Biosynthesis of Self-Assembled Proteinaceous Nanoparticles for Vaccination

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Recent years have seen enormous advances in nanovaccines for both prophylactic and therapeutic applications, but most of these technologies employ chemical or hybrid semi-biosynthetic production methods. Thus, production of nanovaccines has to date failed to exploit biology-only processes like complex sequential post-translational biochemical modifications and scalability, limiting the realization of the initial promise for offering major performance advantages and improved therapeutic outcomes over conventional vaccines. A Nano-B5 platform for in vivo production of fully protein-based, self-assembling, stable nanovaccines bearing diverse antigens including peptides and polysaccharides is presented here. Combined with the self-assembly capacities of pentamer domains from the bacterial AB₅ toxin and unnatural trimer peptides, diverse nanovaccine structures can be produced in common Escherichia coli strains and in attenuated pathogenic strains. Notably, the chassis of these nanovaccines functions as an immunostimulant. After showing excellent lymph node targeting and immunoresponse elicitation and safety performance in both mouse and monkey models, the strong prophylactic effects of these nanovaccines against infection, as well as their efficient therapeutic effects against tumors are further demonstrated. Thus, the Nano-B5 platform can efficiently combine diverse modular components and antigen cargos to efficiently generate a potentially very large diversity of nanovaccine structures using many bacterial species.

In its own right, vaccinology has been undergoing a revolution, and there are now a large number of innovative projects seeking to develop both prophylactic and therapeutic vaccines against diseases such as Hepatitis B, influenza, HIV, and cancers.[4–6] Generally speaking, the major advantages conferred by nanovaccines include improving stability by protecting antigens from premature degradation, providing good adjuvant properties, and assisting in the targeted delivery of an antigen to antigen-presenting cells (APCs).[7] A large variety of nanoscale materials have been deployed in nanovaccine designs. Seminal work with inorganic nanoparticles (NPs, e.g., gold, carbon, and silica) established the capacity of nanovaccine-bound antigens to elicit desired immune responses. Subsequent technologies have elaborated beyond inorganic NPs, for example, use of inorganic/organic hybrid NPs (e.g., PEI adopted silica NPs and biomimetic magnetosomes) to enhance antigen immunogenicity.[8,9] Recently, new types of organic NPs (e.g., lipoprotein-mimicking nanodisks, pickering emulsions, and nanogels) have also received great attention for their applications in vaccines.[10–16] The last decade has seen the widespread application of nanotechnologies to vaccinology to such an extent that there is now a very active research area known as “nanovaccinology.”[1–3] The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adma.202002940.

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These developments have generated structurally flexible materials that enable the fine tuning of physicochemical properties.

The production of almost all of these nanovaccine technologies presently use synthetic (chemical) methods or require hybrid production sequences which couple synthetic methods to some cell-based semi-biosynthesis step. As engineered bacterial and mammal cells should in theory enable the complete biosynthesis of protein, several promising proteinaceous nanovaccines are being developed with virus-like particles, 60-meric nanoparticles, ferritin, encapsulin, and so on.[17–25] In addition to the obvious potential advantages including reduced production times and costs, a fully in vivo production approach also holds promise for capacities that cannot be achieved with previous nanovaccines, including higher biocompatibility and safety. However, this strategy still has to face the difficulty of displaying versatile antigens on these proteinaceous nanoparticles, especially glycan antigens with complex structures.

We envisioned that an all-protein design for nanovaccines would enable the genetic engineering of NPs bearing potentially vast number of antigens, including peptide and also polysaccharide antigens because of the ability to incorporate recently reported protein glycan coupling technologies into the design of an in vivo nanovaccine production concept.[26] Specifically, building from two orthogonal protein-based self-assembly modular domains—the B₅ subunits of bacterial AB₅ family toxins with well-known adjuvant properties and a trimer forming peptide—we have designed the rudiments of a highly versatile,[27] modular nanovaccine platform (Nano-B5) that facilitates combinations of modular parts and antigen cargos to produce, inside cells of diverse bacterial species, a large and expandable diversity of nanovaccine structures (Figure 1a,b). By efficient lymph node draining after vaccination, the antigens bearing on these nanovaccines could be well presented by APCs for T cell activation (Figure 1c). Subsequent mouse and monkey experiments showed strong prophylactic effects against infection as well as efficient therapeutic effects against tumors. Our approach thus offers an attractive platform technology for combining diverse self-adjuvant modular chassis components and antigen cargos to generate various high-performance nanovaccines.

