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Advances and Opportunities in Nanoparticle- and Nanomaterial-Based Vaccines against Bacterial Infections

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As the dawn of the postantibiotic era we approach, antibacterial vaccines are becoming increasingly important for managing bacterial infection and reducing the need for antibiotics. Despite the success of vaccination, vaccines remain unavailable for many pressing microbial diseases, including tuberculosis, chlamydia, and staphylococcus infections. Amid continuing research efforts in antibacterial vaccine development, the advancement of nanomaterial engineering has brought forth new opportunities in vaccine designs. With increasing knowledge in antibacterial immunity and immunologic adjuvants, innovative nanoparticles are designed to elicit the appropriate immune responses for effective antimicrobial defense. Rationally designed nanoparticles are demonstrated to overcome delivery barriers to shape the adaptive immunity. This article reviews the advances in nanoparticle- and nanomaterial-based antibacterial vaccines and summarizes the development of nanoparticulate adjuvants for immune potentiation against microbial pathogens. In addition, challenges and progress in ongoing antibacterial vaccine development are discussed to highlight the opportunities for future vaccine designs.

1. Introduction

Vaccines protect individuals by "training" the immune system with killed or weakened microbes, or crucial immunogenic components of the disease agents, to generate antibodies and cellular responses that can neutralize or eliminate the invading pathogen upon infection. In spite of huge successes gained in battlegrounds against diseases such as smallpox and poliovirus, many challenges remain for the development of effective vaccines against bacterial infections.[1] Unlike viruses, bacteria are highly complicated microbes that carry complex genomes encoding hundreds of proteins. In particular, some are further armed with immune regulators that facilitate the evasion of the microbes from the surveillance the of host immune cells. These bacterial defense mechanisms pose major hurdles for the development of effective vaccines against difficult bacteria such as Chlamydia, *Helicobacter pylori* and *Mycobacterium tuberculosis*. Traditional vaccines typically consist of inactivated or attenuated live pathogens. These vaccines are usually accompanied with adverse reactions and poor effectiveness due to the complexity of bacterial proteins and the presence of potential immunomodulators. Subunit vaccines based on the antigenic components of the target bacteria were later developed to tackle this problem. Instead of whole cells, critical antigens are extracted or purified from the pathogens to elicit necessary protective activities against infection. However, this usually comes at the cost of poor immunogenicity, as many immune-stimulating bacterial components and pathogen-associated molecule patterns (PAMPs) are excluded during the process. Therefore, bacterial immune potentiators, including lipopolysaccharides (LPS), flagellin, and monophosphoryl lipid A (MPLA), are often required to be reintroduced as adjuvants with subunit vaccines to promote an effective immune response toward the target pathogen. In addition, owing to the variation of the required protective immune response against different pathogens and antigenic targets, combinations of antigen and adjuvant must be studied carefully to elicit an appropriate response via the proper immune stimulating pathway.

Advances of nanomaterials in recent years have introduced new toolsets and innovative strategies against bacterial infections.[2] A major advantage of nanomaterials is its ability to be customized with various functionalities to meet the needs for different applications. In vaccine development, nanocarriers can be loaded with a wide array of cargoes, including nucleic acids, synthetic peptides and proteins, to provide the desired antigen source for recognition by immune cells. Nanoformulations can shield cargoes from enzymatic degradation and enable controlled release in target cells. In this regard, vaccine delivery can be vastly improved by nanoformulations as opposed to the conventional antigen-adjuvant mixtures. In addition, immune-stimulating adjuvants can be incorporated within these nanoformulations. Unlike adjuvants in their free form that rapidly diffuse into the circulatory system, adjuvants bearing nanoparticles can be functionalized to localize in specific lymphoid organs. Such targeted delivery can facilitate a more concentrated and sustained response in the local lymph nodes, while minimizing unwanted systemic reactions associated with the dissemination of immune potentiating adjuvants to distal sites. Moreover, the codelivery of adjuvant and

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antigen as a synchronized sequence of activating signals allows precise activation of the immune system and effective elicitation of antigen-specific T cells.[3] The tailorability of nanoparticles make them a versatile platform for improving subunit vaccine efficacy. Detailed discussions on the many of the benefits of nanoparticle vaccines, including improved lymph node accumulation and antigen presentation, can be found in other recent review articles.[4–6] The synthetic flexibility of nanomaterials brings forth many new strategies for improving effectiveness of antibacterial vaccines.

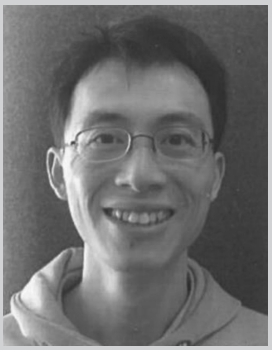
In the present review article, we focus on advances in nanoparticle- and nanomaterial-based antibacterial vaccines and discuss the challenges and opportunities facing vaccine development against some of the most pressing microbial pathogens. We will first review nanoparticulates and nanomaterials that have been applied for antibacterial vaccination, which include outer membrane vesicles (OMVs), self-assembled nanomaterials, inorganic nanoparticles, and polymeric particles (**Figure 1**). As scientists continue to unravel the intricate immunological mechanisms for bacterial containment, we will also examine the different pattern recognition receptors (PRRs) and their corresponding agonists for immunopotentiation. Different PRR agonists containing nanoparticulate adjuvants are reviewed in this section to highlight the opportunities in nanoparticle-mediated immune modulation. Finally, we delve into four specific bacterial pathogens, including *Chlamydia trachomatis*, *H. pylori*, *Staphylococcus aureus*, and *M. tuberculosis*, which are currently drawing major vaccine development efforts as they pose significant public health threats. This section discusses some of the pathogenic and immune evasive mechanisms adopted by bacteria that render conventional vaccines ineffective. Progress and opportunities in nanoparticle-based immunization strategies against these pathogens are also discussed.

2. Nanoformulations for Antibacterial Vaccination

A wide variety of nanomaterial platforms have been applied for antibacterial vaccinations. Some of the frequently used formulations include bacterial outer membrane vesicles, protein or peptide-based materials with self-assembling properties, gold nanoparticles, and polymeric nanoparticles (Figure 1). The following section highlights these antibacterial vaccine nanoformulations and examines the inherent immune potentiating effect observed with these nanovectors. A summary of these formulations is listed in **Table 1**.

2.1. Outer Membrane Vesicles

Outer membrane vesicles are naturally produced proteoliposomes that were first documented in Gram-negative bacteria and were later found in mycobacteria and some Gram-positive bacteria (Figure 1A).[7] These vesicles are generally in the size range of 50–250 nm, and contain proteins, phospholipids, LPS, and periplasmic components. OMVs can play a role in disease pathogenesis as they may carry additional virulence factors such as toxins, cell adhesins, and even immune regulators that interfere with host immune responses.[8] Nonetheless, as OMVs show high resemblance to the antigenic surface of bacteria, they have been examined as a vaccine platform. With their small size that is ideal for efficient lymph node draining and for antigen-presenting cell (APC) uptake, along with potential intrinsic immunostimulating PAMPs, OMVs are suggested to be a suitable platform for vaccine development.



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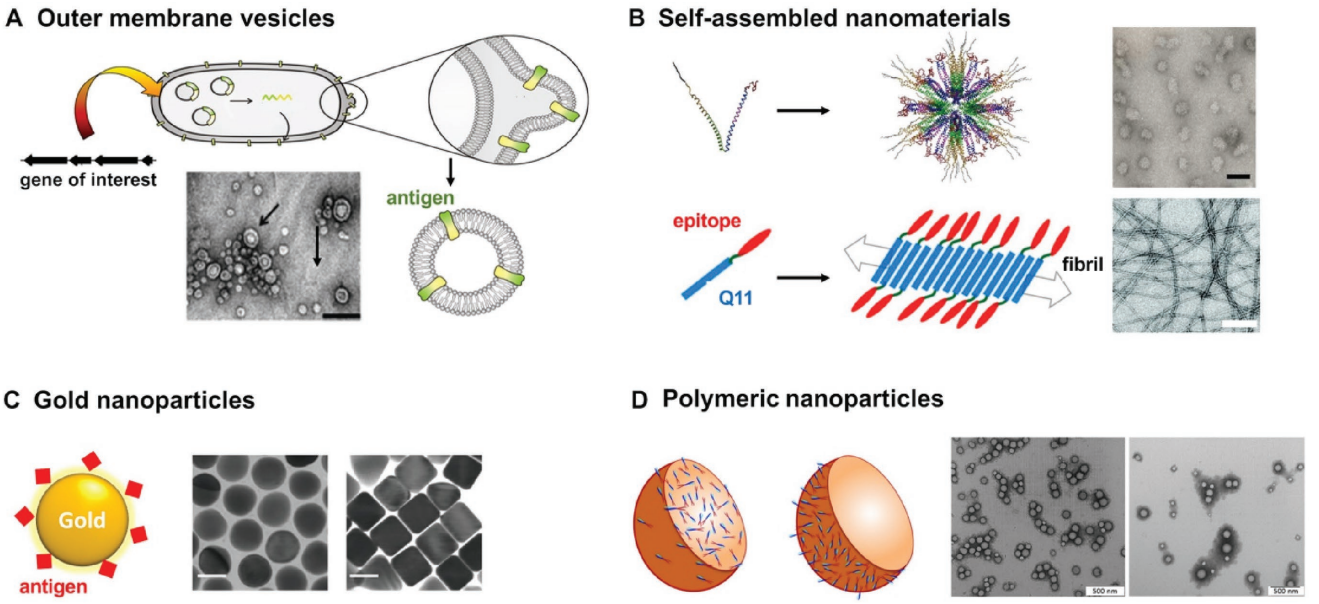

Figure 1. Examples of nanomaterial-based antibacterial vaccines. A) A schematic representing the generation of engineered OMVs from *E. coli* and TEM microscope images highlighting the characteristic bilayered lipid membrane (OMVs indicated by arrows; scale bar = 200 nm). Reproduced with permission.[19] 2014, PLOS. B) Top: Self-assembled nanomaterials based on α-helical rod-like coiled coils (scale bar in the TEM image = 100 nm). Reproduced with permission.[39] Copyright 2012, PLOS. Bottom: Schematic of epitope-bearing Q11 peptides self-assembling into fibrillar aggregates. TEM image shows the fibrillar structure of OVA-Q11 (scale bar = 100 nm). Reproduced with permission.[42,44] 2010, National Academy of Sciences of the United States; 2012, American Chemical Society (United States). C) Schematic representation of a gold nanoparticle and TEM images of spherical and cubic gold nanoparticles (scale bar = 40 nm). Reproduced with permission.[59] 2013, American Chemical Society. D) Schematic representations and TEM images of the peptide-encapsulated (left) and the peptide-coated PLGA nanoparticle (right) (scale bar = 500 nm). Reproduced with permission.[86] 2016, Elsevier.

