS-PAGE after immobilized metal affinity chromatography (**Figure 2A**) and correspond to the expected molecular weight following proteolytic cleavage of native glycans with PNGase-F (**Figure 2B**). After size-exclusion chromatography (SEC), the non-PNGase-treated BOAS were monodispersed (**Figure 2C**). Following SEC, affinity tags were removed with HRV-3C protease; cleaved tags, uncleaved BOAS, and His-tagged enzyme were removed using cobalt

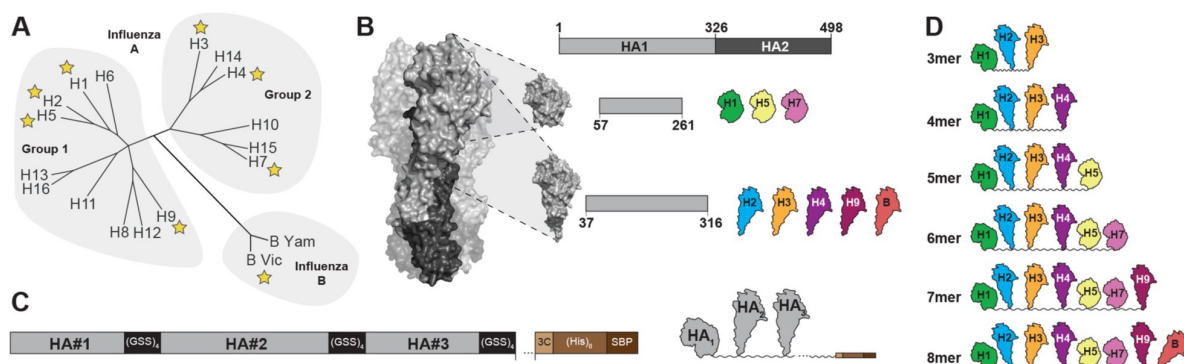


Figure 1

Design of Beads-on-a-string (BOAS) immunogens-

(A) Phylogenetic tree of influenza hemagglutinin (HA) subtypes. Subtypes are further categorized as group 1, group 2 or influenza B, and stars indicate subtypes included in the BOAS. **(B)** Native trimeric HA structure (A/H3/Hong Kong/1968; PDB: 4FNK) is shown on the left, with the HA1 domain in light grey, and HA2 in dark grey. A construct diagram of full-length HA is shown at the top with the locations of head domain segments within HA1. Head domains of H1, H3, and H7 span residues 57-261, and head domains of H2, H3, H4, H9, and B span 37-316 of the HA1 region of HA. **(C)** Construct diagram of HA BOAS. BOAS are assembled by linking HA head domains (light grey) into a single polypeptide chain where HA head domains are separated by a flexible linker with 4 (GSS) repeats. The C-terminus consists of an HRV-3C protease cleavable His tag and streptavidin-binding-protein (SBP) tag for purification and other affinity tags. **(D)** BOAS used in this study.

affinity resin and snap frozen in liquid nitrogen before immunizations. BOAS maintained monodispersity upon thawing, though over time, degradation was observed following longer term (>1 week) storage at 4C (Supplemental Figure 3). This degradation became more significant as BOAS increased in length (Supplemental Figure 3).

We then analyzed the BOAS using negative stain electron microscopy (NS-EM). Individual HAs were readily discernable and equal to the number of components in the respective BOAS (**Figure 2D** and **2E**); we observed that the 3mer could adopt both extended and collapsed, triangular-like conformations (**Figure 2D**), whereas longer BOAS, such as the 8mer, adopted a rosette-like conformation (**Figure 2E**). This is likely a consequence of the flexible GSS linker separating the individual HA head components as well as the addition of significantly more HA head components to the construct.

To assess conformational integrity and ensure that each HA present was properly folded, we assembled a panel of conformation- and subtype-specific mAbs to assay each individual component. This included mAb S5V2-29¹², a broadly-reactive TI-directed positive control antibody and mAbs 5J8^{4,6}, 2G1⁵, K03.12⁵⁴, P2-D9⁵⁵ (Supplemental Figure 4), H5.3⁵⁶, H7.167⁵⁷, and H1209⁵⁸ which are specific to H1, H2, H3, H5, H5, H7 and B subtypes, respectively; an H9 conformation-specific mAb was not available. Each individual HA component was detected using subtype-specific mAb in ELISA, and overall affinity for each component was comparable regardless of BOAS length (**Figure 2F**). Collectively, these data support a plug-and-play platform that can readily exchange HA components while retaining conformational integrity.

Immunogenicity of BOAS

To determine immunogenicity of each BOAS immunogen, we performed a prime-boost-boost vaccination regimen in C5BL/6 mice at two-week intervals with 20µg of immunogen and adjuvanted with Sigma Adjuvant (**Figure 3A**). We compared these BOAS to a control group immunized with a mixture of the eight HA heads present in the 8mer. We then tested serum reactivity to both BOAS immunogen as well as individual full-length HA trimers of each component. Serum titers against the immunogen were detectable after a single prime, and increased following a boost, and stayed relatively level until day 42 (**Figure 3B**). Serum responses varied for differing BOAS lengths, with the 4mer and 5mer BOAS being the most immunogenic (**Figure 3C**). Each of the BOAS, regardless of its length, elicited binding titers to all matched full-length HAs representing individual components (**Figure 3D**). For example, the 6mer, which contains heads of H1, H2, H3, H4, H5, and H7, elicited reactivity to full length matched HAs. Interestingly, some BOAS elicited cross-reactivity to mis-matched components not present in the immunogen. For example, the 3mer, which contained H1, H2, and H3 HA heads, elicited detectable titers to H4 and H5 HAs (**Figure 3D**). Additionally, all groups elicited binding titers to mismatched historical H3 and H1 HAs (**Figure 3D**). When comparing the 8mer and mix groups, the 8mer tended to elicit stronger binding titers to matched and mismatched components than an equal molar mixture of the same components (**Figure 3E**). Similar binding trends were also observed with d28 serum, though the difference between the 8mer and mix groups was more pronounced at d28 (Supplemental Figure 5).

Cross-reactivity of immune responses

To understand potential cross-reactivity observed in the serum analyses, across we used a ConSurf-like^{59,60} method to assess sequence conservation across the eight HA components in the BOAS. Each component of the BOAS was aligned structurally to the H2 head domain and scored at each position for their amino acid conservation at each position on a scale of 1 to 9. These were then pseudo-colored on the structure to visualize conserved and variable epitopes on the head domain surface (**Figure 4A**). We observed regions of both significant variability as well as conservation, notably the TI epitope, as well as the core sialic acid interacting residues in the

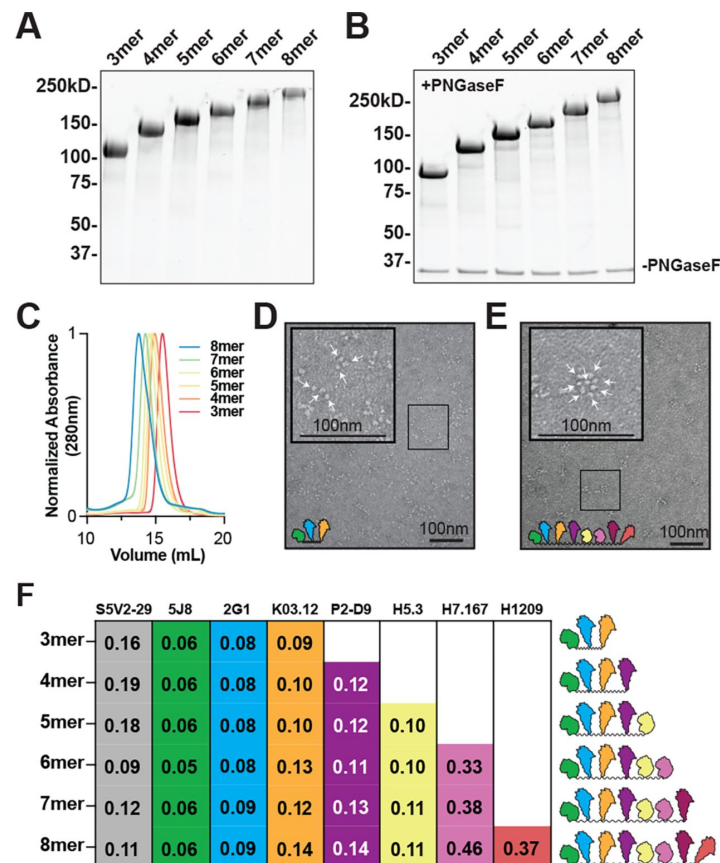


Figure 2

Biochemical Characterization of BOAS-

A) SDS-PAGE analysis of 3mer-8mer BOAS under non-reducing conditions. **B)** SDS-PAGE analysis of 3mer-8mer BOAS under non-reducing conditions following glycan digestion with PNGase-F. **C)** Size exclusion chromatography traces of 3mer-8mer BOAS on an Superdex 200 column in PBS. **D)** Negative stain electron microscopy (NS-EM) images of the 3mer. Arrows point to individual HA monomers. Inset image is 2.5X zoomed enlarged image of boxed area. **E)** Negative stain electron microscopy (NS-EM) images of the 8mer. Arrows point to individual HA monomers. Inset image is 2.5X zoomed enlarged image of boxed area. **F)** Characterization via ELISA of BOAS components with subtype-specific antibodies. Apparent K_D values in μM are reported for each antibody for each BOAS immunogen. Antibodies corresponding to each component are as follows: S5V2-29 (interface, all components except B/Mal/04), 5J8 (RBS, H1), 2G1 (RBS, H2), K03.12 (RBS, H3), P2-D9 (H4), H5.3 (RBS, H5), H7.167 (RBS periphery, H7), H1209 (RBS, B).