We started by expressing a simple module fusion monomer lacking any antigen sequence in standard Escherichia coli DH5α cells periplasmic space, whose oxidation environment is highly conducive to the soluble expression and correct folding of proteins. The construct for the fusion monomer was driven by an isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible promoter and comprised sequences encoding the (19 aa) signal peptide of the DsbA for targeting to the periplasm, a 6× His tag to facilitate purification and detection in cells with antibody-based methods, an 103 aa B₅ subunits of cholera toxin (CTB) as an intra-adjuvant modular, a 4 aa linker peptide (GGSG), and a C-terminal trimer-forming peptide to facilitate self-assembling proteinaceous NP formation.[28] After transforming DH5α cells with this plasmid (pCTBTri, see Table S1 in the Supporting Information) and inducing expression of the modular fusion

![Figure 1. Design for the in vivo production of modular and self-assembling nanovaccines. a) Modular design scheme of the nanovaccines and their versatile application configurations. b) Cell-based fabrication of nanovaccines by expressing a fusion protein containing the B-subunit of AB₅ toxin and a trimer-forming peptide in vivo. Peptide and polysaccharide antigens can be connected to the NP chassis in vivo via, respectively, expression of fusion domains and glycosylation of the 4573 site by a bacterial O-oligosaccharyltransferase. c) Following vaccination, the nanovaccines quickly drain to the lymph node and activate APCs, leading to potent humoral and cellular immune responses.](image-url)
monomer protein with IPTG, transmission electron microscopy (TEM) analysis revealed an obvious morphological difference in the fusion-monomer-expressing cells versus the same strain without IPTG induction (Figure 2a). Specifically, the cells expressing the fusion monomers had substantially enlarged gaps between their inner and outer membrane, clearly indicating that the designed NPs were successfully expressed and localized at the periplasmic space of the bacteria. After treatment of cells with an anti-6×His tag antibody, fluorescence microscopy and super-resolution structured illumination microscopy (SIM) confirmed the presence and location of the fusion monomers in the IPTG-induced cells (Figure 2a; Figure S1, Supporting Information).

Having confirmed that our modular fusion monomers could be inducibly expressed in cells and could apparently preferentially accumulate in the periplasmic space of the bacteria. After treatment of cells with an anti-6×His tag antibody, fluorescence microscopy and super-resolution structured illumination microscopy (SIM) confirmed the presence and location of the fusion-monomers in the IPTG-induced cells (Figure 2a; Figure S1, Supporting Information).

Hence, confirmed that our modular fusion monomers could be inducibly expressed in cells and could apparently preferentially accumulate in the periplasmic space, we lysed IPTG-induced cells and subsequently used Ni²⁺ affinity chromatography and size-exclusive chromatography to purify the 6×His tagged protein products. TEM of the purified extracts revealed formation of NPs of about 20 nm in diameter (Figure 2b). Note that a separate dynamic light scattering (DLS) analysis of purified CTB protein monomers alone indicated that a typical self-assembled CTB pentamers were ≈5 nm in diameter (Figure S2, Supporting Information), suggesting that the typical NPs we observed in the purified modular fusion protein extracts were aggregates of monomers in 3D space. We also used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis to denature the proteinaceous NPs, and Coomassie blue staining confirmed that fusion monomers were the expected 20 kDa in size (Figure 2b). These NPs also showed good colloidal status (verified by DLS analysis in Figure 2c) and temperature stability (Figure S3, Supporting Information), supporting their likely long-term integrity for lymph node delivery in vivo.

To further clarify the assembly properties of the NPs, we explored their assembly using a molecular dynamics simulation
 approach. Briefly, the monomer structure was constructed by linking the CTB and trimer-forming peptide (Tri) via a GGSG peptide. Then the monomer was experienced a 150 ns equilibrium simulation to adjust the relative orientation between CTB and Tri peptide. The structures of pentamers and trimers were constructed by overlapping the backbone elements of this post-equilibrium monomer with that of the crystal structures (PDB ID: 6HSV and 3EFG, respectively), and coupled to assemble into polyhedral particle. 50 ns equilibrium simulations for pentamers and trimers were conducted and 50 frames of the last 1 ns were used to analyze the interaction energy. The results indicated that the interaction energy between the monomers of pentamers and trimers were about $-550$ and $-243$ kcal mol$^{-1}$, respectively, with the electrostatic interactions (ELE) being dominant over van der Waals interactions (vdW) and solvation free (SOL) (Figure 2d).

Having established that one of the B$_5$ toxin CTB-containing modular fusion monomers could be expressed in cells and could assemble into extractable NPs, we next successfully confirmed that variations of the modular design, replacing CTB with the enterotoxigenic E. coli (ETEC) B$_5$ subunits of heat-labile enterotoxin (LTB), as well as a variant bearing Shigella dysenteriae B$_5$ subunits of shiga toxin (StxB), could also be successfully expressed, translocated to the periplasmic space, and purified as stable modular proteinaceous NPs (Figure 2e,f; Figure S4, Supporting Information). Thus, we have established proof-of-concept for our modular fusion protein designs for the in vivo production of self-assembling proteinaceous NPs based on bacterial B$_5$ toxins.