For bacterial vaccines, OMVs can be prepared by processing the culture supernatant of either wild-type or genetic recombinant pathogen strains. Even though wild-type microbes produce vesicles with antigenic contents most similar to the parents, there are safety concerns over the presence of endotoxin, which may induce severe side effects via the interaction with cellular Toll-like receptors (TLRs).[9] One way to deal with this problem is to detoxify the OMVs with detergents like sodium deoxycholate during the extraction process.[10,11] The potency of the OMVs decreases with the removal of the immunoactive LPS such that additional adjuvants have to be included in the vaccine formulation to augment the efficacy. In addition, detergents may cause subtle conformational changes of the antigens on the surface of OMVs. Recombinant microbes that are deficient in toxins have also been developed for producing OMV vaccines.[12,13] These OMVs contain less reactogenic LPS and can thus avoid detergent treatment for better antigen preservation. Moreover, many studies have shown that exogenous antigens can be engineered into attenuated or detoxified bacterial vectors for OMV preparation. *Escherichia coli* is one of the most extensively investigated vectors for producing OMVs containing foreign antigens, such as those from *Streptococcus*, *Pseudomonas aeruginosa*, *Francisella tularensis*, *Acinetobacter baumannii*, and *Yersinia*.[14,15] Other bacterial vectors, including *Neisseria meningitidis*, *Salmonella*, and *Vibrio cholerae*, have also been exploited.[16]

OMVs have been shown in literature to induce good humoral responses that protect against bacterial infection.[8,11,17,18,229] Rosenthal et al. have engineered the probiotic *E. coli* Nissle 1917 bacteria, a commonly used strain for producing OMV vaccines, to express an exogenous green fluorescent protein (GFP). After vaccination, the OMVs harvested from the recombinant bacteria elicited a significantly higher titer of anti-GFP antibodies in mice compared to immunization with GFP alone.[19] Furthermore, in stark contrast to the alum-adjuvanted vaccine that induced mostly Type 2 T helper (Th2) responses, the OMV-GFP vaccinated mice showed a higher ratio of immunoglobulin G2a (IgG2a) to IgG1 titers, suggesting a more Type 1 T helper (Th1)-skewed response. This Th1-biased response is likely attributed to the enrichment of several PAMPs in the OMVs of *E. coli* Nissle 1917, particularly the TLR5 agonist flagellin. Similarly, three antigens from Group A and Group B *Streptococcus* were engineered into *E. coli* respectively for producing OMV vaccines.[15] Not only did these OMVs induce strong humoral responses, they also improved survival of mice against lethal *Streptococcus* challenges. Of note, Th1-associated IgG2a was the predominant isotype of antigen-specific antibodies in the recombinant OMV-vaccinated mice, whereas IgG1 was found to be the major isotype in mice immunized with purified antigen formulated with alum.

The strong immune-stimulating activity of OMVs can be attributed to their intrinsic proinflammatory property due to the presence of bacterial PAMPs. Aside from the above-mentioned TLR5 ligand flagellin, studies have shown that the TLR4 signaling pathway can also be activated by OMVs.[13,15,17] The capacity to engage multiple PRRs upon immunization is central to robust immune responses. The induction of proinflammatory cytokines and the activation of dendritic cells (DCs) in

Table 1. Examples of nanomaterial-based antibacterial vaccines.

Material	Target pathogen	Adjuvant	Humoral response	Cellular response	Ref.
Bacterial outer membrane vesicles					
OMVs	*C. trachomatis*	Alum hydroxide	Yes	N/E[a)]	[229]
OMVs	*Streptococcus* (group B)	Alum hydroxide	Yes	N/E	[15]
OMVs	*S. typhimurium*	N/A	Yes	Yes	[17]
OMVs	*N. meningitidis*	Alum hydroxide	Yes	N/E	[230]
OMVs	*V. cholerae*	N/A	Yes	N/E	[18]
Self-assembled nanomaterials					
Papaya mosaic virus VLP	*L. monocytogenes*	ssRNA	Yes	Yes	[32]
Lipopeptides	*Streptococcus* (group A)	N/A	Yes	N/E	[51]
Peptide β- sheet	*Streptococcus* (group A)	N/A	Yes	N/E	[46]
Lipid Pam	*Streptococcus* (group A)	Pam2, Pam3Cys	Yes	N/E	[54,106]
Hepatitis B VLP	*M. tuberculosis*	N/A	Yes	Yes	[29]
Gold nanoparticles					
Gold NPs	*L. monocytogenes*	Advax	N/E	Yes	[67]
Gold NPs	*P. aeruginosa*	Freud's adjuvant	Yes	N/E	[70]
Gold NPs	*Y. pestis*	Alhydrogel	Yes	N/E	[71]
Gold NPs	*S. pneumoniae*	Quil-A (saponin)	Yes	N/E	[69]
Gold NPs	*E. coli*	N/A	Yes	Yes	[73]
Polymeric nanoparticles					
PPS	*M. tuberculosis*	CpG	N/E	Yes	[173]
PLA	*C. trachomatis*	N/A	Yes	Yes	[222]
PLGA	*C. trachomatis*	N/A	Yes	Yes	[223]
PLGA	*M. tuberculosis*	N/A	N/E	Yes	[168]
PLGA	*L. monocytogenes*	N/A	Yes	Yes	[85]
PLGA	*Streptococcus* (group A)	N/A	Yes	N/E	[86]
PLGA	*Y. pestis*	N/A	Yes	Yes	[231]
PLGA	*Bordetella pertussis*	N/A	Yes	Yes	[232]
PLGA with cell membrane	*S. aureus*	N/A	Yes	N/E	[204,207,209]
PLGA with whole bacteria	*C. trachomatis*	R848	Yes	Yes	[126]

[a)]N/E: not evaluated.

the process provide essential signals for eliciting CD4+ helper T cells, a key mediator in coordinating both arms of immunity. In line with increased secretion of proinflammatory type I interferons, tumor necrosis factor (TNF)-α, and interleukin (IL)-12 as well as upregulated costimulatory molecules in DCs, evidence of OMV-triggered CD4+ T cell responses has been observed in multiple reports.[17,20] Nonetheless, the ability of OMVs in inducing CD8+ T cell responses, which mediates the killing of infected cells, is less explored and remains unclear.

A number of OMV vaccines have long been investigated, and the most successful example is the vaccine for serogroup B meningococcus (MenB).[11,230] The efficacy of the OMV vaccine has been evaluated in Norway, Brazil, Chile, and New Zealand. In these clinical studies, OMV vaccine was shown to effectively induce high levels of antibody response against MenB, with a protective activity up to 87%.[21] Concerning the vaccine safety, the MenB OMV vaccine is prepared in the presence of detergent in order to reduce the content of LPS that could potentially trigger excessive immunological responses. The decreased reactogenicity may in turn hamper the efficacy of the vaccine, such that alum absorption is required to boost its potency. Nonetheless, reports have shown that the MenB-specific antibody declined rapidly after primary immunization.[22] Therefore, up to three booster doses may be necessary to obtain sufficient protection against meningococcal infection.

The MenB OMV vaccine has clearly demonstrated its efficacy in controlling the epidemic in certain regions of the world. However, the vaccine induced immunity mostly targeted the highly variable PorA membrane protein, rendering the protective activity serotype-specific.[23] Future OMV vaccines may need to include multiple meningococcal antigens to increase the strain coverage as a universal vaccine.[24] Furthermore, the production of universal MenB OMV vaccines may be developed in combination with heterologous bacterial vectors, such as LPS-deficient *E. coli*, to meet the increasing demand for vaccine safety.

2.2. Self-Assembled Nanomaterials

Molecules with amphipathic nature can self-assemble into nanostructures in aqueous solutions. Phospholipids such as DC-cholesterol, (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamyl] cholesterol hydrochloride), 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP), and dimethyldioctadecylammonium bromide (DDA) are commonly used in preparing self-assembled liposomes.[25] With the biocompatibility, adjuvanticity, and the cargo loading capacity, liposomes have been extensively investigated for drug delivery and vaccine development. Liposome-based vaccines have made rapid progress in the last few decades. Their characteristics and immune potency have been reviewed in detail elsewhere.[25,26] Here, we focus on nonliposomal nanomaterial vaccines that are composed of other molecules with self-assembling capability.

Nanosized virus-like particle (VLP) is a commonly adopted particulate vaccine system that presents antigens in a virus-mimetic fashion. These particles are highly ordered, spontaneously formed structures that consist of viral capsids or core proteins but lacks the infectious genetic materials. The size of VLPs are in the sub-100 nm range that is suitable for transportation across biological barriers and uptake by immune cells.[6] In addition, owing to their repeating structures, VLPs display self-adjuvanting properties that promote humoral responses.[27] VLP vaccines for viruses, such as hepatitis B virus (HBV), human papillomavirus (HPV), and influenza A virus, have been extensively investigated, and some have successfully reached the market.[28] The effect of using VLP as an antibacterial vaccine carrier was explored by constructing HBV VLPs comprised of mycobacterial CFP-10 and HBV core fusion proteins in a study by Dhanasooraj et al.[29] Without the addition of adjuvant, the CFP-10-containing VLP induced significantly higher antibody and cellular responses against the CFP10 antigen than the VLP and CFP-10 protein mixture. Notably, mice immunized with CFP-10 VLPs exhibited a predominant Th1 type response, i.e., higher IgG2a, and T cell-associated cytokines including interferon (IFN)-γ, TNF-α, and IL-2.