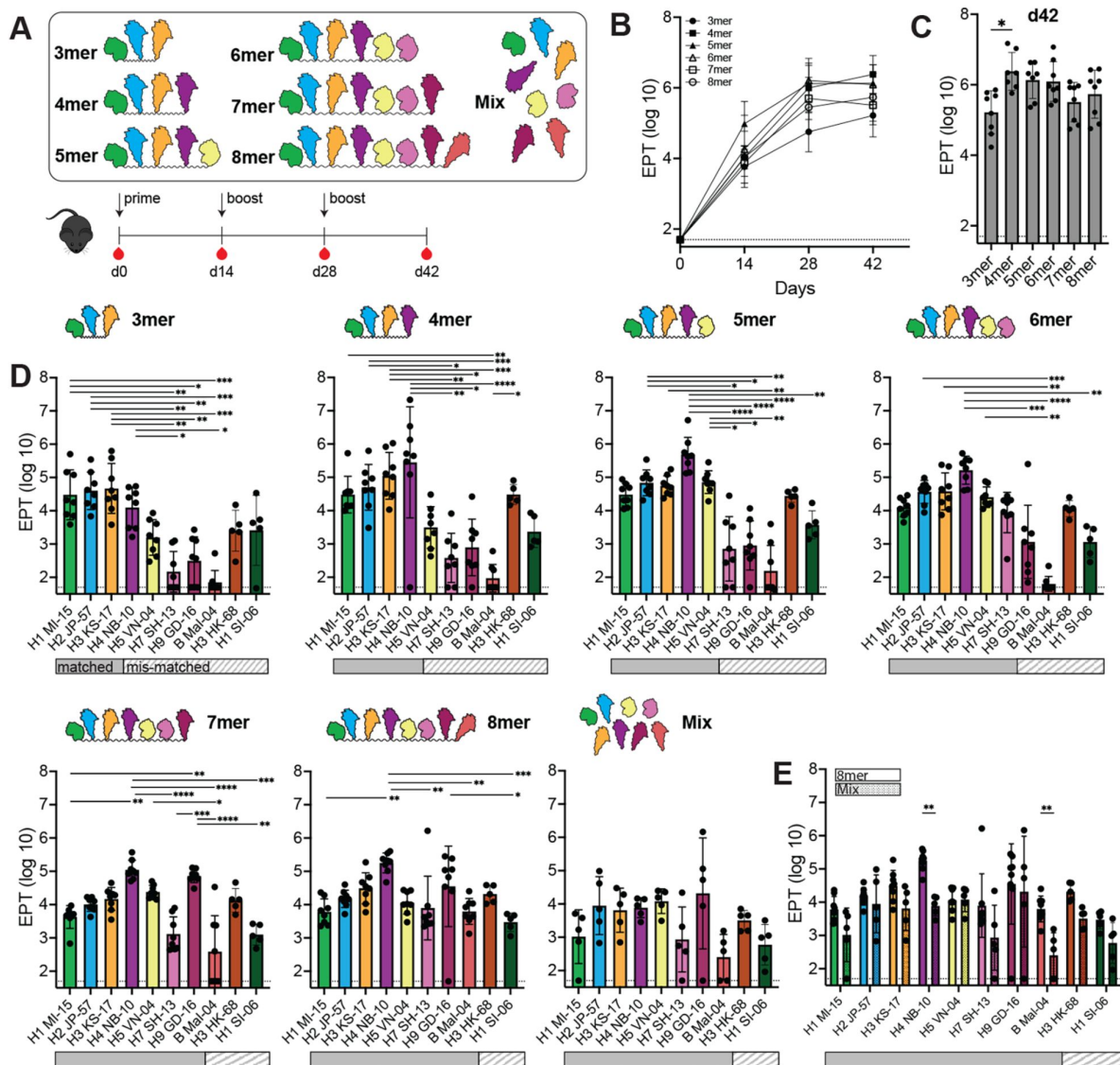


Figure 3

Murine immunizations with 3mer-8mer BOAS-

A) Schematic of immunization regimen with 3mer-8mer BOAS and a Mix control containing an equal molar of the 8 individual HA heads not connected with a linker. Mice were primed with 20µg of immunogen, followed by two homologous boosts two weeks apart. **B)** Immunogenicity of BOAS over time as determined by serum ELISA. Serum reactivity to each immunogen is reported as the endpoint titer (EPT) dilution factor for each group (n=8) and d14, d28, and d42. Data points are an average of n=8 mice and error bars are $\pm$ 1 s.d. **C)** Serum titers to each BOAS at the final time point, d42. Data points for single time point antigen titers are from individual mice (n=8, bars represent mean titers and error bars are $\pm$ 1 s.d., * = $p < 0.05$ as determined by Kruskal-Wallis test with Dunn's multiple comparison post hoc test relative to the 3mer. **D)** Serum titers to full-length HA trimers elicited by 3mer-8mer BOAS. Solid bars below each plot indicate a matched sub-type, and striped bars indicate a mis-matched subtype (i.e. not present in the BOAS). Data points are serum EPTs from individual mice (n=8) and error bars are $\pm$ 1 s.d., * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ as determined by a Kruskal-Wallis test with Dunn's multiple comparison post hoc test.

receptor binding site (RBS), the latter which remained nearly 100% conserved in all BOAS (**Figure 4A**). This was quantitated by taking the average amino acid (AA) conservation score across both the RBS and TI epitopes as well as the entire sequence, and we see that these epitopes retain higher conservation relative to the overall sequence as BOAS length increases. We then determined approximate degrees of focusing to the TI and RBS via a serum competition ELISA using mAbs. All the BOAS maintained, approximately, between 15-35% focusing to the TI epitope, whereas focusing the RBS was more variable (**Figure 4C**). Both RBS focusing (**Figure 4D**) and TI focusing (**Figure 4E**) trended similarly with homology scores for each epitope, indicating epitope conservation may be influencing immune focusing to particular epitopes.

Design of BOAS-conjugated nanoparticles

To further increase avidity affects as well as potential immunogenicity, we split our 8mer BOAS into two 4mer BOAS and attached both in an equal molar ratio to *H. pylori* ferritin nanoparticles (NPs) using SpyTag-SpyCatcher⁵², herein referred to as BOAS-NPs (**Figure 5A**). The two 4mer BOAS efficiently conjugated to nanoparticles as determined by SEC and SDS-PAGE (Supplemental Figure 6). Further biophysical characterization using dynamic light scattering (DLS) showed a shift of from 9.8 ± 1.1 nm to 21.3 ± 2.0 nm and NS-EM showed spherical particles with projections. (**Figure 5D** and **5E**). We verified the presence and structural integrity of each BOAS component on the nanoparticles using an ELISA with the same panel of conformation and subtype-specific mAbs, (**Figure 5E**).

Immunogenicity of BOAS-conjugated nanoparticles

We next immunized mice with BOAS-NP and a SpyCatcher NP control to assess immunogenicity. Mice were immunized with an equal amount of NP relative to the BOAS (20µg), in an equivalent homologous prime-boost-boost regimen (**Figure 6A**). The BOAS-NP was significantly more immunogenic than the SpyCatcher NP control, eliciting titers approximately an order of magnitude greater by d42 (**Figure 6B**). The BOAS NP, however, elicited significantly reduced titers to the SpyCatcher NP scaffold relative to the control (**Figure 6C**). When each group was evaluated for titers against both the BOAS components, the SpyCatcher nanoparticle scaffold, or BOAS-NP (**Figure 6D,E**), the BOAS-NP elicited equivalent titers to each set of BOAS, and greater titers to the decorated nanoparticle relative to the ferritin scaffold (**Figure 6E**). When we examined titers to individual HA components, we observed detectable titers over baseline to all eight HAs present on the BOAS-NPs (**Figure 6F**). When compared to the immunogenicity of the 3mer to 8mer BOAS immunogens, the BOAS-NP showed comparable titers to each component relative to BOAS that contained a given component (**Figure 6G**).

Neutralization of influenza viruses by BOAS-elicited sera

We next determined serum neutralization titers to matched and mis-matched influenza viruses based on the BOAS components (**Figure 7**). We tested serum from mice immunized with all BOAS against H1/Michigan/2015 H1N1 and H3/Kansas/2017 H3N2 viruses, whose HA was present in all the BOAS. These showed varying neutralization titers to both viruses, with all matched BOAS neutralizing virus except for the 7mer and H1N1 virus. Additionally, we tested serum against H5/Vietnam/2004 H5N1 and H7/Shanghai/2013 H7N9 viruses. Neutralization of H5N1 virus was variable, though individual mice from 4mer, 5mer, 6mer, 7mer, 8mer, and BOAS NP groups were able to strongly neutralize this virus. Weak neutralization titers were detected for 6mer, 7mer, and 8mer BOAS. This neutralization was only slightly above the high background neutralization of the negative control NP serum. Serum from the BOAS-conjugated nanoparticle group elicited similar neutralization titers relative to the BOAS alone. In most cases, neutralization titers elicited from the BOAS and BOAS NP cohorts were greater than that from the mix cohort. The scaffold control serum did not neutralize any viruses tested.

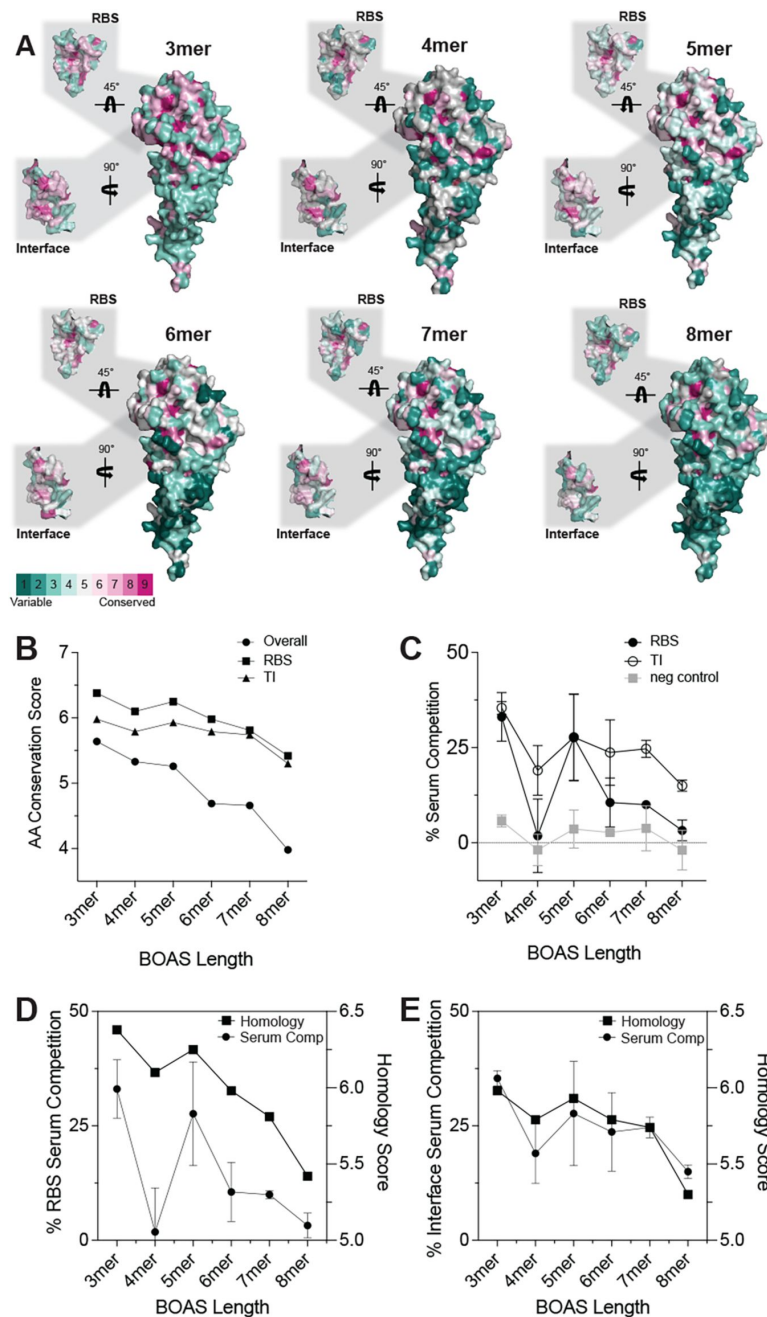


Figure 4

BOAS component epitope homology and serum competition

A) Amino acid conservation of each BOAS projected on the A/H2/Japan/1957 head domain structure (PDB: 2WRE). Variability and conservation scores were determined as the % conservation of each residue at each position for each BOAS composition. Cut-outs are RBS and Trimer interface (TI) epitopes with their respective conservation. **B)** Homology score as determined by the average % conservation of each residue across the entire protein sequence (overall, circle), and the RBS (square) and TI (triangle) epitopes. **C)** Serum competition to RBS and TI epitopes as determined via competition with a cocktail of RBS-directed antibodies, TI-directed antibodies, or a stem-directed negative control antibody. % competition is determined by the relative decrease in serum binding to each BOAS normalized to an untreated control. Each data point represents a mean from $n=3$ mice and error bars are ± 1 s.d. **D)** Trend overlay of % serum competition with RBS-directed antibodies and homology score of the RBS epitope as a function of BOAS length. **E)** Trend overlay of % serum competition with TI-directed antibodies and homology score of the TI epitope as a function of BOAS length.