As the main envisioned application case for our design was the incorporation of diverse antigens onto the NP chassis to exert immune effects, our next elaboration of the design was to incorporate C-terminal antigen domains. While many proteinaceous nanovaccines, such as self-assembly protein nanoparticles or virus-like particles, have been made to it the clinic, the combination of a modular nanoparticle platform with tunable oligosaccharide patterns to mimic pathogen antigens still lacks exploration. Moreover, given our decision to fabricate these proteinaceous NPs in cells, we had the unique opportunity of exploring glycosylated antigens, a capacity typically precluded with synthetic NP fabrication approaches. While we did also generate modular fusion monomer variants with peptide antigens (see below), our initial focus beyond the basic proof-of-concept in vivo fabrication was on glycosylated antigens; specifically on the surface polysaccharide antigens that have a long-term focus of our research group.\cite{31,32}

Our strategy for fabricating modular fusion monomers with an in vivo glycosylated antigen was based on expressing the previously reported 29 amino acid glycosylation sequence 4573 as a C-terminal fusion on CTB fusion monomers in our previously reported 301DWP strain.\cite{31} This is an attenuated form of the bacterial pathogen Shigella flexneri (2a type 301 strain), from which the O antigen ligase and virulence plasmid have been deleted. These cells produce the required pentaoligomeric sugar unit oligosaccharides substrates that are linked together to form the O antigen polysaccharides (OPS) that typically comprise 11–17 repeating pentaoligomeric sugar units.\cite{31} Note that the expression plasmid pPgL-CTBTri4573 (Table S1, Supporting Information) we used for this modular fusion monomer iteration also included the sequence encodingPgIL, an O-oligosaccharyltransferase that could catalyze the conjugation of the OPS to the glycosylation sequence 4573 in periplasmic space. After transformation of 301DWP cells and IPTG induction of recombinant protein expression, we purified the polysaccharide conjugate vaccine NPs (NP-OPS) to enable further characterization. TEM, scanning electron microscopy (SEM), and DLS analyses of the purified extracts revealed NPs of about 25–50 nm in size (Figure 3a), findings in line with our size-exclusion chromatography studies (Figure S5, Supporting Information). TEM/SEM images indicated a certain size distribution because the length of polysaccharide chain itself was not uniform in bacteria. We had no additional intervention in the polysaccharide synthesis process, which could ensure the natural configuration of the polysaccharide antigen.

After thusly confirming the self-assembly of these polysaccharide-conjugated vaccine NPs, we used SDS-PAGE and immunoblotting techniques for further analysis. Note that in these experiments we also produced a modular fusion variant lacking the trimer-forming peptide domains (“CTB-OPS”), which we recombinantly expressed in 301DWP cells alongside PgIL. Staining of SDS gels with Coomassie blue and immunoblotting using an anti-6× His Tag antibody revealed the expected banding patterns for the anticipated populations of variously glycosylated NP-OPS and CTB-OPS (C-OPS) fusion monomers, where the polysaccharide presented a typical ladder and each band in the ladder corresponds to a different number of repeat unit (pentasaccharides). Further, immunoblotting with an antibody against OPS supported the specificity of the PgIL-catalyzed glycosylation of the glycosylation sequence 4573 in both the NP-OPS and C-OPS products and the low molecular weight bands seemed weak mainly because of the titer of anti-OPS antibody was not as high as anti-His antibody (Figure 3b). Of note, such OPS decoration did not compromise the stability of the NPs (Figures S6 and S7, Supporting Information).

After excising the glycosylated bands from these gels and digesting them with proteinase K, a liquid chromatography tandem-mass spectrometry (LC–MS/MS) analysis revealed the expected-charged peptide SAGVA (m/z 404.21); the serine can be covalently bound to polysaccharides. Characterization of the mass-to-charge ratios of the breakdown products from collision induced dissociation verified the chemical structure of the sugar units (Figure 3c), thusly confirming that the polysaccharide conjugated vaccine NPs fabricated in the attenuated S. flexneri cells bear the same OPS sugar units as the natural S. flexneri 2a pathogenic bacterium.\cite{34} Our glycosylated-nanoparticles did not significantly affect CTB’s ability to bind with its known target monosialotetrahexosylganglioside (GM1) (Figure 3d), which is present at the surface of dendritic cells (DCs).\cite{35,36}

We also generated two novel nanovaccines with other peptide and polysaccharide antigens. Specifically, we worked with a known bacterial pentamer unit polysaccharide antigen from Salmonella paratyphi A and with an antigen peptide SL8 (S1NF-KEK) from OVA protein. Upon introduction of the pPgIL-CTBTri4573 plasmid into the previously reported attenuated but OPS-unit-bearing host strain 50971DW/CldIT2,\cite{32} Coomassie blue staining and immunoblotting with antibodies against both the 6× His-tag and the bacterial antigen oligosaccharide
from *S. paratyphi* A was successfully ligated into the NP carrier via its 4573 site (Figure 3e). TEM and DLS analyses indicated that the diameter of these self-assembled proteinaceous NPs had a narrow distribution ranging about 25–50 nm with the peak at 30 nm (Figure 3e). For the peptide, we expressed the pCTBTriSL8 plasmid (Table S1, Supporting Information) for the known tumor model antigen peptide SL8 in DH5α cells. TEM and DLS analyses confirmed the self-assembly of the SL8-bearing NP carrier into ≈20 nm particles (Figure 3f), and an MS/MS analysis confirmed the identity of the C-terminally fused SL8 OVA antigen (Figure S8, Supporting Information). These results together illustrate the versatility of the Nano-B5 platform for generating stable nanovaccines bearing highly structurally diverse antigens.