A similar approach was taken by using self-assembled papaya mosaic virus (PapMV) nanoparticles that consisted of the coat protein of the plant virus and a synthetic noncoding ssRNA as adjuvant. Strong stimulation of the innate immunity by ssRNA-adjuvanted PapMV VLPs was observed in the lungs of nanoparticle-treated mice, which showed high expressions of cytokines and chemokines, increased recruitments of neutrophils and macrophages, and enhanced DC activation.[30] The feasibility of exploiting PapMV VLPs as a vaccine vector was further evaluated by engineering the coat fusion proteins with different influenza virus antigens.[31] Toward antibacterial vaccination, Lebel et al. examined the VLPs' effect on adjuvanting a DC vaccine against *Listeria monocytogenes*.[32] By pre-administering mice with PapMV VLPs before immunization with ovalbumin (OVA)-loaded bone marrow derived dendritic cells (BMDCs), a stronger OVA-specific CD8+ T cell response was detected compared to mice vaccinated with BMDC-OVA alone. With the enhanced cellular immunity, the mice receiving PapMV pretreatment displayed reduced bacterial loads in the spleen and liver following *L. monocytogenes*-OVA challenge. However, as the VLPs induced antivector immunity, the safety and practicality of the particle as a vaccine carrier require further evaluation.

Proteins with self-assembling property can also be employed for vaccine designs. For example, ferritin from *H. pylori* forms an octahedral particle of ≈12 nm in diameter with 24 subunits around a hollow interior.[33] Vaccine antigens can be genetically fused to the N-terminus of ferritins to be displayed on the surface of self-assembled ferritin nanoparticles. Immunization with the hemagglutinin-ferritin nanoparticle elicited higher antibody titers than the licensed inactivated vaccine and effectively protected mice against influenza A virus infection.[34] A number of eukaryotic ribonucleoproteins can also assemble into barrel-shaped nanocapsules with a dimension of ≈35 × 65 nm, offering an internal hollow structure to accommodate vaccine antigen candidates.[35] The vault nanoparticles have been applied to encapsulate chlamydial major outer membrane protein (MOMP). The resulting vaccine enhanced antigen uptake by DCs and triggered the production of proinflammatory cytokines in the absence of adjuvant.[36] Interestingly, the MOMP-loaded vault nanoparticles did not stimulate TLR signaling pathways but triggered the production of IL-1β via the activation of inflammasomes. Following intranasal immunization with vault nanocapsules loaded with either chlamydial MOMP or PmpG proteins, significant antigen-specific antibodies and mucosal cellular responses were developed and reduced bacterial burden was observed in mice challenged with genital chlamydial infection.[36,37]

The concept of self-assembled nanomaterials can go further with a more reductionist approach by using peptides that spontaneously form highly ordered structures.[38] Owing to the characteristics of specific amino acids, certain peptides can assemble into supramolecular structures via noncovalent forces. It has been demonstrated that peptides that are composed of coiled-coil domains can self-assemble into nanoparticles of ≈15–45 nm (Figure 1B).[39,40] By fusing different malarial epitopes to this construct, the peptide nanoparticles induced protective humoral and cellular responses in mice.[39] Another widely studied self-assembling peptide is the Q11 (QQKFQFQFEQQ) that forms beta sheet nanofibers (Figure 1B). Multiple epitopes or chemical groups can be ligated to the ends of the Q11 peptides to integrate into self-assembled fibrillar sheets.[41] The self-adjuvanting activity of the Q11-based vaccine has been shown in mice immunized with OVA-conjugated Q11 nanofibrils, in which strong OVA-specific antibody titers were elicited in the absence of adjuvant.[42] The coupling of OVA and Q11 was found to be critical to the induction of immune responses, as no detectable OVA-specific IgG was observed for the mixture of OVA and Q11. The Q11 backbone was found to be nonimmunogenic as no significant antibody titer was raised against the Q11 sequences upon immunization with complete Freund's adjuvant.[42,43] Owing to the modular design of the Q11 carrier, helper T cell epitopes can also be included in the self-assembled nanofibrils to activate CD4+ T cells to enhance the production of antigen-specific antibodies.[44,45] This system was employed by Azmi et al. to prepare a Q11-based vaccine that contained the J14 epitope derived from the M protein of group A streptococcus.[46]

Another way to construct self-assembled nanoparticles is to couple vaccine antigens to a lipid moiety to form amphiphilic

lipopeptides that spontaneously assemble into micellar structures.[47,48] Hydrophobic moieties such as a dialkyl tail with two palmitic chains or with two 2-aminohexadecanoic acids have been applied for synthesis of self-assembled particle vaccines.[49–51] These lipopeptides have been shown to be self-adjuvanting, capable of eliciting antibodies against a streptococcal epitope as well as T cell responses targeting a model SIINFEKL peptide. Notably, even though these particulate vaccines contained similar long lipid chains, TEM images showed that their morphology varied from short cylindrical micelles ≈5–15 nm in diameter and 25–125 nm in length to 5 nm spherical particles depending on the conjugated epitope sequences.[50,51]

Some lipoproteins derived from bacterial cell walls can self-assemble into particles and for preparing particulate peptide vaccines. Among them, dipalmitoyl-*S*-glyceryl cysteine (Pam2Cys) and tripalmitoyl-*S*-glycerol cysteine (Pam3Cys) are two potent lipid moieties that activate the TLR2/TLR6 and TLR2/TLR1 signaling pathways, respectively.[52] Besides the displayed adjuvant activities, Pam2Cys and Pam3Cys also promote the formation of micelle cores owing to the hydrophobic palmitoyl chains. It is worth noting that the configuration of the acyl chains has critical impact on the shape of palmitoylated peptide aggregates, which can range from vesicles, layers, and tubular networks with sizes varying between 20 and 3 µm.[53] A range of lipopeptide vaccines containing Pam2Cys or Pam3Cys have been tested for viruses and bacteria, including influenza A virus, hepatitis C virus (HCV), group A streptococcus, *L. monocytogenes*, and mycobacteria, and they are effective in eliciting both humoral and cellular responses.[54,55] Together these studies highlight the capability of self-assembling lipopeptides as an adjuvant as well as a vaccine delivery system.

2.3. Gold Nanoparticles

Among different inorganic nanomaterials, gold is most commonly used for nanoparticle vaccine preparations.[56,57] With their capacity for cargo delivery, biocompatibility, and ease of synthesis, gold nanoparticles have been frequently studied as delivery vehicles.[48] They are typically synthesized by reducing gold salts such as $AuCl_3$ to trigger nucleation of gold ions to form particle cores. Based on seed-mediated growth methods, gold nanoparticles of different sizes and shapes, like nanospheres, nanocubes, and nanorods, can be prepared.[58,59] Stabilizing agents or surfactants are absorbed or chemically linked to the surface of the gold nanoparticles for nanoparticle stabilization. These stabilizing molecules also provide an anchor point for further modifications as they can be replaced by other ligands or linked to biological molecules via conjugation reactions (Figure 1C).[57,60] Notably, thiol moieties bind with high affinity to the surface of gold particles; peptides, proteins, and thiol-modified oligonucleotides are therefore readily coupled with gold nanoparticles. Alternatively, amino-group-containing compounds can be attached to the carboxyl ends of the stabilizing molecules that surround the gold particles via EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl) coupling reactions. A wide range of molecules can thus be conjugated to gold nanoparticles, conferring various antigenic and adjuvanting properties.[61,62]

Gold nanoparticles do not induce an antibody response to the vehicle itself, and acute cytotoxicity has rarely been observed to date.[63,64] However, despite mechanisms that remain poorly understood, many reports indicate that gold nanoparticles exhibit adjuvant characteristics as they can promote immune cell recruitment, antigen-presenting cell activation, and cytokine production, which subsequently lead to the induction of humoral and cellular responses.[62,65] The adjuvanticity of the gold nanoparticle is best illustrated in experiments that treat macrophages and BMDCs with gold nanoparticles. Following coincubation, these professional antigen-presenting cells not only exhibited an activated phenotype, but also produced significant levels of proinflammatory cytokines, including TNF-α, IL-6, and IL-12.[59,66,67] These cytokines provide essential immunopotentiating signals that trigger humoral and cellular immunities. It is also worth noting that Niikura et al. found that rod shape gold nanoparticles induced a modest level of IL-1β in BMDCs, but not spherical and cubic particles.[59] In addition to showing distinct immunostimulating properties of gold nanoparticles of different shapes, this result also suggests that gold spheres and cubes are likely to activate the immune system through noninflammasome pathway.

Interestingly, literature suggests that the immunomodulatory property of gold nanoparticles may also be size-dependent.[68] For example, Niikura et al. reported that gold nanoparticles 40 nm in size elicited higher levels of antibodies against the West Nile virus envelope protein than 20 nm ones.[59] Chen et al. observed that gold nanoparticles ranging from 8 to 17 nm induced stronger antibody responses to a foot-and-mouth disease virus peptide, but not particles of 37 and 50 nm.[63] Furthermore, gold nanoparticles with a diameter less than 2 nm have been evaluated for the capability for application as vaccine vehicles, and distinguished humoral and cellular responses were observed.[67,69] These results suggest that gold nanoparticles may have a complicated role in immune potentiation that vary according to their surface properties.

Gold nanoparticles have been used in developing antibacterial vaccines, including *P. aeruginosa*, *L. monocytogenes*, *Streptococcus pneumoniae*, and *Yersinia pestis*.[67,69–72] The adjuvanting activity of gold nanoparticles was clearly demonstrated in the study by Dakterzada et al., showing that gold nanoparticles conjugated with a *P. aeruginosa* antigen elicited an antibody response comparable to the antigen formulated with Freund's adjuvant.[70] A similar observation was made in another study in which the *Yersinia* F1 antigen was conjugated to 15 nm gold nanoparticles.[71] By examining the subclass of anti-F1 antibodies induced by the gold nanoparticle vaccine, the IgG2a titer was found to be selectively enhanced, but not IgG1, compared to mice immunized with soluble F1 antigen. Interestingly, when alhydrogel, an alum-containing adjuvant, was used in combination with gold nanoparticles, the antibody response was further enhanced, albeit skewed toward a more Th2-related response. The high IgG2a titers suggested that gold nanoparticles have an intrinsic adjuvanting property that led to Th1 responses, and this effect that could be counteracted by the presence of a Th2-biased adjuvant, like alum. The capability of activating Th1 responses and T cell immunity is an important feature that mediates protection against intracellular organisms such as *Listeria*. In contrast to subunit vaccines that are generally poor

in generating T cell immunity, gold nanoparticles carrying a listeriolysin O peptide were shown to induce moderate levels of TNF-α and IFN-γ in the sera of immunized mice and protected *Listeria*-infected mice to some degree.[67] When formulating gold nanoparticle vaccines with Advax, a polysaccharide adjuvant that was shown to promote cellular responses, the T cell response and protective activity were further enhanced to a level similar to the peptide-loaded DC vaccine, which is widely adopted for generating T cell immunity.