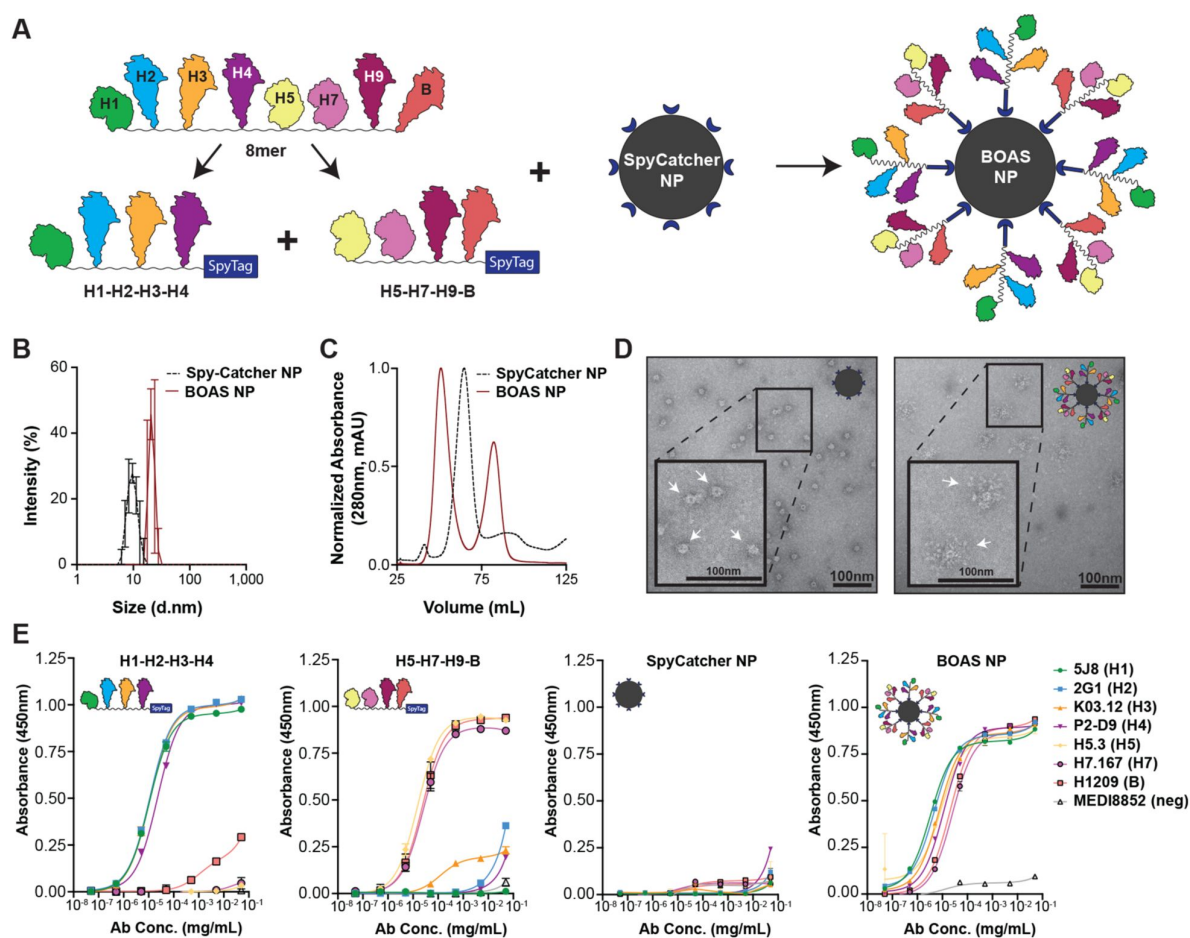


Figure 5

Design of BOAS nanoparticle (NP) immunogens-

A) Schematic of design of BOAS nanoparticles. The eight original 8mer components were split into two 4mers, the first containing H1, H2, H3, and H4 (H1-H2-H3-H4), and the second containing H5, H7, H9, and B (H5-H7-H9-B). Each were expressed with an N-terminal SpyTag, then attached at equal molar ratios to an N-terminally fused SpyCatcher ferritin nanoparticle (SpyCatcher NP). This yields a ferritin nanoparticle with both 4mer BOAS conjugated to its surface (2x4mer BOAS NP). **B)** Dynamic light scattering (DLS) distributions of SpyCatcher NP and 2x4mer BOAS NP. Curves represent an average of 3 technical replicate measurements and error bars are ± 1 s.d. **C)** Size exclusion traces of SpyCatcher NP and 2x4mer BOAS NP on an S400 column. **D)** Representative NS-EM image of SpyCatcher NP. Arrows indicate individual nanoparticles, and the inset is a 2X zoom of the boxed area. Representative NS-EM image of BOAS NP. Arrows indicate individual nanoparticles, and the inset is a 2X zoom of the boxed area. **E)** ELISA of BOAS components, SpyCatcher NP, and 2x4mer BOAS NP with structure-dependent subtype-specific antibodies and a negative control stem-directed antibody, MEDI8852. Antibodies corresponding to each component are as follows: 5J8 (RBS, H1), 2G1 (RBS, H2), K03.12 (RBS, H3), P2-D9 (H4), H5.3 (RBS, H5), H7.167 (RBS periphery, H7), H1209 (RBS, B). ELISA data points are technical duplicates and error bars are ± 1 s.d.

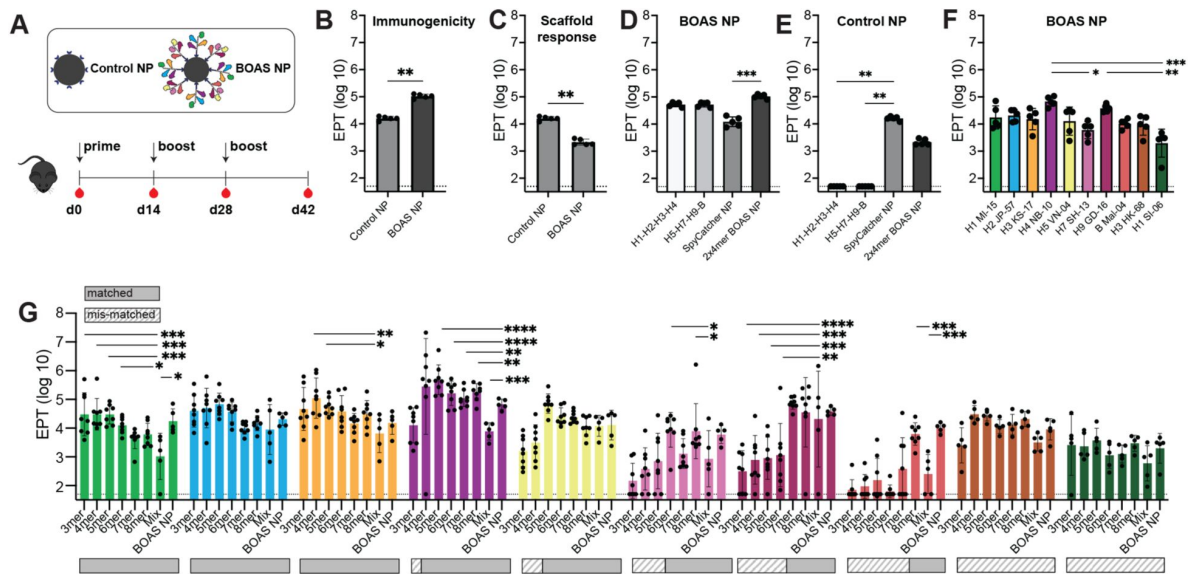


Figure 6

Murine Immunizations with BOAS NP

A) Schematic of immunization regimen with 2x4mer BOAS NP and SpyCatcher NP control. Mice were primed with 20 μ g of NP, followed by two homologous boosts two weeks apart. **B)** Serum reactivity to each immunizing antigen at d42. ** $p < 0.01$ as determined by Mann-Whitney test. **C)** Serum reactivity to Control NP scaffold by both groups. ** $p < 0.01$ as determined by Mann-Whitney test. **D)** Serum reactivity elicited by BOAS NP group ($n=5$) at d42 to both individual BOAS components (H1-H2-H3-H4 and H5-H7-H9-B), Control NP, and BOAS NP. *** $p < 0.001$ as determined by Kruskal-Wallis test with Dunn's multiple comparison post hoc test. **E)** Serum reactivity elicited by Control NP group ($n=5$) at d42 to both individual BOAS components (H1-H2-H3-H4 and H5-H7-H9-B), Control NP, and BOAS NP. ** $p < 0.01$ as determined by Kruskal-Wallis test with Dunn's multiple comparison post hoc test. **F)** Serum reactivity elicited by BOAS NP group ($n=5$) to individual full-length HA components of BOAS at d42. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ as determined by Kruskal-Wallis test with Dunn's multiple comparison post hoc test. **G)** Serum reactivity to each matched and mis-matched individual full-length HA component of BOAS (H1/MI/15 light green, H2/Jp/57 blue, H3/KS/17 light orange, H4/NB/10 purple, H5/VN/04 yellow, H7/SH/13 pink, H9/GD/16 maroon, B/Mal/04 salmon, H3/HK/68 dark orange, and H1/SI/06 dark green) by 3mer-8mer BOAS and BOAS NP. Solid bars below each plot indicate a matched sub-type, and striped bars indicate a mis-matched subtype (i.e. not present in the BOAS or NP). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ as determined by Kruskal-Wallis test with multiple comparisons relative to the mix control group. In all plots, data points for single time point antigen titers are from individual mice ($n=5$ for BOAS NP and Mix cohorts, $n=8$ for 3mer-8mer BOAS studies), bars represent mean titers and error bars are ± 1 s.d.