**It is well-established that the immune response achieved for a particular vaccine agent is strongly influenced by the duration over which the antigen persists, as well as by an antigen’s (size-related) ability to accumulate in lymph nodes**.\[37,38\] We used Cy7-SE fluorescent dye to covalently label three antigens: the NP-OPS nanovaccine, the aforementioned tripeptide lacking C-OPS, and OPS. Each of these was injected into the tail base of BALB/c mice. The intensity of the signal for OPS decayed quickly, and very little signal was observed in lymph nodes (Figure 4a,b; Figure S9, Supporting Information). In contrast, and reflecting
Figure 4. The nanovaccine enhances lymph node targeting and elicits potent immune activation. a,b) Representative images and corresponding quantitative fluorescence analysis of different vaccines (labeled with Cy7-SE) at injection sites \((n = 4)\) (a) and dLNs \((n = 3)\) (b) in supine and prone position respectively at different time points. c) Expression of the T cell recognition signals (MHC-I and MHC-II) and the co-stimulatory markers (CD40 and CD80) on DCs (MHC-I\(^+\), MHC-II\(^+\), CD40\(^+\), or CD80\(^+\) among the CD11c\(^+\) cell population) in dLNs 24 h postvaccination in mice \((n = 4)\). d) DCs were cultured in vitro and treated with different stimuli (PBS, OPS, C-OPS, and NP-OPS); 24 h later, RNA-seq was performed; the differential accumulation patterns of transcripts for known immune activation indicator genes is shown as a heat map. e) KEGG pathway analysis of numerous immune-related pathways in the NP-OPS group compared with control group. f) The release profile of Th1-skewed cytokines and Th2-skewed cytokines \((n = 3)\). g) The proportion of CD8\(^+\) T cells among total dLN cells and the proportion of granzyme B\(^+\) CD8\(^+\) T cells among the CD8\(^+\) T cells at 3 days postvaccination \((n = 4)\). h) The proportion of CD4\(^+\) T cells among total dLN cells at 3 days postvaccination \((n = 4)\) and the proportion of Tfh (CXCR5\(^+\) PD-1\(^+\) among the CD4\(^+\) cell population) and GC (GL7\(^+\) CD95\(^+\) among the B220\(^+\) cell population) in dLNs at 7 days postvaccination \((n = 5)\). Data are presented as means ± s.d. Each group was compared with NP-OPS by one-way analysis of variance (ANOVA) with Dunn’s multiple comparison test: ****\(P < 0.0001\), ***\(P < 0.001\), **\(P < 0.01\), and *\(P < 0.05\).
its CTB-pentamer-mediated increase in stability and increased size upon self-assembly, the signal for C-OPS persisted significantly longer than the OPS signal, and revealed a stronger accumulation pattern in draining lymph nodes (dLN), although this accumulation began to fade after the 6 h postinjection time point. Although these increases over OPS were substantial, dramatic further increases in both injection-site retention time and lymph-node accumulation were observed for NP-OPS, outcomes attributable to the increased stability and more than 6× increase in particle size facilitated by the trimer-forming peptide domain of the fusion Nano-B5 platform. It bears emphasis that analysis of the total signal intensity throughout the time course experiment revealed a more than 14× increase in the lymph-node-specific accumulation of NP-OPS over OPS.

We sacrificed some of the antigen-treated mice at 24 h postinjection, and in line with our observation of lymph node-specific accumulation, flow cytometry analysis of dissected dLNs revealed similar trends for the three antigen types in DCs expressing MHC-II as well as the co-stimulatory molecules CD40 and CD80 (Figure 4c). Of note, the expression of MHC-I was increased, which could be attributed to the ability of early-endosome and lysosome escape after being efficiently internalized by DCs (Figure 4c; Figure S10, Supporting Information). These results clearly suggest that using the Nano-B5 platform to package antigens can successfully increase antigen-presentation in mice. Given the dramatic immunostimulatory effects we observed upon injection of NP-OPS into mice, we next conducted global transcriptome profiling and found