Gold nanoparticles have also been formulated with bacterial OMVs to modulate immunity against an *E. coli*. In a study by Gao et al., *E. coli* OMV-coated gold nanoparticles were prepared using 30 nm particles.[73] As compared to the OMV formulation, the OMV-coated gold nanoparticles induced enhanced maturation of dendritic cells in vaccinated mice. The nanoparticle formulation also induced higher levels of IFN-γ and interleukin-17 (IL-17), suggesting the promotion of Th1 and Type 17 T helper (Th17) biased cellular immune responses. The unique immune-potentiating properties of gold nanoparticles warrant further investigations on their biological mechanism.

2.4. Polymeric Nanoparticles

Progress in the field of nanomaterials has greatly facilitated the design of polymeric structures that mimic important features of pathogens. In contrast to other subunit vaccine preparations, polymeric nanoparticles provide a versatile delivery platform. Polymeric nanoparticles are composed of a polymer-based matrix that serve as a vehicle for the antigen and the adjuvant. Biocompatible, biodegradable, and nontoxic polymers such as poly(D,L-lactic-*co*-glycolic acid) (PLGA) and chitosan are generally chosen as the carrier matrix although other materials like polystyrene (PS) and polyethyleneimine have also been used.[74–76] Using techniques including emulsion-evaporation or nanoprecipitation, polymers can be formulated into spherical nanoparticles that provide the basis for conjugating or encapsulating cargoes of interest.[75] Depending on the polymer and the surface modification, the size of nanoparticle can range from 10 nm to 1 μm in diameter, and the zeta potential can be either positive, negative, or neutral.[77] As recognition by immune cells is largely determined by these physicochemical properties, polymeric nanoparticles can therefore be optimized to target cross-presenting phagocytic cells to prime immune responses. In addition, the release of internal loads can also be controlled through appropriate formulations.[76] With the added benefit of controllable release, polymeric nanoparticles are able to be fine-tuned for optimal immune responses.[76,78] The incorporation of surface linkers also permits functionalization with targeting molecules, antigens, and adjuvants, which can further improve vaccination potency.[79]

Polymeric nanoparticles can carry and deliver antigens either through interior encapsulation or surface association. Interestingly, the method of antigen coupling has been shown to influence the outcome of immune responses. Compared to encapsulation that preferentially activates CD4+ T cells, antigen-coated nanoparticles have been shown to induce a stronger cytotoxic CD8+ T cell response. Consistent with the level of CD4+ T cell activation, higher levels of antigen-specific IgG were observed in mice immunized with surface-conjugated antigens.[80,81] It was suggested that the differential activation of T cells by these two formulations was associated with distinct intracellular trafficking of antigen-laden nanoparticle in dendritic cells.[80] By tracking a model ovalbumin protein with a fluorescent dye, the surface-conjugated antigen was found to be cleaved from the carrier in the endosome, thus potentially escaping into the cytoplasm for major histocompatibility complex (MHC) class I (MHC-I) presentation and CD8+ T cell recognition. In comparison, antigens encapsulated within nanoparticles are likely to survive to the late lysosomal stage, where they enter the exogenous antigen processing pathway that primarily induce CD4+ T cell responses. Nonetheless, several studies have reported induction of CD8+ T cell responses by particle-encapsulated antigens.[82] Further investigations on antigen trafficking and processing following nanoparticle delivery are thus warranted.

To obtain protective immunity, adjuvant systems that augment the immunostimulating activity are typically included in the polymeric nanoparticle formulation. Depending on their characteristics, adjuvants can be formulated in the same way as the antigen candidate by either encapsulation, incorporation, or conjugation with the polymeric particle. Through careful arrangement, polymeric nanoparticles can be constructed to have delicate designs that resemble the structure of pathogens.[83] Like viruses, important vaccine antigens can be displayed on the particle surface, while the immunopotentiating adjuvants are contained within. Or similar to bacteria, adjuvants that trigger cellular PRRs can be decorated on the outside of antigen-loaded nanoparticles. More importantly, the poor immunogenicity commonly faced by subunit vaccines can be improved by the pathogen-mimetic nanoparticles, which ensure simultaneous delivery of antigen and immunostimulating signals to host cells.[19,84]

Owing to their biocompatibility, PLGA or PLA-based nanoparticles have been extensively explored for vaccine development. With a slower release kinetics of the encapsulated OVA antigen, PLGA nanoparticles induced a higher and sustained antibody response than liposomes of similar size.[85] It was also noted that the polymeric vaccine elicited a protective immunity against listeria infection with the highest bacterial clearance compared to liposomes and alum. The effective protection was contributed by the robust cellular immunity mounted by the nanoparticle, as vaccination with the alum-formulated antigen had no effect at all in reducing bacterial loads in spite of exhibiting the highest level of antigen-specific IgG. The effect of controllable release was also explored by Marasini et al. in a study comparing PLGA nanoparticles with two different antigen-coupling methods (Figure 1D).[86] Despite having similar size and cellular uptake efficiency, the antigen-encapsulated nanoparticle had a stronger effect on promoting DC maturation and elicited higher antibody titers against a group A streptococcus J14 epitope as compared to the antigen-coated counterpart. The improved potency was attributed to the antigen protection and sustained release of the entrapped peptides.

Mucosal vaccination using PLGA/PLA nanoparticles via intranasal or intratracheal routes has also been investigated and proven to be effective in mediating protective responses against respiratory bacterial infections such as *Y. pestis*.[87,231,232] Polymeric particles have also been engineered to overcome the

harsh conditions facing oral vaccine administration as vaccines may be diluted, denatured or degraded in the gastrointestinal tract before reaching the target.[88] For example, a multicoated nanoparticle has been engineered with a pH and trypsin responsive outer layer consisted of poly[(methyl methacrylate)-*co*-(methyl acrylate)-*co*-(methacrylic acid)] (PMMMA) for targeted delivery to the large intestine.[89] As phase transits from the acid gastric environment to the weak alkaline small intestine, the PMMMA shell will disintegrate and subsequently reveal the antigen-contained PLGA core that is suitable for cellular uptake in the large intestine. The idea of encapsulating vaccines in this condition-responsive shell was evaluated using the surface immunogenic protein from group B streptococcus. Compared to plain PLGA nanoparticles which mostly accumulated in the liver, particles with the intelligent PMMMA coat were primarily directed to the large intestine and spleen, and resulted in 100% protection against streptococcus challenge.

In addition to be used as a vaccine vehicle, synthetic polymeric nanoparticles can also serve as a carrier for immunomodulators to help shape desirable immune responses. For example, Kasturi et al. prepared a PLGA nanoparticle encapsulating MPLA and R837, which are agonists of TLR4 and TLR7 respectively.[90] The combinatorial nanoformulation was able to significantly enhance the immunogenicity of antigens, including hemagglutinin from influenza H5N1 virus and the protective antigen of *Bacillus anthracis*. The work presents an example of using nanocarriers to modulate the immune system via activation of multiple PRRs. Such immune-modulatory capability via coordinated delivery of PRR agonists offers a compelling approach toward designing advanced vaccine formulation against ongoing microbial threats.

3. Nanoparticulate Pattern Recognition Receptor Agonists in Antibacterial Immunity

Current antibacterial vaccines in clinics function primarily by eliciting humoral responses. For instance, vaccines against tetanus, diphtheria, pneumococci, meningococci, and *Haemophilus influenzae* mediate protection via the induction of immunoglobulins, which can neutralize toxin factors or facilitate antibody-mediated phagocytosis and complement activation.[91] However, humoral responses alone have been shown to offer inadequate protection against many microbial pathogens, and the role of cellular immune responses is being increasingly recognized and incorporated into antibacterial vaccine development. Among the components of the cellular immune response, CD4+ helper T cells, are central to antibacterial immunity, and they can be divided into several subpopulations that possess functions including enhancing B cell proliferation and antibody production, promoting cytotoxic T lymphocyte (CTL) development, and recruiting and activating macrophages and neutrophils. Activated CD4+ T cells proliferate and differentiate into immune effectors that can directly and indirectly clear bacterial infections (**Figure 2**). Specialized subsets of Th cells are presently categorized into four major subpopulations, including Th1, Th2, Th17, and regulatory T cells (Treg).[92] Of these subpopulations, Th1 and Th17 are thought to be the primary contributors to antimicrobial defense. While Th1 cells facilitate the clearance of intracellular pathogens by enhancing cell-mediated immunity via IFN-γ and IL-2, Th17 cells promote immunity against extracellular bacteria by inducing antimicrobial peptides and recruiting neutrophils and macrophages.[93] Th17 cells are also identified as an integral component in mucosal antibacterial immunity.[94] Through production of IL-17A, IL-17F, and IL-22, Th17 cells are implicated in host mucosal defense against several mucosa-resident pathogens, including *P. aeruginosa, M. tuberculosis*, *S. aureus*, and *H. pylori*; increased levels of Th17 cells have been correlated with lower bacterial burden and reduced dissemination.[95] These findings have shifted design rationales for antibacterial vaccines with increasing focus being placed on the activation of cellular immune responses. In particular, activating cellular PRRs with molecular adjuvants presents a compelling immunomodulatory strategy that has been broadly adopted to enhance the immunogenicity of subunit bacterial antigens.