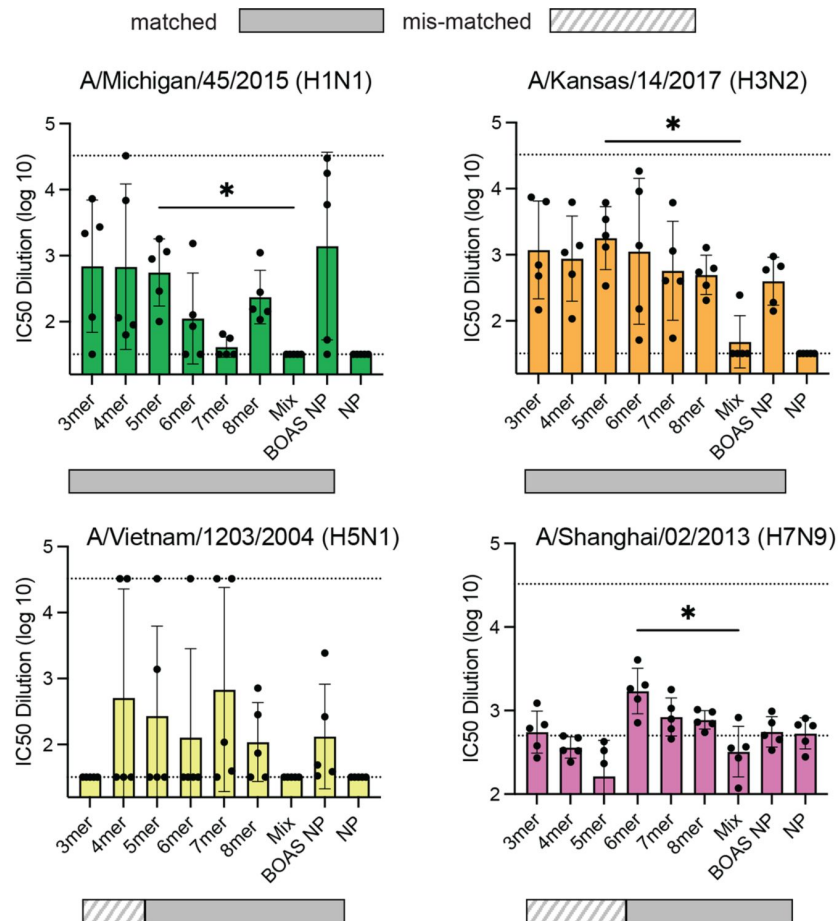


Figure 7

Microneutralization titers to matched and mis-matched virus-

Microneutralization of matched and mis-matched psuedoviruses: H1N1 (green, top left), H3N2 (orange, top right), H5N1 (yellow, bottom left), and H7N9 viruses (pink, bottom right) with d42 serum. Solid bars below each plot indicate a matched sub-type, and striped bars indicate a mis-matched subtype (i.e. not present in the BOAS). NP negative controls were used to determine threshold for neutralization. Upper and lower dashed lines represent the first dilution (1:32) (for H1N1, H3N2, and H5N1) or neutralization average with negative control NP serum (H7N9), and the last serum dilution (1:32,768), respectively, and points at the dashed lines indicate IC50s at or outside the limit of detection. Individual points indicate IC50 values from individual mice from each cohort (n=5). The mean is denoted by a bar and error bars are ± 1 s.d., * = $p < 0.05$ as determined by a Kruskal-Wallis test with Dunn's multiple comparison post hoc test relative to the mix group.

Discussion

Here we engineered “beads-on-a-string” (BOAS) that included tandemly linked, antigenically distinct HA heads as a single construct. This platform allows a mixing-and-matching of up to eight distinct HA heads from both influenza A and B viruses. Furthermore, we showed that the order and number of HA heads can vary without losing reactivity to conformation-specific mAbs *in vitro*, highlighting the flexibility of this platform. Mice immunized with BOAS had comparable serum reactivity to each individual component; though relative binding and neutralization titers varied between immunogens; this is likely a consequence of length and/or composition. Further oligomerization for increased multivalent display was accomplished by conjugating two 4mer BOAS inclusive of eight distinct HA heads to a ferritin nanoparticle via SpyTag/SpyCatcher ligation. Similar to the BOAS, these conjugated nanoparticles elicited similar titers to all eight HA components and could neutralize matched viruses.

Thus, tandemly linking HA heads is a robust method for displaying multiple influenza subtypes in a single protein-based immunogen. Binding titers were elicited to all components present in the immunogen, and there was no significant correlation between HA position within the BOAS (*i.e.*, terminal or internal) and immunogenicity. However, relative immunogenicities of each HA varied despite the guaranteed equimolar display of each subtype. There were qualitatively immunodominant HAs, notably H4 and H9, and these were relatively consistent across BOAS in which they were a component. This effect was reduced in the mix cohort. Further studies using the modularity of the BOAS could further deconvolute relative immunodominances of HA subtypes.

Despite similar binding titers across multiple BOAS lengths, expression levels and neutralization titers were quite variable. While all 3mer to 8mer BOAS could be overexpressed, expression inversely correlated with overall length. To mitigate this, multiple BOAS (*e.g.*, two 4mers) or conjugation to protein-based nanoparticles, as was done here, could be used to ensure coverage of each desired HA subtype. Furthermore, neutralization titers were quite variable across different BOAS lengths despite similar binding titers. This may be related to multiple factors including homology, stability, and accessibility of neutralizing epitopes for different BOAS lengths. Notably, for longer BOAS we observed degradation following longer term storage at 4C, which may reflect their overall stability. Studies manipulating BOAS composition at intermediate lengths could optimize neutralizing responses to particular influenzas of interest.

Based on the immunogenicity of the various BOAS and their ability to elicit neutralizing responses, it may not be necessary to maximize the number of HA heads into a single immunogen. Indeed, it qualitatively appears that the intermediate 4-, 5-, and 6mer BOAS were the most immunogenic and this length may be sufficient to effectively engage and crosslink BCR for potent stimulation. These BOAS also had similar or improved binding cross-reactivity to mis-matched HAs as compared to longer 7- or 8mer BOAS. Notably, the 3mer BOAS elicited detectable cross-reactive binding titers to H4 and H5 mismatched HAs in all mice. This observed cross-reactivity could be due to sequence conservation between the HAs, as H3 and H4 share ~51% sequence identity, and H1 and H2 share ~46% and ~62% overall sequence identity with H5, respectively (Supplemental Figure 7). Additionally, the degree of surface conservation decreased considerably beyond the 5mer as more antigenically distinct HAs were added to the BOAS. These data suggest that both antigenic distance between HA components and BOAS length play a key role in eliciting cross-reactive antibody responses, and further studies are necessary to optimize BOAS valency and antigenic distance for a desired response.

Potential enrichment of antibodies targeting the conserved RBS and TI epitopes may also be contributing to observed cross-reactivity. Both epitopes are relatively conserved across all BOAS lengths (**Figure 4C**), and the two BOAS showing the most cross-reactivity, the 3mer and 5mer, elicit a significant portion of the serum response toward both RBS and TI epitopes as determined via a serum competition assay with available epitope-directed antibodies (**Figure 4B**). Notably, this proportion is approximate, as at the time of reporting, antibodies that bind the receptor binding site of all components were not available. RBS-directed antibodies to the H4 and H9 component were not available, and the RBS-directed antibodies used targeting the other HA components have different footprints around the periphery of the RBS. Additionally, there are currently no reported influenza B TI-directed antibodies in the literature. Therefore, this may be an underestimate of the serum proportion focused to the conserved RBS and TI epitopes. Isolated TI-directed antibodies, in particular, can engage more than nine unique subtypes across both group 1 and 2 influenzas^{10,12}, and our monomeric head-based BOAS immunogens, have the otherwise occluded TI epitope exposed^{10–12}. Furthermore, we have previously shown that this TI epitope, when exposed, is immunodominant in the murine model¹⁴. Further studies with different combinations of HAs could aid in understanding how length and composition influences epitope focusing. For example, a BOAS design with a cluster of group 1 HAs followed by a cluster of group 2 HAs, rather than our roughly alternating pattern could impact which HAs are in close proximity to one other or could be potentially shielded in certain conformations, and thus could affect antigenic results. Combining the BOAS platform with other immune-focusing approaches³² such as hyperglycosylation^{14,31,61,62} or resurfacing^{30,63} could enhance cross-reactive responses. Additionally, modifying linker spacing and rigidity can also be used as a mechanism to enhance BCR cross-linking and thus enhance cross-reactive B cell activation and elicitation⁶⁴.

BOAS can be further multimerized via conjugation to a surface of a nanoparticle. Interestingly, this only had a marginal effect on immunogenicity. The BOAS conjugated-nanoparticle elicited titers of $\sim 10^5$ (**Figure 6B**), whereas the best BOAS alone reached an order of magnitude greater (**Figure 3C**). This appears in contrast with other studies where attaching an antigen to a nanoparticle scaffold enhanced immunogenicity and neutralization potency^{38,65–68}. One recent example designed quartets of antigenically distinct SARS-like betacoronavirus RBDs coupled to an mi3-VLP scaffold via a similar SpyCatcher/SpyTag system and showed increased binding and neutralization titers following conjugation to the NP compared to quartet alone⁴⁸. This discrepancy may be in part due to the larger mi3 nanoparticle⁶⁹ which displays 60 copies of the antigen rather than the 24 copies displayed on the ferritin nanoparticle used in this study. Nevertheless, HA-specific responses were similar whether the BOAS were conjugated to the nanoparticle or not, indicating that HA proximity to the NP surface did not impact responses to each component. This observation is consistent with betacoronavirus quartet nanoparticles as well. Additionally, BOAS conjugation to the nanoparticle significantly reduced the scaffold-directed response to near baseline. The addition of the large BOAS projections to the surface of the nanoparticle likely masked the immunogenic scaffold epitopes⁷⁰.

Collectively, this study demonstrates the versatility of the BOAS platform to present multiple HA subtypes as a single immunogen. This “plug-and-play” approach can readily exchange HAs to elicit desired immune responses. BOAS are potentially advantageous over other multivalent display platforms such as protein-based nanoparticles, which can produce off-target responses due to their inherent immunogenicity³⁸. Furthermore, when genetic fusions of the antigen to nanoparticles is not possible, SpyCatcher/SpyTag (or another suitable conjugation approach) must be used, further contributing to scaffold-specific responses as well as additional multi-step manufacturing and purification challenges. Not only does our BOAS platform circumvent these potential caveats, but because this is a single polypeptide chain, this immunogen could readily be formulated as an mRNA lipid nanoparticle (LNP)⁷¹. The BOAS platform forms the basis for next-generation influenza vaccines and can more broadly be readily adapted to other viral antigens.

Materials and methods

Beads-on-a-string (BOAS) immunogen and full-length hemagglutinin (HA) expression and purification

Hemagglutinin (HA) head for BOAS immunogens were designed based on the following sequences from the following subtypes: H1 (H1/A/Michigan/45/2015) (GenBank: AMA11475.1), H2 (H2/A/Japan/305/1957) (GenBank: AAA43185), H3 (H3/A/Kansas/14/2017) (GenBank: AVG71503), H4 (H4/A/American Black Duck/New Brunswick/00464/2010) (GenBank: AGG81749), H5 (H5/A/Viet Nam/1203/2004) (GenBank: ADD97095), H7 (H7/A/Shanghai/01/2014) (GenBank: AHK10800), H9 (H9/A/Guangdong/MZ058/2016) (GenBank: AOR17625.1), and B (B/Malaysia/2506/2004) (GenBank: ABU99194). Head subdomains from these HAs were used in the BOAS immunogens, and full-length soluble ectodomain (FLsE) trimers were used in ELISAs. Additional H1 (H1/A/Solomon Islands/3/2006) and H3 (H3/A/Hong Kong/1/1968) FLsEs were used in ELISAs as mismatched, antigenically distinct HAs for all BOAS. Sequences were codon optimized using IDT and subcloned into pVRC vectors for expression in mammalian cells. For BOAS immunogens, all HA heads were separated by a “GA(GSS)₄AS” spacer. Specific head domain regions for each subtype were selected based on expression levels in expi293F cells. All protein sequences contained a C-terminal 8x-His tag for purification with an upstream 3C-protease cleavage site. Full-length HA trimers contained an additional C-terminal foldon (Fd) trimerization domain for expression as soluble trimers. BOAS immunogens and HA trimers were transfected in expi293F cells using Expifectamine transfection reagent and enhancers based on manufacturer’s instructions. Five days post-transfection, supernatant was harvested and clarified via centrifugation, then purified on a TALON cobalt resin via the 8x-His Tag. Recovered proteins were then purified on a Superdex 200 (S200) Increase 10/300 GL (for trimeric HAs) or Superose 6 Increase 10/300 GL (for BOAS) size-exclusion column in Dulbecco’s Phosphate Buffered Saline (DPBS) within 48 hours of cobalt resin elution. Pooled fractions of BOAS were then treated with HRV-3C protease to remove tags for 16 hours at 4°C, then passed over a TALON cobalt resin to remove any remaining protease, cleaved tags, and uncleaved protein. Trimeric HAs were used for ELISAs with purification tags present.