Figure 5. The nanovaccine elicits robust prophylactic effects against bacterial infection. a) Immunization schedule for the evaluation of prophylactic performance. b) The IgG subtype titers (IgG1, IgG2a, IgG2b, and IgG3) against the LPS of S. flexneri 2a strain 301 were measured in the serum of BALB/c mice immunized with PBS, OPS, OPS+Al (aluminum hydroxide), C-OPS, or NP-OPS (n = 10). c) Serum bactericidal activity was measured for samples treated with the different dilutions of serum indicated in (b) (n = 3). The dotted line represents the 50% bactericidal rate. d) Mice vaccinated as described in (a) were infected intraperitoneally with \( \approx 4.35 \times 10^7 \) CFU mouse \(^{-1} \) (5 × LD\(_{50}\)) of S. flexneri 2a strain 301 cells two weeks after receiving the final immunization, and their survival times were monitored (n = 10). e) The bactericidal activity in immunized mice was visualized by intraperitoneal injection of luciferase-labelled S. flexneri 2a strain 301 (introduced plasmid pXen-18 into strain 301), and luciferase activity was measured at different time points. Data are presented as means ± s.d. Each group was compared with NP-OPS by one-way ANOVA with Dunn’s multiple comparison test: \( ****P < 0.0001, ***P < 0.001, **P < 0.01, \) and survival analysis was calculated by Log-rank test: \( ****P < 0.0001 \) and \( **P < 0.01 \).
(a) Time course of E.G7 inoculation and vaccination. E.G7 inoculation was performed on Day 0, and vaccination was performed on Day 3. Analysis of tumor growth and survival was performed on Days 7, 23, and 39.

(b) Tumor volume over time in different groups. PBS, SL8, SL8+AS04, and NP-SL8 were compared. The tumor volumes were measured every 3 days for 25 days. The percentage of live and dead cells was also calculated.

(c) Survival rate of different groups. The survival rates were calculated based on the number of live and dead cells. The differences in survival rates were statistically significant (***p < 0.001).

(d) Immunohistochemical staining of DAPI, KI67, and TUNEL in different groups. The staining results showed a significant decrease in tumor cell proliferation and an increase in apoptosis in the SL8+AS04 and NP-SL8 groups.

(e) Tumor weight of different groups. The tumor weights were measured and compared. The differences in tumor weight were statistically significant (**p < 0.01).

(f) Immunohistochemical staining of DAPI and CD8 in different groups. The staining results showed a significant increase in CD8+ T cells in the SL8+AS04 and NP-SL8 groups.

(g) Flow cytometry analysis of CD8+ T cells in different groups. The percentage of CD8+ T cells was calculated. The differences in CD8+ T cell percentages were statistically significant (****p < 0.0001).

(h) Flow cytometry analysis of Granzyme B and IFN-γ in CD8+ T cells in different groups. The percentage of Granzyme B+ and IFN-γ+ T cells was calculated. The differences in the percentages of Granzyme B+ and IFN-γ+ T cells were statistically significant (**p < 0.01).
that the NP-OPS treated mice exhibited major increases in the expression of 33 well-known immune activation indicator genes, including antigen-presentation and processing related genes like H2-K1 and CTSB, as well as both CD40 and CD80 (Figure 4d; Figure S11, Supporting Information). Further, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed obvious enrichment for genes from numerous immune-related pathways in the NP-OPS treated mice. And cytokine-focused analyses of the both RNA-seq (Figure 4e,f) and in vitro data (Figure S12, Supporting Information) again emphasizing that both Th1 and Th2 responses are elevated upon exposure to the NP-OPS nanovaccine.

NP-OPS treatment enlarged dLNs (Figure S13, Supporting Information) resulting from increased T cell proliferation (Figure S14, Supporting Information). We analyzed the proportions of CD8$^+$ T cells from dLNs and observed—consistent with all of the preceding results indicating strong immunostimulatory effects with our NP-OPS nanovaccine—a marked and significant increase in the proportion of CD8$^+$ T cells in the NP-OPS treated mice (Figure 4g). Moreover, we observed a huge increase in the proportion of cells positive for Granzyme B in the NP-OPS mice, results suggesting strong cell lysis capacity for therapeutic vaccinations (Figure 4g). These experiments also revealed that the NP-OPS mice had large increases in their proportions of humoral immunoresponse CD4$^+$ T cells (Figure 4h), T-Follicular Helper (Thh) cells, and B cells in Germinal center (GC) (Figure 4h; Figure S15, Supporting Information), results suggesting the apparent suitability of using NP-OPS nanovaccine in prophylactic vaccination applications.