There are three major families of PRRs: Toll-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These PRRs are located at various cellular domains, with TLRs distributed on either the plasma membrane or in the endosomal compartment, and RLRs and NLRs in the cytoplasm. Together these PRRs sense and respond to different levels of danger signals, which range from proteins, nucleic acids, lipids, and carbohydrates that are commonly found on foreign microorganisms. Other PRRs, include C-type lectin receptors (CLRs)[96] and cytosolic dsDNA sensors (CDSs),[97] also function to modulate the innate immune response through their unique signaling pathways. Following the activation of specific innate immune receptors, the signals downstream of these PRRs can function in either synergistic or antagonistic manner.[98] The cooperation of multiple PRRs have been shown to contribute to host defense against bacteria.[99] For instance, studies based on transgenic mice deficient in specific TLR genes have shown that the synergy between TLR2 and TLR4 is critical to the control of *Salmonella typhimurium*,[100] whereas the dual activation of TLR2 and TLR9 plays an important role in controlling the infection of *M. tuberculosis*.[101] These findings offer support that antibacterial vaccines may be rationally designed with tailored molecular adjuvants to promote the needed effector responses. While the interplays among different PRR pathways remain poorly defined and warrant continued studies, cooperative immune activation using multiple PRR agonists have been demonstrated in numerous studies.[90,102] Continuing adjuvant research offers the hope that immune potentiation may be tailored by adjusting adjuvant combinations and delivery. Through controlled activation of specific innate immune pathways, production of cytokine milieu may be modulated to shape adaptive immunity, thereby achieving the desired immune profile for optimal antibacterial defense.[103]

The discovery and development of PRR-activating molecules have also brought forth numerous nanoparticle-based immunological adjuvants, which are designed to improve the potency and safety of immune-stimulating factors. Nanoparticulate agonists have been prepared to target several major PRRs, including TLR2, 3, 4, 7, 8, 9, RIG-I, NOD1, and CDSs (**Figure 3**). In the following section, we review the different

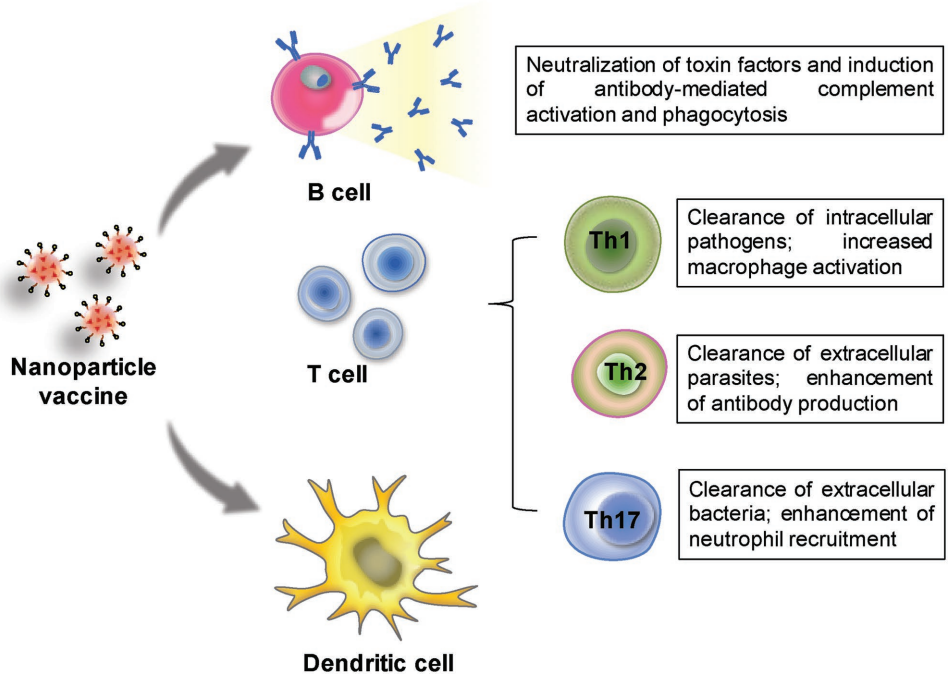

Figure 2. Role of nanoparticles in orchestration of immune and adaptive responses in treating bacterial infections. The efficacy of antibacterial vaccine relies on sustenance of host immune response that integrates humoral response to antigen presentation and cross-priming. B cells can directly recognize pathogenic antigen and develop into antibody secreting plasma cells and memory cells while CD4+ and CD8+ T cells are activated by DCs that present pathogenic antigen together with cytokines and costimulatory molecules.

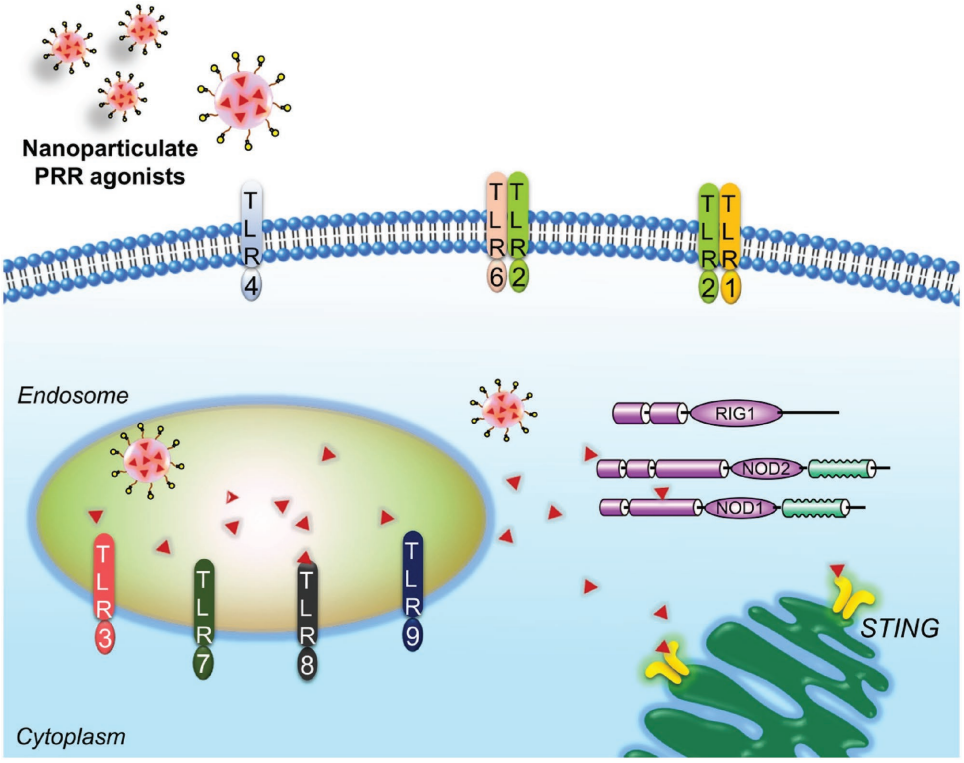

Figure 3. Nanoparticulate agonists targeting various PRRs. The figure summarizes the major PRRs that have been discussed in this paper, their cellular locations, and the design considerations for nanoparticle-mediated adjuvant delivery.

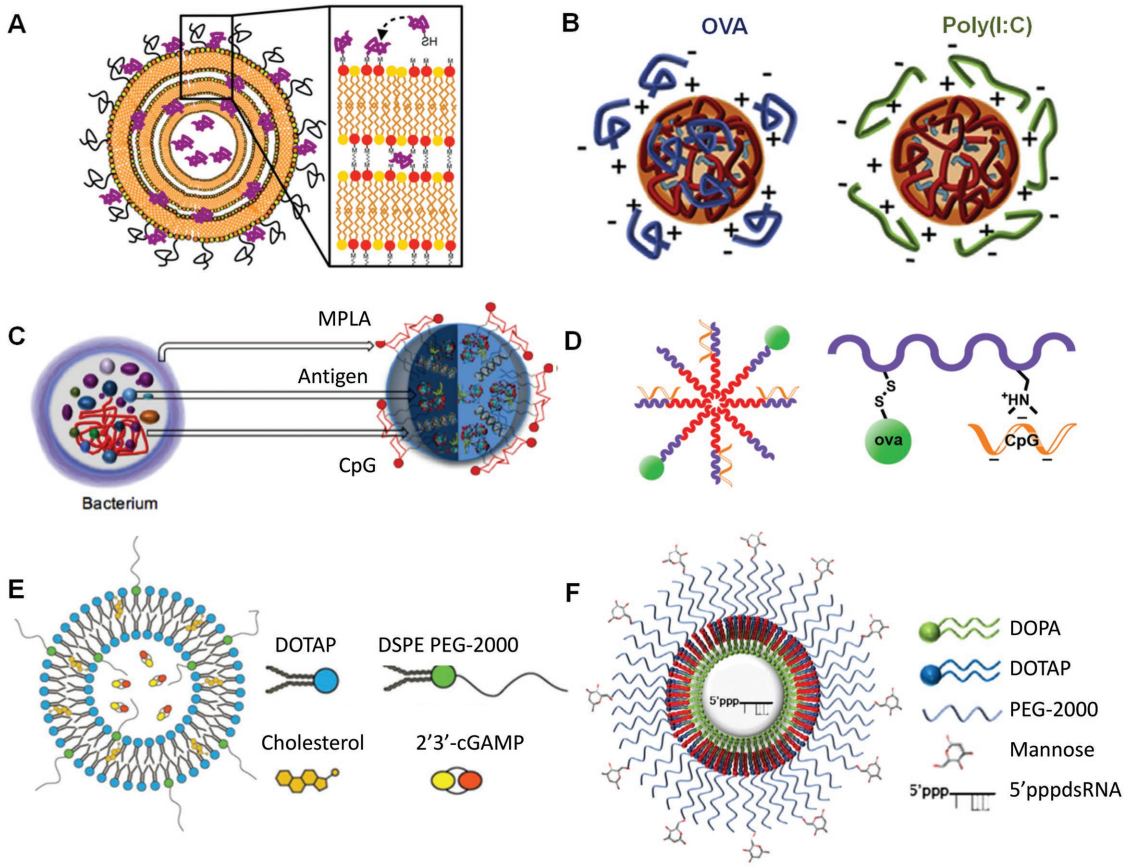

Figure 4. Various nanoparticle designs encapsulating TLR agonists. A) Interbilayer-crosslinked multilamellar vesicles (ICMVs) encapsulating TLR4 agonist MPLA and malaria antigen VMP001 in their aqueous cores as synthetic vaccines. Reproduced with permission.[112] 2012, National Academy of Sciences of the United States of America. B) Poly(g-glutamic acid) (g-PGA)-based synthetic vaccine nanoparticles (SVNP) encapsulating model tumor antigen (OVA) and toll-like receptor 3 (TLR3) agonist (poly(I:C). Reproduced with permission.[123] 2017, Elsevier. C) PLGA-based pathogen mimicking nanoparticles encapsulating OVA along with TLR9 agonist (CpG) and a TLR4 agonist (MPLA). Reproduced with permission.[138] 2016, Elsevier. D) pH-responsive nanoparticle vaccines for dual delivery of a model antigen and a TLR9 agonist. Reproduced with permission.[133] 2013, American Chemical Society. E) Liposomes synthesized from DOTAP and cholesterol for the delivery of a STING agonist (2′3′-cGAMP). Reproduced with permission.[147] 2017, John Wiley and Sons. F) Cancer peptide antigen conjugated lipid calcium phosphate (LCP) nanoparticles containing RIG-1 agonist 5′pppdsRNA. Reproduced with permission.[152] 2017, Elsevier.

nanoparticulate agonists and their PRR targets with emphasis on the advantages of nanoparticles over conventional adjuvant formulations. We separate the PRRs into three different categories—plasma-membrane bound, endosomal, and cytoplasmic PRRs—to highlight the delivery requirement for effective PRR activation. As the agonists for different PRRs can vary greatly in their physicochemical properties, different nanoparticle compositions for specific agonist encapsulation are also emphasized (**Figure 4**). This section provides an overview on current nanoparticle agonists and aims to offer design inspirations toward better antibacterial vaccine formulations.