IgG expression and purification

All antibody variable regions were codon optimized for mammalian cell expression and subcloned into pVRC vectors containing either heavy or light (kappa or lambda) with humanized constant regions. Equimolar heavy and light chain plasmids were co-transfected into HEK293F cells using polyethylenimine (PEI). Five days following transfection, supernatants were harvested, clarified via centrifugation, and purified on a Protein G resin. Purified antibodies were then buffer exchanged into DPBS for use in ELISA assays.

Nanoparticle Assembly

C-terminally SpyTagged BOAS and N-terminal SpyCatcher nanoparticles were subcloned into pVRC expression vectors, then expressed in expi293F cells and purified via TALON cobalt resin as above. SpyTagged BOAS were further purified via size-exclusion chromatography on a Superose 6 column, as above, followed by cleavage of the C-terminal His tag via 3C HRV protease to yield a C-terminal SpyTag. SpyCatcher nanoparticles were purified on a HiPrep Sephacryl S400 column in DPBS. Purified SpyCatcher nanoparticles were then mixed with an equimolar ratio mixture of each 4mer BOAS (H1, H2, H3, H4 and H5, H7, H9,B) at a 1.2 molar excess relative to the nanoparticle overnight at 4°C. The mixture was then re-purified over an S400 column, concentrated, aliquoted, and snap-frozen in liquid nitrogen until use.

Negative Staining procedure for TEM

5 μ l of the sample was adsorbed for 1 minute onto a carbon-coated grid made hydrophilic by a 20 second exposure to a glow discharge (25mA). Excess liquid was removed with filter paper (Whatman #1), the grid was then floated briefly on a drop of water (to wash away phosphate or salt), blotted again on a filter paper and then stained with 0.75% uranyl formate or 1% uranyl acetate for 20-30 seconds. After removing the excess stain with a filter paper the grids were examined in a JEOL 1200EX Transmission electron microscope or a TecnaiG² Spirit BioTWIN and images were recorded with an AMT 2k CCD camera.

Immunizations

C57BL/6 mice (Jackson Laboratory) (n=8 per group for 3-, 4-, 5-, 6-, 7-, and 8mer cohorts; n=5 for BOAS NP, NP, and mix cohorts) were immunized with 20 μ g of BOAS immunogens of varying length and adjuvanted with 50% Sigmas Adjuvant for a total of 100 μ L of inoculum. Immunogens and adjuvant were administered intramuscularly (IM) at day 0, day 14, and day 28. Serum samples were collected at days 0, 14, 28, and 42 (prior to immunogen administration). All experiments were conducted in 6-10 week old female mice under the institutional IACUC protocol (2014N000252).

Serum ELISAs

High-binding 96-well plates (Corning) were coated with 200ng per well of BOAS immunogen, nanoparticle, or trimeric HAs overnight at 4°C in DPBS. The following day, plates were blocked with 1% BSA in PBS-T (DPBS+0.1% Tween-20) for 1 hour at RT, rocking. Diluted serum in DPBS were generated starting at a 1:50 dilution, followed by 10-fold serial dilutions for a total of 7 dilutions. After one hour, blocking buffer was discarded, and 40 μ L of diluted serum was added and incubated for 1 hour at RT, rocking. Serum was then discarded, and plates were washed 3 times with PBS-T, after which 100 μ L of HRP-conjugated anti-mouse secondary antibody (Abcam) at a 1:20,000 dilution was added and incubated for 1 hour at RT rocking. Following secondary antibody incubation, plates were again washed 3 times with PBS-T. Slow TMB development solution was then added to plates and incubated for 30-40 mins at RT, then stopped with an equal volume of 0.2M sulfuric acid stop solution. Developed plates were then analyzed via absorbance measurements at 450nm. Each condition was blank subtracted with a secondary only control and plotted in GraphPad Prism 10 (v10.0.2). Serum endpoint titer (EPT) were determined using a non-linear regression (sigmoidal, four-parameter logistic (4PL) equation, where x is concentration) to determine the dilution at which dilution the blank-subtracted 450nm absorbance value intersect a 0.1 threshold. Serum titers for individual mice against respective antigens are reported as log transformed values of the EPT dilution.

Serum Competition ELISAs

Serum competition ELISAs were performed following a similar protocol to serum ELISAs described above. Following blocking with BSA in PBS-T, blocking solution was discarded and 40 μ L of either DPBS (no competition control), a cocktail of humanized antibodies targeting the RBS and periphery (5J8, 2G1, K03.12, H5.3, H7.167, H1209), a cocktail of humanized TI-directed antibodies (S5V2-29, D1 H1-17/H3-14, D2 H1-1/H3-1), or a negative control antibody (MEDI8852) were added at a concentration of 100 μ g/mL per antibody. Plates were incubated with competing antibodies or controls for 1 hour at RT. Serum from mice from respective BOAS cohorts were diluted by 1:5,000 (3mer and 6mer) or 1:10,000 (4mer, 5mer, 7mer, and 8mer) in DPBS, then 40 μ L added on top of competing antibody for a final dilution of 1:10,000 (3mer and 6mer) or 1:20,000 (4mer, 5mer, 7mer, and 8mer). These dilutions were selected based on a dilution range in serum ELISAs in which serum reactivity behaved linearly. Plates were incubated with serum and competing antibodies for an additional 1 hour at RT, washed 3 times with PBS-T, then incubated with an HRP-

conjugated human/bovine/horse cross-adsorbed anti-mouse secondary antibody (Southern Biotech) at a 1:20,000 dilution for 1 hour at RT. The remaining wash and development steps were performed as in the serum ELISA.

Microneutralization Assays

Serum microneutralization assays using R3 viruses followed the published protocol in Creanga et al.⁵¹ Briefly, MDCK-SIAT1-PB1/H5/H7 cells were thawed into D10 media with penicillin and streptomycin (P/S) and 10% heat-inactivated fetal bovine serum (FBS) three days prior to infection. Cells were seeded two days later at 1.5×10^4 cells/well in flu media (Opti-MEM + 0.01% FBS + P/S + 0.3% Bovine Serum Albumin (BSA) + Ca/Mg) overnight at 37°C. Serum was also treated with receptor destroying enzyme (RDE) II at a 1:3 dilution overnight at 37°C, followed by inactivation at 56°C for 30-60 mins. Treated serum was then diluted in media at 1:3 for a final starting dilution of 1:32. On the day of infection, cells were treated with 60µL of virus in flu media + TPCK-trypsin for 1 hour at 37°C with 5% CO₂. After one hour, media was removed via aspiration and cells were treated with 100µL treated serum. The following day, fluorescent foci were counted on a Zeiss Celldiscoverer 7 + LSM900 + Airyscan 2 (CD7+LSM900) to determine neutralization. Counts were normalized to a cell control and untreated virus control, and IC50 values for neutralization were determined via a fit to a 4PL non-linear regression in technical duplicate in Prism v10.0.2 (GraphPad).

Homology Analyses

Homology analyses on HA BOAS was conducted using a manual method analogous to Consurf.^{59,60} HA head domain structures (or closely related variants) were aligned in Pymol to the H2 JP-57 structure (PDB: 2WRE). Structures used for alignments were as follows: H1 (PDB: 6XGC), H3 (PDB: 4O5N), H4 (PDB: 5XL3), H5 (PDB: 6CFG), H7 (PDB: 6FYU), H9 (PDB: 1JSI), B (PDB: 4FQJ). Any mis-matches in specific amino acids were adjusted using the mutagenesis wizard in Pymol. Following structural alignment, each position on the HA head domain was scored for % aa identity at each position on a scale of 1-9, where 1 = 10-19% identical, 2 = 20-29% identical ... to 9 = 90-100% identical, which we refer to as a “conservation score”. This was repeated for each composition of BOAS (i.e. 3mer, 4mer, 5mer, ... 8mer). Each position on the H2 structure was then pseudo colored based on the conservation score. Average amino acid conservation scores were defined as the average of conservation score over the whole sequence (overall), or of amino acids within a given epitope. TI epitope amino acids were defined as residues 60, 86-106, and 212-232 (H1 numbering), and RBS epitope amino acids were defined as residues 94-96, 128-146, 153-161, 182-194, and 222-229 (H1 numbering).

Statistical Analysis

Significance for ELISAs and microneutralization assays were determined using Prism (GraphPad Prism v10.2.3). ELISAs comparing serum reactivity and microneutralization and comparing >2 samples were analyzed using a Kruskal-Wallis test with Dunn’s post-hoc test to correct for multiple comparisons. Multiple comparisons were made between each possible combination or relative to a control group, where indicated. ELISAs comparing two samples were analyzed using a Mann-Whitney test. Significance was assigned with the following: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$. Where conditions are compared and no significance is reported, the difference was non-significant.

Author information

Author Contributions

DTL and AGS designed research; DTL, FAMN, MLV, DPM, and LR performed research; DTL and AGS analyzed data; DTL and AGS wrote the paper. All authors edited and commented on the paper.

Competing financial interest

DTL and AGS have filed a provisional patent covering the work described here.

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Additional files

Supplemental Figures (Revised) [↗](#)

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Reviewer #2 (Public review):

Summary:

The authors describe a "beads-on-a-string" (BOAS) immunogen, where they link, using a non-flexible glycine linker, up to eight distinct hemagglutinin (HA) head domains from circulating and non-circulating influenzas and assess their immunogenicity. They also display some of their immunogens on ferritin NP and compare the immunogenicity. They conclude that this new platform can be useful to elicit robust immune responses to multiple influenza subtypes using one immunogen and that it can also be used for other viral proteins.

Strengths:

The paper is clearly written. While the use of flexible linkers has been used many times, this particular approach (linking different HA subtypes in the same construct resembling adding beads on a string, as the authors describe their display platform) is novel and could be of interest.

Comments on revisions:

The authors have addressed most comments. Some mistakes/issues remain:

TI should be defined earlier on line 61 not on line 196

No legend for Figure 3E - it looks like this is where the authors did the first immunization with the "mix" to compare to the BOAs but strangely they do not mention this in the response to reviewers letter and only mention fig 6G and 7

Maybe add "mix" to the title of Figure 3?

In Figure 6G they do show the response to the mix but do not mention it in the immunizations for that figure. Also weird because obviously the mix is not a NP while this figure addresses NP format.

Line 796 - pseudo viruses

The authors should add some clarification in the paper as they did in response to reviewers.