Strongly encouraged by these obvious immunostimulatory effects for the nanovaccine produced with our self-assembling Nano-B5 platform, and after verifying the in vivo safety (Figures S16 and S17, Supporting Information) and the endotoxin levels (Figure S18, Supporting Information) of these proteinaceous NPs, we next used a previously established animal evaluation model to assess the humoral immune response performance against a highly lethal bacterial infection with S. flexneri 2a 301.[11] BALB/c mice were immunized with one of five treatments (PBS control, OPS, OPS + Al (aluminum hydroxide, having been used in various human vaccines), C-OPS, or NP-OPS) on days 0, 14, and 28. Blood was sampled on day 38 to facilitate quantitation of antibodies against S. flexneri lipopolysaccharide (LPS) and for use in vitro bactericidal assays (Figure 5a). The bacterial pathogen challenge was administered on day 42, followed by monitoring of mouse survival. Enzyme-linked immunosorbent assay (ELISA)-based measurement of the titers for the 4 anticipated antibody subtypes against S. flexneri 2a 301 LPS in serum samples revealed increases upon both C-OPS and NP-OPS immunization, with the increases being most profound for the NP-OPS treated mice. Note that the addition of an aluminum adjuvant alongside free OPS resulted in no observable increase in titers for the four examined antibodies against bacterial oligosaccharides (Figure 5b).

Consistent with these observed increases in antibodies against bacterial antigens, the bacterial assays we performed using serum samples from each of the immunization program experimental groups showed that only blood from the C-OPS and NP-OPS substantially reduced the viability of S. flexneri 2a 301, again with the nanovaccine treated sample exhibiting the most pronounced killing effects (50% bacterial titer was about 1:1800, higher than 1:500 of C-OPS) (Figure 5c). On day 42, mice were abdominally challenged with 4.35 × 10$^7$ CFU (5 times of a half lethal dose) of S. flexneri 2a strain 301. All of the mice from the PBS, OPS, and OPS+ adjuvant groups were unprotected, dying within three days of pathogen challenge. And while the C-OPS group had a 40% protection rate, fully 100% of the NP-OPS nanovaccine treated mice survived in this strong pathogen challenge (Figure 5d). Supporting the strong anti-pathogen effects of immunization with our nanovaccine in a high dose of S. flexneri intraperitoneally to investigate the prophylactic effect of systemic infection, a low dose (1/2 of a half lethal dose) was intradermally injected to further evaluate the clearance of local infection. Luciferase-based analysis of the pathogen in mice abdomens clearly revealed reduced pathogen signal intensity in NP-OPS immunized animals, even from the initial monitoring time point at 2 h (Figure 5e). These results together demonstrate that NP-OPS significantly outperforms C-OPS and OPS (with or without adjuvant) in eliciting humoral immune responses against bacterial polysaccharide antigens.

Work from the tumor vaccination field has illustrated that although using peptides as tumor antigens can confer strong specificity and high safety levels, such antigen epitopes alone face the critically limiting problem of eliciting very poor immunogenicity. Given the strong apparent antigen presentation performance of our Nano-B5 platform nanovaccine, we subsequently assessed cellular immune response performance of using the aforementioned NP-SL8 nanovaccine to treat tumor model C57BL/6 mice bearing subcutaneously injected OVA expressing E.G7 cells in their right chests. These experiments also evaluated the potential antitumor activity of SL8, C-SL8 (fused SL8 at C-terminal of CTB), and SL8+AS04 (an adjuvant having been approved for commercial use). Tumor cells were injected on day 0, and the candidate NP antitumor vaccine was administered on days 3 and 7 (Figure 6a). Assessment of tumor volume was based on vernier caliper measurements conducted.
once every two days, and the predetermined endpoint was defined as a tumor volume 1500 mm³ (at which time mice were sacrificed). There were stark differences in the tumor progression outcomes for the different experimental groups: all of the PBS control and SL8 alone animals and 7 of 8 C-SL8 animals had reached the endpoint by day 23 (Figure 6b; Figure S19 and Table S2, Supporting Information). Strikingly, whereas none of the NP-SL8 group mice had reached the endpoint by day 23, 4 of the 10 SL8+AS04 group mice had reached the endpoint by this time point (Figure 6b). Moreover, 7 of the NP-SL8 mice were apparently tumor-free, and only 3 of the 10 mice in this treatment group reached the endpoint by day 39 of the experiment (Figure 6c).