3.1. Nanoparticulate Agonists for Plasma Membrane Bound PRRs

A majority of the membrane bound PRRs senses components on the surfaces of pathogens, which are comprised primarily of lipopolysaccharides or lipopeptides. Surface PRRs identified to this date include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11. Of these TLRs, TLR2 and TLR4 are commonly targeted by commercial vaccine adjuvants. These surface PRR agonists and their nanoformulations are reviewed below.

3.1.1. TLR1, 2, and 6

Among the plasma membrane bound TLRs, TLR2 is unique in its ability to modulate downstream signaling cascades by forming heterodimers with TLR1 or TLR6.[104] Triacylated or diacylated lipopeptides are among the primary agonists for TLR2/TLR1 and TLR2/TLR6 heterodimers, respectively.[105] In Section 2.2, we discussed several TLR2 ligands, including Pam2Cys and Pam3Cys, which possess self-assembling properties owing to their amphiphilic nature. To facilitate adjuvant/antigen codelivery, the TLR2 ligands have also been physically attached to other nanocarrier systems for delivery. For example, Moyle et al. developed a chemical synthesis method

for site-specific incorporation of a molecularly defined number of fatty acid TLR2 ligands onto engineered recombinant protein antigens.[106] Through the conjugation approach, three synthetic TLR2 ligands, including lipid core peptide, Pam2Cys, and Pam3Cys, were attached to multiple streptococcal proteins. These lipoproteins self-assembled in aqueous buffer yielding nanoparticle formulations with nanoscale dimensions. Upon subcutaneous immunization, the nanovaccines stimulated a strong humoral response against the streptococcal antigens. Notably, the authors showed that the immunogenicity of the construct was influenced by the conjugation approach, suggesting that the presentation of the TLR2 ligands in the nanoformulations has an effect on immune activation.

TLR2 ligands have also been conjugated to chitosan-based nanoparticles for improving DNA vaccines. By conjugating Pam3Cys to a chitosan derivative via a polyethylene glycol (PEG) linker, Heuking et al. showed that the resulting nanoparticles induced enhanced secretion of interleukin-8 (IL-8) and TNF-α by differentiated THP-1 cells when compared to nonfunctionalized chitosan nanoparticles.[107] Direct conjugation to PEG has also been explored as a strategy to enhance lymphatic drainage of TLR2 ligands. In a study by Sekiya et al., PEG-conjugated R4Pam2Cys was constructed for enhancing subunit vaccine potency.[108] Even though the authors observed no perceptible effect of the nanoformulation in inducing DC maturation markers and cytokine profile upon in vitro transcriptome analysis, in vivo administration showed a faster and more efficient trafficking of the construct from the injection site to the draining lymph node. Enhanced uptake by migratory DCs was also observed, leading to higher CD8+ T cell proliferation in vivo.

3.1.2. TLR4

The involvement of TLRs in LPS recognition and subsequent signal transduction in mammals was first elucidated with TLR4.[109] Upon activation by LPS, TLR4 acts either through myeloid differentiation primary response protein 88 (MyD88)-dependent or MyD88-independent pathways to induce proinflammatory cytokines. Commercially available agonists of TLR4, including LPS derived from various bacterial pathogens and MPLA, are commonly adopted in vaccine formulations for immune potentiation.

Owing to the presence of lipid groups, TLR4 agonists are commonly incorporated into the hydrophobic domain of polymer or lipid-based nanocarriers. For instance, in an effort to improve the potency of hepatitis B vaccine, Chong et al. incorporated MPLA with a hepatitis B antigen (HBcAg) into PLGA nanoparticles.[110] The nanometer size of the PLGA particles facilitated uptake by DCs and macrophages, resulting in enhanced processing and presentation of the encapsulated antigen and adjuvant. The platform showed induced cellular immune responses in the local lymph nodes and spleen. Notably, addition of the MPLA significantly enhanced the antigen-specific Th1 immune responses with heightened IFN-γ production by cytotoxic T lymphocytes. The sustained release profile of MPLA and the antigen/adjuvant codelivery afforded by the nanocarrier contributed to the enhanced adjuvanticity that was absent in the formulation with soluble antigen and adjuvant.

Another nanoformulation of MPLA was developed and studied by Moon et al. using multilayered vesicle (MLV) with covalently crosslinked lipid bilayers (Figure 4A).[111,112] In mice immunization studies using OVA as a model antigen, the interbilayer crosslinked multilamellar vesicles (ICMVs) containing MPLA showed robust antibody titers compared to liposomes and ICMVs without MPLA. The authors also demonstrated an induction of antigen-specific CD8+ T cells by the ICMV formulation, while administration of a mixture of soluble OVA and MPLA induced little cellular immune responses. The platform was further applied to a malarial antigen in another study.[112] ICMVs containing a VMP001 antigen and MPLA induced potent, long-lasting IgG titers. In contrast, soluble compositions with MPLA required at least 10 times the antigen to elicit even a subdued humoral response that waned over time. ICMV formulations also elicited a higher number of antigen-specific CD4+ T cells as compared to its soluble counterpart. A systematic evaluation showed that the MPLA nanoformulations allowed for significant dose sparing, which offers the advantage of decreased risk of reactogenicity, enhancing its potential as a safe vaccine candidate.

3.2. Nanoparticulate Agonists for Endosomal PRRs

When cells are infected by microbes, they encounter viral and bacterial genetic materials that can hijack the cellular protein synthesis machinery for disease pathogenesis. Distinct immune pathways have thus evolved to detect infection by sensing these internalized components. In the endosome, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and DNA are recognized by endosomal TLRs that include TLR3, TLR7, TLR8, TLR9, and TLR13.[113] Common agonists against the endosomal TLRs and their nanoformulations are reviewed below.

3.2.1. TLR3

TLR3 is activated primarily by dsRNA.[114] It has also been found to recognize endogenous intra-cellular ssRNA, sequence-independent small interfering RNA (siRNA), and short hairpin RNA (shRNA).[115] Unlike all other TLRs, TLR3 does not signal through MyD88 but via the adaptor protein TRIF.[116] TLR3 signaling is mediated by phosphorylation of tyrosine residues in the TIR domain[117] and phosphatidylinositol-3 kinase[118] that activates nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and MAPKs to induce proinflammatory cytokines such as type I interferons (IFN-α and β). Polyinosinic–polycytidylic acid (poly(I:C)) is commonly employed to stimulate TLR3.[119] In addition to producing proinflammatory cytokines and chemokines, poly(I:C) induces stable maturation of DCs. As a double stranded RNA construct, however, poly(I:C) can be degraded by nucleases in the body. It is thus often administered at formidably high doses that invoke risks of toxicity and autoimmune responses. Many polymeric formulations have therefore been developed to deliver poly(I:C) for more effective immune stimulation.[120]

In one example, poly(I:C) was encapsulated in PLGA microparticles (MP) for intra-lymph nodal vaccination.[121] Upon administration, the formulation exhibited sustained release of poly(I:C) in the lymph nodes over several days. The size of the particles resulted in increased adjuvant uptake by lymph-node-resident APCs and extended the activation of DCs relative to soluble adjuvant or smaller poly(I:C)-loaded NPs. The study also showed that enhanced persistence of poly(I:C) correlated with enhanced humoral and T cell responses to a model antigen which was not evident even with a lofty dose of soluble adjuvant. Delivery systems containing poly(I:C) were further extended to nanoparticles. Toward promoting cellular responses against human papillomavirus (HPV), Rahimian et al. synthesized a polymeric nanoparticle encapsulating a 27 amino acids long synthetic peptide analogous to viral protein E6 and E7 using a biodegradable polymer poly (D,L-lactic-*co*-hydroxymethyl glycolic acid) (pLHMGA).[122] Coencapsulation of poly(I:C) was a critical feature for the vaccine design since the peptide itself is weakly immunogenic. Upon subcutaneous delivery, these particles showed prolonged persistence in lymph nodes where antigen-specific immune responses were primed and subsequent expansion of CD8+ T cells was observed. While soluble poly(I:C) also increased the immunogenicity of the antigen-loaded nanoparticles, the nanoformulations coencapsulating the adjuvant considerably alleviated safety concerns by reducing the systemic concentrations of poly(I:C).

In a similar study by Kim et al., lymph node targeting synthetic vaccine nanoparticles (SVNPs) were made by loading poly(I:C) to amine-functionalized poly(γglutamic acid) (PGA) nanoparticles.[123] Poly(I:C) readily associated with the positively charged nanoparticles via electrostatic complexation, and a separate set of antigen-loaded nanoparticles were prepared and administered in combination (Figure 4B). APCs treated with SVNP-OVA (encapsulating OVA) and SVNP-IC (encapsulating poly(I:C)) showed higher secretion of proinflammatory cytokines (TNF-α and IL-6) and type I interferons than with soluble mix of adjuvant and OVA. In addition to activating innate NK cells, the SVNPs also induced CD8+ T cell responses that conferred antigen-specific cellular immunity. The study highlights that adjuvant and antigen nanoparticles may be prepared separately and administered together for vaccination. Such strategy may reduce the complexity and manufacturing challenge in preparing antigen/adjuvant coformulated nanoparticles.

3.2.2. TLR7/8

TLR7 and TLR8 are phylogenetically similar and play a role in sensing viral ssRNA and short dsRNA. Synthetic oligo-ribonucleotides and small-molecule agonists such as imidazoquinolines have been developed for TLR7/8 activation, serving as promising vaccine adjuvant candidates.[113] However, low-molecular-weight imidazoquinoline derivatives including R837 (imiquimod) and R848 (resiquimod) are associated with instability and poor cellular uptake.[124] For these small-molecule agonists, chemical conjugation has been the primary approach for nanoparticle incorporation as imidazoquinoline derivatives can be modified with functional groups for polymer attachment. Nanocarriers prepared with the polymer-adjuvant conjugates can then facilitate delivery to the endosomal compartment for immune stimulation.[4,125,126] Kim et al., for instance, chemically synthesized an adjuvant nanoparticle by conjugating imidazoquinoline to the surface of 30 nm iron-based nanoparticles.[127] The authors showed efficient internalization of these nanocomplexes by immature DCs, which were subsequently activated and expressed costimulatory markers. When combined with OVA protein these adjuvant-nanocomplexes led to antigen-specific cytotoxic T cell responses and increased proliferation of adoptively transferred antigen-specific T cells.