<https://doi.org/10.7554/eLife.97364.2.sa2>

Reviewer #3 (Public review):

This work describes the tandem linkage of influenza hemagglutinin (HA) receptor binding domains of diverse subtypes to create 'beads on a string' (BOAS) immunogens. They show that these immunogens elicit ELISA binding titers against full-length HA trimers in mice, as well as varying degrees of vaccine mismatched responses and neutralization titers. They also compare these to BOAS conjugated on ferritin nanoparticles and find that this did not largely improve immune responses. This work offers a new type of vaccine platform for influenza vaccines, and this could be useful for further studies on the effects of conformation and immunodominance on the resulting immune response.

Overall, the central claims of immunogenicity in a murine model of the BOAS immunogens described here are supported by the data.

Strengths included the adaptability of the approach to include several, diverse subtypes of HAs. The determination of an optimal composition of strains in the 5-BOAS that overall yielded the best immune responses was an interesting finding and one that could also be adapted to other vaccine platforms. Lastly, as the authors discuss, the ease of translation to an mRNA vaccine is indeed a strength of this platform.

One interesting and counter-intuitive result is the high levels of neutralization titers seen to vaccine-mismatched, group 2 H7 in the 5-BOAS group that differs from the 4-BOAS with the addition of a group 1 H5 RBD. At the same time, no H5 neutralization titers were observed for any of the BOAS immunogens, yet they were seen for the BOAS-NP. Uncovering where these immune responses are being directed and why these discrepancies are being observed would be informative future work.

There are a few caveats in the data that should be noted:

(1) 20 ug is a pretty high dose for a mouse and the majority of the serology presented is after 3 doses at 20 ug. By comparison, 0.5-5 ug is a more typical range (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6380945/>, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9980174/>). Also, the authors state that 20 ug per immunogen was used, including for the BOAS-NP group, which would mean that the BOAS-NP group was given a lower gram dose of HA RBD relative to the BOAS groups.

(2) Serum was pooled from all animals per group for neutralization assays, instead of testing individual animals. This could mean that a single animal with higher immune responses than the rest in the group could dominate the signal and potentially skew the interpretation of this data.

(3) In Figure S2, it looks like an apparent increase in MW by changing the order of strains here, which may be due to differences in glycosylation. Further analysis would be needed to determine if there are discrepancies in glycosylation amongst the BOAS immunogens and how those differ from native HAs.

Comments on revisions:

The authors have addressed all concerns upon revision.

<https://doi.org/10.7554/eLife.97364.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Reviews:

Reviewer #1 (Public Review):

Summary:

In this manuscript by Thornlow Lamson et al., the authors develop a "beads-on-a-string" or BOAS strategy to link diverse hemagglutinin head domains, to elicit broadly protective antibody responses. The authors are able to generate varying formulations and lengths of the BOAS and immunization of mice shows induction of antibodies against a broad range of influenza subtypes. However, several major concerns are raised, including the stability of the BOAS, that only 3 mice were used for most immunization experiments, and that important controls and analyses related to how the BOAS alone, and not the inclusion of diverse heads, impacts humoral immunity.

Strengths:

Vaccine strategy is new and exciting.

Analyses were performed to support conclusions and improve paper quality.

Weaknesses:

Controls for how different hemagglutinin heads impact immunity versus the multivalency of the BOAS.

Only 3 mice were used for most experiments.

There were limited details on size exclusion data.

We appreciate the reviewer's comments and have made the following changes to the manuscript.

(1) We recognize that deconvoluting the effect of including a diverse set of HA heads and multivalency in the BOAS immunogens is necessary to understand the impact on antigenicity. Therefore, we now include a cocktail of the identical eight HA heads used in the 8-mer and BOAS nanoparticle (NP) as an additional control group. While we observed similar HA binding titers relative to the 8-mer and BOAS NP groups, the cocktail group-elicited sera was unable to neutralize any of the viruses tested; multivalency thus appears to be important for eliciting neutralizing responses

(2) We increased the sample size by repeated immunizations with n=5 mice, for a total of n=8 mice across two independent experiments.

(3) We expanded the details on size exclusion data to include:

a) extended chromatograms from Figure 2C as Supplemental Figure 3.

b) additional details in the materials and methods section (lines 370-372):

"Recovered proteins were then purified on a Superdex 200 (S200) Increase 10/300 GL (for trimeric HAs) or Superose 6 Increase 10/300 GL (for BOAS) size-exclusion column in Dulbecco's Phosphate Buffered Saline (DPBS) within 48 hours of cobalt resin elution."

Reviewer #2 (Public Review):

Summary:

The authors describe a "beads-on-a-string" (BOAS) immunogen, where they link, using a non-flexible glycine linker, up to eight distinct hemagglutinin (HA) head domains from circulating and non-circulating influenzas and assess their immunogenicity. They also display some of their immunogens on ferritin NP and compare the immunogenicity. They conclude that this new platform can be useful to elicit robust immune responses to multiple influenza subtypes using one immunogen and that it can also be used for other viral proteins.

Strengths:

The paper is clearly written. While the use of flexible linkers has been used many times, this particular approach (linking different HA subtypes in the same construct resembling adding beads on a string, as the authors describe their display platform) is novel and could be of interest.

Weaknesses:

The authors did not compare to individuals HA ionized as cocktails and did not compare to other mosaic NP published earlier. It is thus difficult to assess how their BOAS compare.

Other weaknesses include the rationale as to why these subtypes were chosen and also an explanation of why there are different sizes of the HA1 construct (apart from expression). Have the authors tried other lengths? Have they expressed all of them as FL HA1?

We appreciate the reviewer's comments. We responded to the concerns below and modified the manuscript accordingly.

(1) We recognize that including a "cocktail" control is important to understand how the multivalency present in a single immunogen affects the immune response. We now include an additional control group comprised of a mixture of the same eight HA heads used in the 8-mer and the BOAS nanoparticle (NP). While this cocktail elicited similar HA binding titers relative to the 8-mer and BOAS NP immunogens (Fig. 6G), there was no detectable neutralization any of the viruses tested (Fig. 7).

(2) In the introduction we reference other multivalent display platforms but acknowledge that distinct differences in their immunogen design platforms make direct comparisons to ours difficult—which is ultimately why we did not use them as comparators for our *in vivo* studies. Perhaps most directly relevant to our BOAS platform is the mosaic HA NP from Kanekiyo *et al.* (PMID 30742080). Here, HA heads, with similar boundaries to ours, were selected from historical H1N1 strains. These NPs however were significantly less antigenic diverse relative to our BOAS NPs as they did not include any group 2 (e.g., H7, H9) or B influenza HAs; restricting their multivalent display to group 1 H1N1s likely was an important factor in how they were able to achieve broad, neutralizing H1N1 responses. Additionally, Cohen *et al.* (PMID 33661993) used similarly antigenically distinct HAs in their mosaic NP, though these included full-length HAs with the conserved stem region, which likely has a significant impact on the elicited cross-reactive responses observed. Lastly, we reference Hills *et al.* (PMID 38710880), where authors designed similar NPs with four tandemly-linked betacoronavirus receptor binding domains (RBDs) to make "quartets". In contrast to our observations, the authors observed increased binding and neutralization titers following conjugation to protein-based NPs. We acknowledge potential differences between the studies, such as the antigen and larger VLP NP, that could lead to the different observed outcomes.

(3) We intended to highlight the "plug-and-play" nature of the BOAS platform; theoretically any HA subtype could be interchanged into the BOAS. To that end, our rationale for selecting the HA subtypes in our proof-of-principle immunogen was to include an antigenically diverse

set of circulating and non-circulating HAs that we could ultimately characterize with previously published subtype-specific antibodies that were also conformation-specific. In doing so, these diagnostic antibodies could confirm presence and conformation integrity of each component. We intentionally did not include HA subtypes that we did not have a conformation-specific antibody for.

The different sizes of HA head domains was determined exclusively by expression of the recombinant protein. We have not attempted expression of full-length HA1 domains. Furthermore, we have not attempted to express the full-length HA (inclusive of HA1 and HA2) in our BOAS platform. The primary reason was to avoid including the conserved stem region of HA2 which may distract from the HA1 epitopes (e.g., receptor binding site, lateral patch) that can be engaged by broadly neutralizing antibodies. Additionally, the full-length HA is inherently trimeric and may not be as amenable to our BOAS platform as the monomeric HA1 head domain.

Reviewer #3 (Public Review):

This work describes the tandem linkage of influenza hemagglutinin (HA) receptor binding domains of diverse subtypes to create 'beads on a string' (BOAS) immunogens. They show that these immunogens elicit ELISA binding titers against full-length HA trimers in mice, as well as varying degrees of vaccine mismatched responses and neutralization titers. They also compare these to BOAS conjugated on ferritin nanoparticles and find that this did not largely improve immune responses. This work offers a new type of vaccine platform for influenza vaccines, and this could be useful for further studies on the effects of conformation and immunodominance on the resulting immune response.

Overall, the central claims of immunogenicity in a murine model of the BOAS immunogens described here are supported by the data.

Strengths included the adaptability of the approach to include several, diverse subtypes of HAs. The determination of the optimal composition of strains in the 5-BOAS that overall yielded the best immune responses was an interesting finding and one that could also be adapted to other vaccine platforms. Lastly, as the authors discuss, the ease of translation to an mRNA vaccine is indeed a strength of this platform.

One interesting and counter-intuitive result is the high levels of neutralization titers seen in vaccine-mismatched, group 2 H7 in the 5-BOAS group that differs from the 4-BOAS with the addition of a group 1 H5 RBD. At the same time, no H5 neutralization titers were observed for any of the BOAS immunogens, yet they were seen for the BOAS-NP. Uncovering where these immune responses are being directed and why these discrepancies are being observed would constitute informative future work.

There are a few caveats in the data that should be noted:

(1) 20 ug is a pretty high dose for a mouse and the majority of the serology presented is after 3 doses at 20 ug. By comparison, 0.5-5 ug is a more typical range (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6380945/>, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9980174/>). Also, the authors state that 20 ug per immunogen was used, including for the BOAS-NP group, which would mean that the BOAS-NP group was given a lower gram dose of HA RBD relative to the BOAS groups.

We agree that this is on the “upper end” of recombinant protein dose. While we did not do a dose-response, we now include serum analyses after a single prime. The overall trends and reactivity to matched and mis-matched BOAS components remained similar across days d28 and d42. However, the differences between the BOAS and BOAS NP groups and the mixture group were more pronounced at d28, which reinforces our observation that the multivalency

of the HA heads is necessary for eliciting robust serum responses to each component. These data are included in Supplemental Figure 5, and we've modified the text (lines 185-187) to include;

“Similar binding trends were also observed with d28 serum, though the difference between the 8mer and mix groups was more pronounced at d28 (Supplemental Figure 5).”

Additionally, we acknowledge that there is a size discrepancy between the BOAS NP and the largest BOAS, leading to an approximately ~15-fold difference on a per mole basis of the BOAS immunogen. The smallest and largest BOAS also differ by ~2.5-fold on a per mole basis; this could favor the overall amount of the smaller immunogens, however because vaccine doses are typically calculated on a mg per kg basis, we did not calculate on a molar basis for this study. Any promising immunogens will be evaluated in dose-response study to optimize elicited responses.

(2) Serum was pooled from all animals per group for neutralization assays, instead of testing individual animals. This could mean that a single animal with higher immune responses than the rest in the group could dominate the signal and potentially skew the interpretation of this data.