We conducted a variety of postmortem analyses of these tumor model mice to further explore the potential mechanisms through which the nanovaccine conferred its antitumor effects. Immunofluorescence analysis of TUNEL and Ki67 signals in tumor tissues revealed sequentially increased extents of apoptosis in the PBS, SL8, SL8+AS04, and NP-SL8 treatment groups, and this was accompanied by sequential decreases in cell proliferation, respectively (Figure 6d). Further supporting the antitumor effects, the tumor weights of the SL8+AS04 and NP-SL8 group mice were dramatically reduced, with the nanovaccine group mice exhibiting the lowest overall tumor weights and this was accompanied by sequential decreases in cell proliferation, respectively (Figure 6d). Further supporting the antitumor effects, the tumor weights of the SL8+AS04 and NP-SL8 group mice were dramatically reduced, with the nanovaccine group mice exhibiting the lowest overall tumor weights (Figure 6e). We also observed significantly increased T cell infiltration in the tumors of the SL8+AS04 and NP-SL8 mice. Intriguingly, although CD8⁺ T cell infiltration was increased to a similar extent in both groups from immunofluorescence (Figure 6f) and flow cytometry (Figure 6g) results, a subsequent analysis of the Granzyme B and IFN-γ content in these CD8⁺ T cells clearly suggested that the cell lysis capacity of the T cells of mice treated with our nanovaccine was markedly increased over T cells from SL8+AS04 mice (Figure 6h). We proposed such a robust enhancement on cellular immune response could be mainly attributed to two aspects. Considering the immune response achieved for a particular vaccine agent is strongly influenced by the antigen’s ability of retention and size-related lymph nodes targeting, our stable nanosized vaccines (25–50 nm) could greatly promote the endocytosis of loaded antigens by APCs residing in the lymph nodes.17,18 Meanwhile, previous studies have reported CTB have the capacity to enhance MHC-I presentation, due to GM1 binding-dependent cytoplasm transportation.19,20 After self-assembly, multiple CTB pentamers in each nanoparticle could provide more opportunity for GM1 recognition, which not only remained the capacity of escaping into the cytoplasm, but also promoted MHC-I presentation for CD8⁺ T cell activation.

Inspired by the extremely promising results we observed in diverse mouse models and seeking to bring our nanovaccine platform closer to clinical relevance, we undertook evaluations of our nanovaccine bearing S. paratyphi A OPS described above (Figure 3e) in nonhuman primates. Briefly, 15 cynomolgus monkeys with age of 3–3.5 years and an average weight of 3.5 ± 0.5 kg were selected. The vaccines for S. paratyphi A including OPS, C-OPS, C-OPS+adjuvant (aluminum hydroxide), C-OPS+adjuvant (AS04), and NP-OPS were administered intramuscular (i.m.) into the upper arm. The dose of polysaccharide was 5 μg per injection and immunizations were performed on days 0, 14, and 28 (Figure 7a). We first used Cy5-labeled NP-OPS to assess the delivery of the nanovaccine. At 48 h after injection, dLN was surgically removed and analyzed with fluorescence imaging: the extent of lymph-node accumulation was obviously strong from the prepared dLN (Figure 7b). Having confirmed its ability to accumulate in primate lymph node, we next performed ultrasonography assessment for dLNs on days 0, 21, and 35 and showed that the NP-OPS vaccination caused the strongest immune response, resulting in a 3× increase of dLN volume. This increase was larger than that observed for OPS and for either of the C-OPS+adjuvant preparations (Figure 7c). Thus, our NP-OPS clearly outperforms conventional vaccine preparations in terms of immune activation in monkeys.

To examine antibody production upon vaccination of the monkeys, blood was sampled before the first vaccination and 7 days after each of the three rounds of injections, ELISA-based measurement of the titers for the IgG antibody against S. Paratyphi A strain CMCC 50973 LPS in serum samples revealed increases upon both C-OPS and NP-OPS immunization, with the increases being most profound for the NP-OPS treated monkeys (Figure 7d). For further verification, we performed bactericidal assays using serum samples from monkeys immunized with the five different vaccines and bactericidal titer was defined as the dilution of test serum that resulted in a 50% killing of bacteria. We detected huge increases (up to 100-fold) for the bactericidal titer of blood samples from the nanovaccine treated monkeys but not conventional vaccination groups (Figure 7e), again confirmed that NP-OPS could induce strong humoral immune response and produce protective antibody against pathogen.

To evaluate the effect of cellular immune response, we first detected the activities of CD8⁺ T cells in blood circulation by flow cytometry. The results showed that Granzyme B content in CD8⁺ T cells was significant increase in NP-OPS group compared with other groups (Figure 7f). In addition to the analysis of the amount, we then used lactate dehydrogenase (LDH) releasing assay to detect in vitro killing activity of cytotoxic T lymphocytes (CTLs). The CD8⁺ T cells from each monkey were separated by Fluorescence activated Cell Sorting and coincubated with target cells (cynomolgus monkey skin cells which have been stimulated by C-OPS). The releases of LDH in medium were detected after 24 h incubation and the results showed that the killing effect against the target cells in NP-OPS group was strongest than others, again indicating a potent cell lysis ability (Figure 7g). Moreover, safety evaluation results showed that few abnormal were found on levels of cytokines (IL-6, TNF-α, and IFN-γ) (Figure 7h) and biochemical indexes (ALT, AST, ALP, BUN, and LDH) (Figure 7i) postvaccination in serum. Collectively, these results support that the nanovaccine induces safe and effective immune responses in nonhuman primates.