As TLR7/8 have inherent physicochemical properties that may differ from the building blocks of carrier systems, conjugation of these adjuvants to polymers could influence the dynamics of polymer assembly. This influence was taken into consideration by Lynn et al. in designing an adjuvant based polymeric structure to enhance humoral and cellular responses against protein antigens.[128] By modulating the amount of TLR7/8 agonists conjugated to *N*-(2-hydroxypropyl)methacrylamide (HPMA), the authors systematically evaluated the influence of the agonist density on particle formation, in vivo kinetics of cellular uptake by different APCs, and the resulting innate immune responses. They demonstrated that these constructs enhanced the intensity and duration of innate immune activation in draining lymph nodes and reduced systemic distribution and toxicity. The benefits of the polymer conjugates were also extended to soluble antigens and temperature-responsive polymers to promote antigen-specific adaptive immune responses.

Along the lines of stimuli-responsive polymers, Nunh et al. developed pH sensitive nanogels as a delivery vehicle that enriched molecular adjuvant at the site of administration and the draining lymph node.[129] Using amphiphilic block copolymers consisting of methoxy triethylene glycol methacrylate (mTEGMA) and pentafluorophenyl methacrylate (PFPMA) as the nanoparticle backbone, the authors covalently ligated a TLR7/8 agonist 1-(4-(aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine (IMDQ) to the polymers. With the nanoparticulate agonist, the authors demonstrated increased adjuvant stability and antigen/adjuvant codelivery to the same antigen-presenting cells in the draining lymph node. Moreover, IMDQ nanogels confined the release of type I IFNs to the draining lymph nodes leading to enhanced local recruitment of monocytes and reduced systemic toxicity. The functional implication of the design strategy was a higher number of IFN-γ secreting CD4+ and CD8+ T cells in addition to a 10-fold higher level of IgG2c compared to soluble IMDQ.

3.2.3. TLR9

TLR9 is activated by unmethylated cytosine-phosphate-guanine (CpG) dinucleotides of bacterial or viral origin.[130] This enables researchers to design molecularly well-defined synthetic TLR9 agonists with oligodeoxynucleotides (ODNs) of 8–30 bases in length containing multiple CpG motifs. Immune potentiation by CpG ODNs induces innate immune activation of DCs that leads to upregulation of chemokine receptor CCR7 and secretion of Th1-promoting chemokines and cytokines

including macrophage inflammatory protein-1, IFN-γ-inducible protein-10 (IP-10), and other IFN-inducible genes. These signals ultimately enhance T cell trafficking to the draining lymph nodes resulting in the enhancement of adaptive immune responses at a later stage.[131] CpG ODNs are perhaps one of the most widely applied adjuvant in nanoparticle vaccine development, and its nanoformulations have been demonstrated to enhance its efficacy by preventing degradation by nucleases and improving cellular uptake.[132] Multiple approaches have been applied to associate CpG with nanocarriers, including electrostatic complexation,[133,134] chemical conjugation,[135,136] and physical encapsulation.[137] Attempts at pathogen mimicry have also been made with vaccine nanoparticles that combine CpG with other TLR ligands (Figure 4C), which demonstrated synergistic innate immune potentiation and effective priming of CD8+ T cells.[138,139] These formulations have been applied in a variety of vaccination settings against cancer and infectious disease.

To enhance CpG delivery and release to the endosomal compartment, Wilson et al. described a pH-responsive, endosomolytic polymeric micelle prepared with pyridyl disulfide ethyl methacrylate (PDSEMA) (Figure 4D).[133] By covalently ligating antigens via disulfide bonds and electrostatically adsorbing CpG, the 23 nm nanocarriers significantly enhanced the immunogenicity of the antigen target, resulting in an increase in CD4+ IFN-γ+ responses as well as inducing a balanced IgG1/IgG2c humoral response. A similar dual delivery approach was demonstrated with PLGA carriers, which encapsulated OVA with surface adsorbed CpG molecules.[140] The system demonstrated a slow release profile of the adsorbed CpG that ushered robust CTL responses in mice, evident from the release of IFN-γ from antigen-specific CD8+ T cells. In a different approach for CpG delivery, Liu et al. conjugated phosphorothioate-stabilized CpG oligos to a lipophilic tail for albumin hitch-hiking.[135] The amphiphilic CpG efficiently associated with serum album and accumulated in the draining lymph nodes upon subcutaneous administration. The functional application of this platform was explored by combining the amphiphilic CpG with peptides. The conjugate formulation significantly enhanced the expansion of antigen-specific, cytokine-producing CD8+ T cells and their cytolytic activity as compared to unmodified peptide/CpG immunizations.

Toward vaccination against microbial pathogens, nanoparticulate CpG has been explored by Kachura et al. for preparing anthrax vaccine.[141] The authors cross-linked CpG ODN molecules to sucrose polymer Ficoll to engineer nanoparticles that have been previously characterized to augment IFN-α production from human plasmacytoid dendritic cells (pDCs). When primates were immunized against recombinant protective antigen (rPA) from *B. anthracis* with the Ficoll nanoparticles, a rapid induction of protective toxin neutralizing antibody titers was observed. Following a single immunization, the primates showed protection against a high-dose aerosolized anthrax challenge. In contrast, monomeric adjuvants were found to dissipate rapidly upon systemic administration and showed low induction of humoral responses. With less systemic reactogenicity, the Ficoll nanoparticles also proved to be safer than the free adjuvant.

3.3. Nanoparticulate Agonists for Cytoplasmic PRRs

Rounding out the different PRR categories are danger sensors that are located inside the cytoplasm. These cytoplasmic PRRs detect microbial proteins or genetic materials upon cellular invasion by pathogens. Cytoplasmic PRRs can be broadly classified into three families based on their structures: NLRs, RIG-I, and ER bound interferon (IFN)-inducible proteins (stimulator of interferon genes; STING). Owing to the technical challenge in intracellular delivery, adoption of agonists against cytoplasmic PRRs are generally less common. However, application of nanocarriers has shown much promise in improving cytoplasmic PRR activation for vaccine development.

3.3.1. NOD

The NLR family includes 22 members identified in humans and more than 30 in mice. NOD1 and NOD2 are sensors of peptidoglycan moieties from Gram-positive and Gram-negative bacteria that enter the cells through membrane defect or phagocytosis. Through cooperation with TLRs, their activation modulates inflammatory and apoptotic response to facilitate the activation of immune response.[142]

In order to deliver NOD ligands, Pavot et al. used a PLA-based nanocarrier to encapsulate both NOD1 (CL235 [tetradecanoyl-δ-D-glutamyl-(L)-meso-lanthionyl-(D)-alanine]) and NOD2 ligands (CL365 [6-*O*-stearoyl-*N*-glycolyl-murabutide]).[143] The acyl chains on these ligands facilitated their incorporation into the PLA matrix. Upon administration in mice with a surface adsorbed HIV antigen, the NLR-activating nanoparticles induced mucosal and systemic immune responses. The authors also showed efficient uptake of the PLA NPs by human and mouse DCs in vitro. Although the mechanism of immune activation was not examined in detail, the vaccination results showed promise with efficient activation of autologous T cells from HIV-positive patients. These results highlight the nanoparticulate NLR agonist as a promising candidate for promoting both cellular and mucosal immunity.

3.3.2. STING

STING is a transmembrane adapter protein on the endoplasmic reticulum (ER) responsible for cytosolic sensing of dsDNA from viral, bacterial or parasitic infections. Cytosolic DNA is detected by sensor cyclic-guanosine monophosphate adenosine monophosphate synthase (cGAS) that catalyzes the synthesis of various cyclic dinucleotides (CDN), including cyclic GMP-AMP (cGAMP) and cyclic di-GMP (cdGMP), which act as secondary messengers and agonists of STING.[144] Cytosolic DNA sensing activates STING pathway in DCs, leading to their maturation and production of type I interferons and other cytokines.[145] An increasing number of CDNs that directly activate STING have been identified and are emerging as adjuvant candidates to boost humoral as well as cellular immunity. However, the poor penetration of CDNs across cellular membrane remains a major challenge in their application. Therefore, vehicles for

intracellular delivery of STING agonists are being explored for vaccine development.

In recent studies, liposomes have been used to enhance the intracellular delivery STING agonists. Hanson et al. demonstrated that a liposomal carrier enhanced intracellular and lymphatic delivery of cdGMP, leading to enhanced cellular and humoral immunity while reducing the risk of systemic toxicity.[146] Compared to free cdGMP formulation, antigens formulated with liposomal cdGMP showed a 30-fold enhancement in antibody induction. Several other formulations have also been developed for CDN delivery. For instance, cationic liposomes have been applied with the aim of increasing encapsulation efficiency of negatively charged cGAMP (Figure 4E). Such constructs showed increased uptake relative to soluble cGAMP and induction of IFN-β in an in vitro as well as an in vivo setup.[147,148]

3.3.3. RIG-I

RIG-I, a part of the RIG-I-like receptor family that includes MDA5 and LGP2, is activated by dsRNA produced during viral replication.[149] RIG-I-mediated viral sensing is primarily triggered by 5′-triphosphate bearing RNA.[150] When activated, RIG-I and MDA5 subsequently activate NF-κB and IFN regulatory factors 3 and 7 (IRF3/7) to induce antiviral type I IFNs. The pleiotropic effect of type I IFNs is critical in shaping the antiviral and antibacterial cellular immunity.[151]

Owing to the difficulty in accessing RIG-I in the cellular cytoplasm, efforts to deliver RIG-I agonist 5'-triphosphate dsRNA with nanocarriers were recently made by Goodwin and Huang using a lipid-coated calcium phosphate (LCP) nanoparticle (Figure 4F).[152] Using a mouse colon cancer peptide antigen (p-AH1-A5) as a vaccine candidate, the authors demonstrated excellent antigen-specific immunity with the RIG-I nanoparticle vaccine. Interestingly, in a head-to-head comparison with nanoformulations encapsulating CpG and cGAMP, the nanoparticulate RIG-I agonist yielded the highest cytotoxic T lymphocyte response. While direct comparisons among different adjuvants is difficult as optimal dosing and delivery methods may vary, the study highlights some of the functional differences among PRR agonists. By demonstrating the capacity to deliver three different types of agonists using the same nanocarrier, the work also suggests the possibility of codelivering multiple agonists simultaneously for cooperative immune activation.