We repeated the neutralization assays with data points for individual mice. There does appear to be variability in the immune response between mice. This is most noticeable for responses to the H5 component. We are currently assessing what properties of our BOAS immunogen might contribute to the variability across individual mice.

(3) In Figure S2, it looks like an apparent increase in MW by changing the order of strains here, which may be due to differences in glycosylation. Further analysis would be needed to determine if there are discrepancies in glycosylation amongst the BOAS immunogens and how those differ from native HAs.

There does appear to be a relatively small difference in MW between the two BOAS configurations shown in **Figure S2**. This could be due to differences in glycosylation, as the reviewer points out, and in future studies, we intend to assess the influence of native glycosylation on antibody responses elicited by our BOAS immunogens.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Major Concerns

(1) From Figure 2D-E, it looks like BOAS are forming clusters, rather than a straight line. Do these form aggregates over time? Both at 4 degrees over a few days or after freeze-thaw cycle(s)? It is unclear from the SEC methods how long after purification this was performed and stability should be considered.

Due to the inherent flexibility of the Gly-Ser linker between each component we do not anticipate that any rigidity would be imposed resulting in a “straight line”. Nevertheless, we appreciate the reviewers concern about the long-term stability of the BOAS immunogens. To address this, we include 1) the extended chromatograms from Figure 2C as Supplemental Figure 3 to show any aggregates present, 2) traces from up to 48 hours post-IMAC, and 3) chromatograms following a freeze-thaw cycle. Post-IMAC purification there is a minor (<10% total peak height) at ~9mL corresponding to aggregation. Note, we excluded this aggregation for immunizations. Post freeze-thaw cycle, we can see that upon immediate (<24hrs) thawing, the BOAS maintain a homogeneous peak with no significant (<10%) aggregation or

degradation peak. However, after ~1 week post-freeze-thaw cycle at 4C, additional peaks within the chromatogram correspond to degradation of the BOAS.

We modified the materials and methods section to state (lines 370-372)

“Recovered proteins were then purified on a Superdex 200 (S200) Increase 10/300 GL (for trimeric HAs) or Superose 6 Increase 10/300 GL (for BOAS) size-exclusion column in Dulbecco’s Phosphate Buffered Saline (DPBS) within 48 hours of cobalt resin elution.”

We commented on BOAS stability in the results section (lines 142-148)

“Following SEC, affinity tags were removed with HRV-3C protease; cleaved tags, uncleaved BOAS, and His-tagged enzyme were removed using cobalt affinity resin and snap frozen in liquid nitrogen before immunizations. BOAS maintained monodispersity upon thawing, though over time, degradation was observed following longer term (>1 week) storage at 4C (Supplemental Figure 3). This degradation became more significant as BOAS increased in length (Supplemental Figure 3).”

We also included in the discussion (lines 277-279):

“Notably, for longer BOAS we observed degradation following longer term storage at 4C, which may reflect their overall stability.”

(2) Figures 3-4 and 6-7, to make conclusions off of 3 mice per group is inappropriate. A sample size calculation should have been conducted and the appropriate number of mice tested. In addition, two independent mouse experiments should always be performed. Moreover, the reliability of the statistical tests performed seems unlikely, given the very small sample size.

We agree that additional mice are necessary to make assessments regarding immunogenicity and cross-reactivity differences between the immunogens. To address this, we repeated the immunization with 5 additional mice, for a total of n=8 mice over two independent experiments. We incorporated these data into Figure 3B-D, as well as an additional Figure 3E (see below). We also now report the log-transformed endpoint titer (EPT) values rather than reciprocal EC50 values and added clarity to statistical analyses used. We have added the following lines to the methods section

lines 427-431:

“Serum endpoint titer (EPT) were determined using a non-linear regression (sigmoidal, four-parameter logistic (4PL) equation, where x is concentration) to determine the dilution at which dilution the blank-subtracted 450nm absorbance value intersect a 0.1 threshold. Serum titers for individual mice against respective antigens are reported as log transformed values of the EPT dilution.”

lines 406-408:

“C57BL/6 mice (Jackson Laboratory) (n=8 per group for 3-, 4-, 5-, 6-, 7-, and 8mer cohorts; n=5 for BOAS NP, NP, and mix cohorts) were immunized with 20µg of BOAS immunogens of varying length and adjuvanted with 50% Sigmas Adjuvant for a total of 100µL of inoculum.”

lines 482-490:

“Statistical Analysis

Significance for ELISAs and microneutralization assays were determined using Prism (GraphPad Prism v10.2.3). ELISAs comparing serum reactivity and microneutralization and comparing >2 samples were analyzed using a Kruskal-Wallis test with Dunn’s post-hoc test to

correct for multiple comparisons. Multiple comparisons were made between each possible combination or relative to a control group, where indicated. ELISAs comparing two samples were analyzed using a Mann-Whitney test. Significance was assigned with the following: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$. Where conditions are compared and no significance is reported, the difference was non-significant.”

(3) One critical control that is missing is a homogenous BOAS, for example, just linking one H1 on a BOAS. Does oligomerization and increasing avidity alone improve humoral immunity?

We agree that this is an interesting point, However, to address the impact of oligomerization and avidity on humoral immunity, we now include an additional control with a cocktail of HA heads used in the 8mer. We have incorporated this into Figure 3A, 3D and 3E, Figure 6G, and Figure 7.

Additionally, we have added the following lines in the manuscript:

lines 38-40:

“Finally, vaccination with a mixture of the same HA head domains is not sufficient to elicit the same neutralization profile as the BOAS immunogens or nanoparticles.”

lines 105-106:

“Additionally, we showed that a mixture of the same HA head components was not sufficient to recapitulate the neutralizing responses elicited by the BOAS or BOAS NP.”

lines 169-172:

“To determine immunogenicity of each BOAS immunogen, we performed a prime-boost-boost vaccination regimen in C5BL/6 mice at two-week intervals with 20µg of immunogen and adjuvanted with Sigma Adjuvant (Figure 3A). We compared these BOAS to a control group immunized with a mixture of the eight HA heads present in the 8mer.”

lines 265-267:

“There were qualitatively immunodominant HAs, notably H4 and H9, and these were relatively consistent across BOAS in which they were a component. This effect was reduced in the mix cohort.”

(4) While some cross-reactivity is likely (Figure 6G), there is considerable loss of binding when there is a mismatch. Of the antibodies induced, how much of this is strain-specific? For example, how well do serum antibodies bind to a pre-2009 H1?

We agree with the reviewer that there is a considerable loss of binding when there is a mismatched HA component. To better understand this and incorporate a mismatched strain into our analysis of the 8mer and BOAS NP, we looked at serum binding titers to a pre-2009 H1, H1/Solomon Islands/2006, and an antigenically distinct H3, H3/Hong Kong/1968. We have incorporated this data into Figures 3D, 3E, 6F and 6G. We observed relatively high titers against both a mismatched H1 and H3, indicating that the BOAS maintain high titers against subtype-specific strains that are conserved over considerable antigenic distance. However, this was similar in the mixture group, indicating that this may not be specific to oligomerization of BOAS immunogens.

We added the following to the methods section:

lines 357-361

“Head subdomains from these HAs were used in the BOAS immunogens, and full-length soluble ectodomain (FLsE) trimers were used in ELISAs. Additional H1 (H1/A/Solomon Islands/3/2006) and H3 (H3/A/Hong Kong/1/1968) FLsEs were used in ELISAs as mismatched, antigenically distinct HAs for all BOAS.”

Minor Concerns

(1) Line 44-46, the deaths per year are almost exclusively due to seasonal influenza outbreaks caused by antigenically drifted viruses in humans, not those spilling over from avian sp. and swine. For accuracy, please adjust this sentence.

We have adjusted lines 45-48 to say “This is largely a consequence of viral evolution and antigenic drift as it circulates seasonally within humans and ultimately impacts vaccine effectiveness. Additionally, the chance for spillover events from animal reservoirs (e.g., avian, swine) is increasing as population and connectivity also increase.”

(2) Figure 4D-E, provide a legend for what the symbols indicate, or simply just put the symbol next to either the homology score and % serum competition labels on the y-axis.

We have included a legend in Figures 4D,E to distinguish between homology score and % serum competition

(3) I am a bit confused by the data presented in Figure 7. The figure legend says the two symbols represent technical replicates. How? Is one technical replicate of all the mice in a group averaged and that's what's graphed? If so, this is not standard practice. I would encourage the authors to show the average technical replicates of each animal, which is standard.

We thank the reviewer for their suggestion, and we have revised Figure 7 such that each symbol represents a single animal for n=5 animals. We have also adjusted the figure caption to the following:

“Figure 7: Microneutralization titers to matched and mis-matched virus- Microneutralization of matched and mis-matched psuedoviruses: H1N1 (green, top left), H3N2 (orange, top right), H5N1 (yellow, bottom left), and H7N9 viruses (pink, bottom right) with d42 serum. Solid bars below each plot indicate a matched sub-type, and striped bars indicate a mis-matched subtype (i.e. not present in the BOAS). NP negative controls were used to determine threshold for neutralization. Upper and lower dashed lines represent the first dilution (1:32) (for H1N1, H3N2, and H5N1) or neutralization average with negative control NP serum (H7N9), and the last serum dilution (1:32,768), respectively, and points at the dashed lines indicate IC50s at or outside the limit of detection. Individual points indicate IC50 values from individual mice from each cohort (n=5). The mean is denoted by a bar and error bars are ± 1 s.d., * = $p < 0.05$ as determined by a Kruskal-Wallis test with Dunn’s multiple comparison post hoc test relative to the mix group.”

(4) Paragraphs 298-313, multiple studies are referred to but not referenced.

We have added the following references to this section:

(38) Kanekiyo, M. et al. Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* 498, 102–106 (2013).

(48) Hills, R. A. et al. Proactive vaccination using multiviral Quartet Nanocages to elicit broad anti-coronavirus responses. *Nat. Nanotechnol.* 1–8 (2024) doi:10.1038/s41565-024-01655-9.

- (65) Jardine, J. et al. Rational HIV immunogen design to target specific germline B cell receptors. *Science* 340, 711–716 (2013).
- (66) Tokatlian, T. et al. Innate immune recognition of glycans targets HIV nanoparticle immunogens to germinal centers. *Science* 363, 649–654 (2019).
- (67) Kato, Y. et al. Multifaceted Effects of Antigen Valency on B Cell Response Composition and Differentiation In Vivo. *Immunity* 53, 548-563.e8 (2020).
- (68) Marcandalli, J. et al. Induction of Potent Neutralizing Antibody Responses by a Designed Protein Nanoparticle Vaccine for Respiratory Syncytial Virus. *Cell* 176, 1420-1431.e17 (2019).
- (69) Bruun, T. U. J., Andersson, A.-M. C., Draper, S. J. & Howarth, M. Engineering a Rugged Nanoscaffold To Enhance Plug-and-Display Vaccination. *ACS Nano* 12, 8855–8866 (2018).
- (70) Kraft, J. C. et al. Antigen- and scaffold-specific antibody responses to protein nanoparticle immunogens. *Cell Reports Medicine* 100780 (2022) doi:10.1016/j.xcrm.2022.100780.

Reviewer #2 (Recommendations For The Authors):

Can the authors define "detectable titers"?