Medically, our work shows that our nanovaccines are safe in primates and mice and outperform conventional vaccine preparations in stimulating more efficient immune response. As a technology, it bears special emphasis that the polysaccharide antigens in our nanovaccines were synthesized by the bacteria themselves, (i.e., without artificial activation or modification), which ensures their natural conformation and maintains the fidelity of the antigen, which is conducive to the stimulation of specific immunity responses. In the future, we will further test the prophylactic ability of our nanovaccines against multidrug
Figure 7. Evaluation of nanovaccine performance in cynomolgus monkeys. a) Treatment schedule for the evaluation on cynomolgus monkeys. b) Delivery of nanovaccine to draining lymph nodes (dLN). Nanovaccine was labeled with Cy5-SE dye, and dLN was imaged 48 h after injection to monkeys. Scale bar: 1 cm. c) dLN sizes after immunization with different vaccines. The size of dLN was measured by ultrasonography on days 0, 21, and 35, and changes of dLN volume \((D1 \times D2^2)/2\) were tracked. d) IgG titers against the LPS of \(S. Paratyphi\) A strain CMCC 50973. The titers were measured in sera samples from monkeys 7 days after each round of immunization with OPS, C-OPS, C-OPS+AI (aluminum hydroxide), C-OPS+AS04, or NP-OPS. e) Antibody induced serum bactericidal effect against \(S. paratyphi\) A. Bactericidal activity of the different dilution serum pre- and postimmunization in each group was measured and the increase of bactericidal titer based on the preimmune serum was calculated. f) Analysis of Granzyme B expression in CD8\(^+\) T cells in bloods. g) CTL lysis capacity to infected cells. CD8\(^+\) T cells in blood were sorting out on day 35 from each immunized monkey and then incubated with target cells (cynomolgus monkey skin cells which have been stimulated by C-OPS). After 24 h incubation, the percent of specific lysis of target cells was evaluated according to the releases of LDH. h) Safety evaluation of immunization with the different vaccines. The levels of cytokines (IL-6, TNF-\(\alpha\), and IFN-\(\gamma\)) (h) and biochemical indexes (AST, ALT, BUN, LDH, and ALP) (i) were measured on days 0, 7, 14, and 35. \(n = 3\) and data are presented as means \(\pm\) s.d. Each group was compared with NP-OPS by one-way (in (d), (f), and (g)) or two-way (in (e)) ANOVA with Dunn’s multiple comparison test: ****\(P < 0.0001\), ***\(P < 0.001\), **\(P < 0.01\), and *\(P < 0.05\).
resistant bacteria and intracellular bacteria that are difficult to treat with antibiotics, such as Acinetobacter baumannii, Klebsiella pneumoniae, Staphylococcus aureus, and Brucella abortus. Meanwhile, we will prepare to display specific tumor antigen epitope (such as MUC-1) and even neoantigen on the nano-B5 platform and investigate its antitumor efficacy on more physiological relevant tumor model. In addition, we have confirmed the successful connection of huge polysaccharide chains (over 30 kDa) to the nanoparticles, which thus shed a light on the display of longer peptides and even protein domains. Considering the outbreak of SARS-CoV-2 worldwide and the serious threat to public health, we believe our Nano-B5 chassis can also be readily used as a tool for the development of an effective anti-viral nanovaccine by displaying the receptor binding domain of Spike S1 protein onto the nanoparticles.

In looking toward additional iterations of the Nano-B5 concept, we anticipate incorporation of N-linked glycosylation systems like PglS-catalyzed introduction of eukaryotic glycoprotein antigens and another O-linked glycosylation system using PglS-catalyzed methods to introduce capsular polysaccharides.[41,42] These and other elaborations should considerably expand the application prospects and enable preparation of sugar antigen vaccines or other medical applications, for example, by incorporating sugar structures with adjuvant effects which have recently been reported to further enhance the immune response of HIV vaccines.[41] Moreover, the nanoparticles can be efficiently expressed in the periplasmic space of bacteria, which is a valuable supplement to the traditional cytoplasmic expression technology.

Meanwhile, the all-peptide design of the Nano-B5 concept should facilitate the elaboration of multiple-antigen and dLN targetable nanovaccines, provided that the B5 pentamer and trimer-forming peptide self-assembly components can be suitably engineered to function properly with ever-more-complex modular multiple-antigen and designs. There are also opportunities for expanding the chassis itself. For example, the trimerization could be replaced with other polymeric forms, which would change the size and geometry, of the chassis particles and enable different arrangements of antigens orientation combinations and may support yet superior delivery and/or stimulation configurations.

Besides, considering that these designs are ultimately encoded as stable DNA, they should be suitable for immediate testing and application in a potentially huge number of cellular chassis, which themselves can be engineered to support the functionalization and biochemical elaboration elements for further specificity and improved vaccine performance. Finally, it bears mention that our platform is a genuinely living production process, and is thus evolvable; attendantly, any problems or therapeutic performance of any designed nanovaccines with our concept could be subjected to directed-evolution-based improvement strategies.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

AB5 toxins, biosynthesis, conjugate vaccines, nanovaccines, self-assembled proteins

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