Maybe add a threshold value of reciprocal EC on the figure for each plot.

We recognize the reviewers concern with reporting serum titers in this way, and we have adjusted our reported titers as endpoint titers (EPT) with a dotted line for the first detectable dilution (1:50). We have also adjusted the methods section to reflect this change:

(lines 427-431)

“Serum endpoint titer (EPT) were determined using a non-linear regression (sigmoidal, four-parameter logistic (4PL) equation, where x is concentration) to determine the dilution at which dilution the blank-subtracted 450nm absorbance value intersect a 0.1 threshold. Serum titers for individual mice against respective antigens are reported as log transformed values of the EPT dilution.”

It also appears that not all X-mer elicits an immune response against matched HA, e.g. for the 7 and 8 -mer. Not sure why the authors do not mention this. It could be due to too many HAs, not sure.

We apologize for the confusion, and agree that our original method of reporting EC50 values does not reflect weak but present binding titers. Upon further analysis with additional mice as well as adjusting our method of reporting titers, it is easier to see in Figure 3D that all X-mer BOAS do indeed elicit binding detectable titers to matched HA components.

It will be nice to add a conclusion to the cross-reactivity - again it appears that past 6-mer there has been a loss in cross-reactivity even though there are more subtypes on the BOAS.

Also, the TI seemed to be the more conserved epitope targeted here.

(Of note these two are mentioned in the discussion)

We have updated the results section to include the following:

(lines 281-294)

“Based on the immunogenicity of the various BOAS and their ability to elicit neutralizing responses, it may not be necessary to maximize the number of HA heads into a single immunogen. Indeed, it qualitatively appears that the intermediate 4-, 5-, and 6mer BOAS were the most immunogenic and this length may be sufficient to effectively engage and crosslink BCR for potent stimulation. These BOAS also had similar or improved binding cross-reactivity to mis-matched HAs as compared to longer 7- or 8mer BOAS. Notably, the 3mer BOAS elicited detectable cross-reactive binding titers to H4 and H5 mismatched HAs in all mice. This observed cross-reactivity could be due to sequence conservation between the HAs, as H3 and H4 share ~51% sequence identity, and H1 and H2 share ~46% and ~62% overall sequence identity with H5, respectively (Supplemental Figure 6). Additionally, the degree of surface conservation decreased considerably beyond the 5mer as more antigenically distinct HAs were added to the BOAS. These data suggest that both antigenic distance between HA components and BOAS length play a key role in eliciting cross-reactive antibody responses, and further studies are necessary to optimize BOAS valency and antigenic distance for a desired response.”

Figure 5E, the authors could indicate which subtype each mab is specific to for those who are not HA experts. (They have them color-coded but it is hard to see because very small).

The authors also do not explain why 3E5 does not bind well to H1, H2, H3, H4 4-mer BOA, etc...

We apologize for the lack of clarity in this figure. We updated Figure 5E to include the subtype it is specific for as well as listing the antibodies and their subtype and targeted epitope in the figure caption.

Minor

Figure 1B zoom looks like the line is hidden to the structure - should come in front

We adjusted the figure accordingly.

Line 127 - whether the order

Corrected

What is the rationale for thinking that a different order will lead to a different expression and antigenic results?

We thank the reviewer for this question. We did not necessarily anticipate a difference in protein expression based on BOAS order. We, however, wanted to verify that our platform was indeed “plug-and-play” platform and we could readily exchange components and order. We do, however, hypothesize that a different order may in fact lead to different antigenic results. We think that the conformation of the BOAS as well as physical and antigenic distance of HA components may influence cross-linking efficiency of BCRs and lead to different antigenic results with different levels of cross-reactivity. For example, a BOAS design with a cluster of group 1 HAs followed by a cluster of group 2 HAs, rather than our roughly alternating pattern could impact which HAs are in proximity to each other or could be potentially shielded in certain conformations, and thus could affect antigenic results. We expand on this rationale in the discussion in lines 310-314:

“Further studies with different combinations of HAs could aid in understanding how length and composition influences epitope focusing. For example, a BOAS design with a cluster of group 1 HAs followed by a cluster of group 2 HAs, rather than our roughly alternating pattern

could impact which HAs are in close proximity to one other or could be potentially shielded in certain conformations, and thus could affect antigenic results.”

Maybe list HA#1 HA#2 HA#3 instead of HA1, HA2, HA3 to make sure it is not confounded with HA2 and HA2

We agree that this may be confusing for readers, and have adjusted Figure 1C to show HA#1, HA#2, etc.

For nsEM, do the authors have 2D classes and even 3D reconstructions? Line 148-149: maybe or just because there are more HAs.

We did not obtain 2D class or 3D reconstructions of these BOAS. However, we do agree with the reviewer that the collapsed/rosette structure of the 8mer BOAS may be a consequence of the additional HA heads as well as the flexible Gly-Ser linkers between the components. We have added clarify to our statement in the discussion to read:

lines 154-156:

“This is likely a consequence of the flexible GSS linker separating the individual HA head components as well as the addition of significantly more HA head components to the construct.”

Line 153 "interface-directed" - what does this mean?

We apologize for any confusion- we intend for “interface-directed” to refer antibodies that engage the trimer interface (TI) epitope between HA protomers. We have adjusted the manuscript to use the same terminology throughout, i.e. trimer interface or its abbreviation, TI.

For Figure 2 F - do you have a negative control? Usually one does not determine an ELISA KD, it is not very accurate but shows binding in terms of OD value.

We did include a negative control, MEDI8852, a stem-directed antibody, though it was not shown in the figure because we observed no binding, as expected. This negative control antibody was also used in Figure 5E for characterizing the BOAS NPs, and also shows no binding. We recognize that in an ELISA the KD is an equilibrium measurement and we do not report kinetic measurements as determined by a method such as bio-layer interferometry (BLI), and have this adjusted the figure caption to denote the values as “apparent K_D values”.

Line 169 - reads strangely, "BOAS-elicited serum, regardless of its length, reacted The length is the one of the Immunogen, not the serum

We agree that this statement is unclear, and we have modified the sentence to read:

lines 177-178:

“Each of the BOAS, regardless of its length, elicited binding titers to all matched full-length HAs representing individual components (Figure 3D).”

What is the adjuvant used (add in results)?

We used Sigma adjuvant for all immunizations, and have included this information in the results section:

lines 169-171:

“To determine immunogenicity of each BOAS, we performed a prime-boost-boost vaccination regimen in C5BL/6 mice at two-week intervals with 20µg of immunogen and adjuvanted with Sigma Adjuvant (Figure 3A).”

This information is also included in the methods section in lines 406-412.

| *Line 178 - remove " across"*

We have removed the word “across” in this sentence and replaced it with “on” (line 194)

| *Trimer- interface, and interface epitopes are used exchangeably - maybe keep it as trimer interface to be more precise*

As stated above, we have adjusted the manuscript to use the same term throughout, *i.e.*, trimer interface or its abbreviation, TI.

| *Line 221 - no figure 6H (6G?)*

We apologize for this typo and have corrected to Figure 6G (line 231)

Reviewer #3 (Recommendations For The Authors):

(1) Since 20 ug x3 doses is quite a high amount of vaccine, differences between immunogens may become blurred. Thus, it may be informative to compare post-prime serology for all immunogens or select immunogens to compare to the post-3rd dose data.

We agree with the reviewer that this is on the upper end of vaccine dose and thus we explored the serum responses after a single boost. The overall trends and reactivity to matched and mis-matched BOAS components remained similar across days d28 and d42. However, the differences between the BOAS and BOAS NP groups and the mixture group were more pronounced at d28, which bolsters our claim that the presentation of the HA heads is important for eliciting strong serum responses to all components. We have included this data in Supplemental Figure 5, and have acknowledged this in the text:

lines 185-187:

“Similar binding trends were also observed with d28 serum, though the difference between the 8mer and mix groups was more pronounced at d28 (Supplemental Figure 5).”

| *(2) Significance statistics for all immunogenicity data should be added and discussed; it is particularly absent in Figures 3D and 7.*

We have added statistical analyses to Figure 3 and Figure 7 to reflect changes in immunogenicity. We have also added the following to the methods section:

lines 482-490:

“Statistical Analysis

Significance for ELISAs and microneutralization assays were determined using either a Mann-Whitney test or a Kruskal-Wallis test with Dunn’s post-hoc test in Prism (GraphPad Prism v10.2.3) to correct for multiple comparisons. Multiple comparisons were made between each possible combination or relative to a control group, where indicated. Significance was assigned with the following: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** =

$p < 0.0001$. Where conditions are compared and no significance is reported, the difference was non-significant.”

(3) Figure 2F: the figure has K03.12 listed for the H3-specific mAb and in the main text, but the caption says 3E5 - is the 3E5 in the caption a typo? 3E5 is listed for the competition ELISAs as an RBS mAb, but its binding site is distal to the RBS at residues 165-170 (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9787348/>), H7.167 binds in the RBS periphery and not directly within the RBS, and the epitope for P2-D9 is undetermined/not presented. This could mean that there is actually a higher proportion of RBS-directed antibodies than what is determined from this serum competition data. Also, reference to these as 'RBS-directed' in the serum competition methods section should be revised for accuracy.

We sincerely apologize for this error and the resulting confusion. 3E5 in the caption is incorrect and should be K03.12 (<https://www.rcsb.org/structure/5W08>) and does engage the receptor binding site. We also apologize for the oversight that H7.167 is in the RBS periphery and not directly in the RBS. The additional P2-D9 in the panel of RBS-directed antibodies was also in error, as we do not believe it is RBS-directed, but is indeed H4 specific. We also included a reference to the paper and immunogen that elicited this antibody. We agree that this indicates that there could be a higher proportion of RBS-directed antibodies in the serum and have modified the text in the results and methods sections to read:

lines 300-306:

“Notably, this proportion is approximate, as at the time of reporting, antibodies that bind the receptor binding site of all components were not available. RBS-directed antibodies to the H4 and H9 component were not available, and the RBS-directed antibodies used targeting the other HA components have different footprints around the periphery of the RBS. Additionally, there are currently no reported influenza B TI-directed antibodies in the literature. Therefore, this may be an underestimate of the serum proportion focused to the conserved RBS and TI epitopes.”

lines 435-439:

“Following blocking with BSA in PBS-T, blocking solution was discarded and 40µL of either DPBS (no competition control), a cocktail of humanized antibodies targeting the RBS and periphery (5J8, 2G1, K03.12, H5.3, H7.167, H1209), a cocktail of humanized TI-directed antibodies (S5V2-29, D1 H1-17/H3-14, D2 H1-1/H3-1), or a negative control antibody (MEDI8852) were added at a concentration of 100µg/mL per antibody.”

(4) Only nsEM data is shown for the 3-BOAS and 8-BOAS, where differences in morphology were seen between these longer and shorter proteins. Including nsEM images for all BOAS immunogens may show trends in morphology or organization that could correlate with immune responses, e.g. if the 5-BOAS also forms a higher proportion of rosette-like structures, while the 4-BOAS is still a mix between extended and rosette-like, this could be a factor in the better immune responses seen for 5-BOAS.

We appreciate the reviewer’s suggestion for further analysis of morphology between the intermediate BOAS sizes. We agree that the relationship between BOAS length and morphology should be explored more in depth, and we intend to do so in future studies and to also vary linker length and rigidity.

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