4. Challenges and Opportunities in Antibacterial Vaccination

The previous sections summarize the different nanoparticle platforms and nanoparticulate adjuvants to illustrate a broad range of engineering toolsets for advancing antibacterial vaccine designs. In the following section, we discuss several major bacterial diseases and the progress and challenges in their vaccine development. In particular, four major microbial pathogens, including *M. tuberculosis*, *H. pylori*, *S. aureus*, and *C. trachomatis*, are examined (**Figure 5**). Each of these pathogens possesses distinctive vaccination challenges that correspond to the microbe's antigenic signature, immune-evasive mechanism, and site of infection. In tackling these challenges, innovative nanoformulations have been designed to boost antibacterial defense by controlling immune modulation, enabling mucosal delivery, and facilitating nondisruptive toxoid preparation. Many nanoparticle- and nanomaterial-based vaccination strategies have demonstrated enhanced potency and improved safety profiles. With the aim of capturing the vast opportunities in materials-based vaccination approaches, we survey the vaccine nanoformulations that have shown promises against these major bacterial diseases.

4.1. M. tuberculosis

Mycobacterium species have been associated with a range of diseases throughout human history and remain a major health issue to this day. In particular, *M. tuberculosis*, which is the microbial agent responsible for tuberculosis (TB), has been estimated to infect more than 2 billion people globally, and is among the leading cause of infectious diseases only second to HIV.[153,225] Tuberculosis is an airborne infectious disease. Following inhalation of aerosol droplets containing mycobacteria, the bacilli reach the alveoli in the lower respiratory airways where they are mostly engulfed by alveolar macrophages.[154] The bacilli express a variety of mycobacterial factors interfere with normal intracellular vacuole trafficking, phago-lysosomal fusion, and maturation to escape killing by host immune cells.[155] At the same time, a series of granulomatous responses will be initiated by alveolar macrophages to limit the spread of the mycobacteria to other organs.[156] This response leads to local accumulation of macrophages, lymphocytes, and epithelioid cells, resulting in the formation of granulomas known as the pathological hallmark of *M. tuberculosis* infection. Although the granulomas temporarily contain the bacteria, the ultimate collapse of these structures causes persisting bacteria to reactivate. Contribute to the survival and pathogenesis of *M. tuberculosis* is the bacteria's unique cell wall composition, which contains a wide range of ligands for cellular PRRs.[157] Even though these mycobacterial PAMPs result in recognition by immune cells, they are capable of modulating host responses through fine-tuning the downstream signaling pathways to promote bacterial persistence.

A few antibiotics are available for the control of mycobacterial infection, yet treatment of TB is facing an imminent challenge with increasing reports of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis.[158] As the World Health Organization has set the goal to end the TB epidemic by 2035, development of an effective and safe TB vaccine is of high priority. It is generally agreed that successful TB vaccines need to induce T cell responses, particularly polyfunctional CD4+ T cells that express IFN-γ, TNF-α and IL-2,[159] and whole bacteria vaccines are generally applied to induce these responses.[160] To date, the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine is the only licensed vaccine against TB, but its protective efficacy varies among individuals and is negligible toward pulmonary tuberculosis in adults.[160,161] Recombinant BCG, including strains that express Th1 cytokines, additional *M. tuberculosis*-specific antigens, and listeriolysin, have been

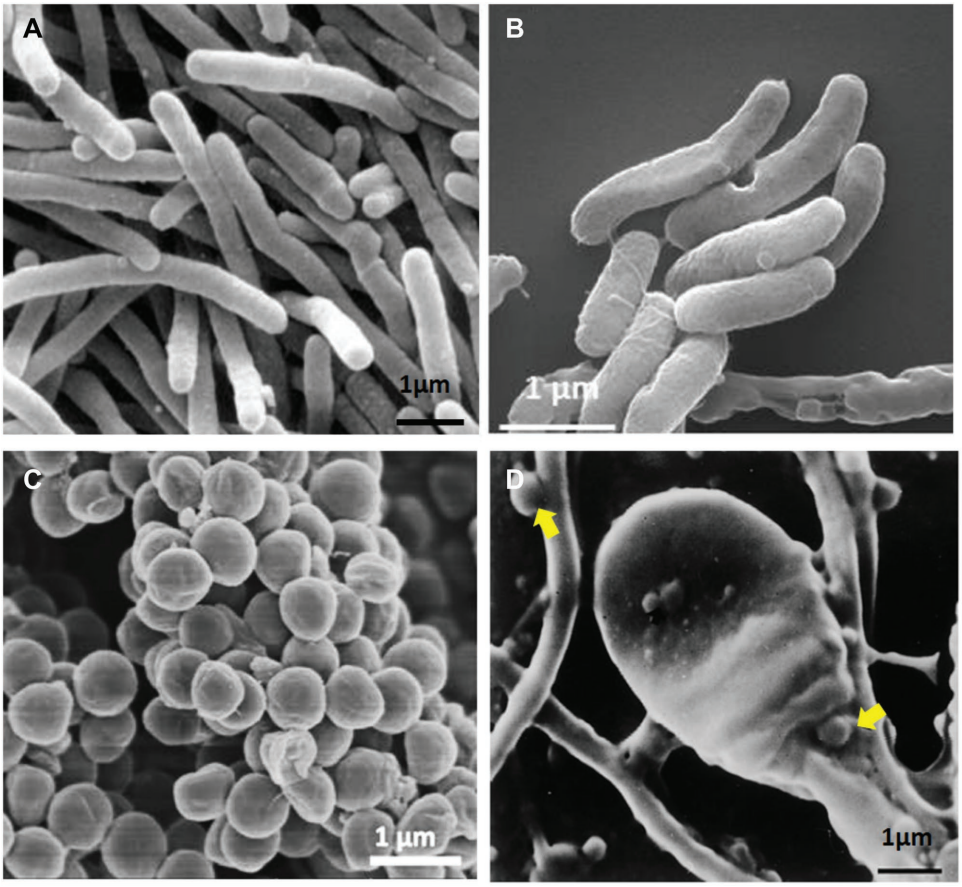

Figure 5. Scanning electron microscope images showing morphology of A) *M. tuberculosis*. Reproduced with permission.[225] 2004, John Wiley and Sons. B) *H. pylori*. Reproduced with permission.[226] 2017, Elsevier. C) *S. aureus*. Reproduced with permission.[227] 2014, Nature publishing group. D) *C. trachomatis* indicated by yellow arrows. Reproduced with permission.[228] 2002, John Wiley and Sons.

engineered to improve the immunogenicity of BCG.[162] To further enhance immune responses to Ag85 and ESAT-6 proteins, which are immunodominant TB antigens that are missing in BCG vaccines, nonpathogenic *M. vaccae*, and an attenuated clinical isolate of *M. tuberculosis* are also being investigated as TB vaccine candidates.[163] These whole bacteria vaccines are now in the Phase III and the Phase I trial, respectively.[164] While these whole bacteria vaccines have demonstrated promising efficacy, safety concerns remain to individuals who may be immunocompromised. Subunit TB vaccines, which promise better safety profiles, thus continue to draw research interest and developmental efforts from scientists and engineers.

4.1.1. Nanoparticle Vaccines against M. tuberculosis

A wide range of immunodominant mycobacterial antigens, includes Ag85A/B, ESAT-6, TB10.4, Mtb8.4, and MPT83, have been examined for their protective efficacy against *M. tuberculosis* infection.[165,166] To enhance the immunogenicity of these subunit antigens, synthetic polymeric particles have been broadly applied. PLA or PLGA-based nanoparticles bearing mycobacterial proteins or antigen-encoding DNA were constructed in several studies and demonstrated the ability to induce antigen-specific antibodies, IFN-γ-secreting CD4+ T cells, and even CD8+ T cell-mediated cytotoxicity.[167,168] A majority of these PLGA nanoparticle vaccines target a single antigen, and such single-antigen approach is generally deemed inadequate to provide broad-spectrum protection against mycobacterial infection. In addressing this issue, Cai et al. produced PLGA particles that contained DNA encoding three mycobacterial antigens: Ag85B, MPT-64, and MPT-83.[169] After intramuscular vaccination, elevated humoral and cellular responses against all three antigens were observed in mice receiving the PLGA-formulated DNA vaccine. In particular, a single dose of PLGA-encapsulated DNA reduced the bacterial loads in the lungs and spleen following virulent *M. tuberculosis* infection, and the protection was comparable the BCG vaccine. Since most TB patients acquire mycobacterial infection via the respiratory route, aerosolized particle vaccines have also been prepared for pulmonary delivery to elicit anti-TB immunity in the lungs.[170] The pulmonary delivery approach was investigated in studies by Lu et al. using aerodynamic PLGA particles encapsulating Ag85B antigen and trehalose-6,6-dibehenate adjuvant for airway delivery.[171,172] In a guinea pig model, insufflation of the PLGA particle vaccine resulted in anti-TB protection, albeit inferior to BCG vaccination.[171] Given that the BCG vaccine has been widely used in many developing countries and there

is a need to prolong its protective activity, the authors subsequently explored the effect of employing the pulmonary PLGA vaccine as a booster for BCG. This heterologous vaccination strategy with subcutaneous BCG priming and intra-tracheal PLGA-Ag85B boosting not only effectively reduced the bacterial burden in both the lungs and spleen, but also led to significantly smaller histopathological changes in the lungs of the *M. tuberculosis*-challenged animals as compared to single BCG-immunization and homologous BCG prime-boost regime.

The importance of the pulmonary immune response in protecting against *M. tuberculosis* infection was further highlighted in another study using polypropylene sulfide (PPS) nanoparticles with surface-conjugated Ag85B.[173] Compared to the intradermal immunization route, pulmonary vaccination with the Ag85B PPS nanoparticle led to a significant reduction of bacteria burden in the lungs of mice with *M. tuberculosis* aerosol challenge. Ballester et al. suggested the protective efficacy was attributed to the PPS nanoparticle-induced Th17 responses in the lungs and spleen of the pulmonary immunized mice, which is absent in mice receiving the same nanoparticle vaccine via the intradermal route.[166,174] With increasing evidence suggesting that IL-17A-secreting CD4+ T cells may enhance the recruitment and activation of neutrophils and IFN-γ-producing CD4+ T cells that help control mycobacterial growth, it will be of interest to promote Th17 responses on top of polyfunctional Th1-CD4+ T cells to improve protection against mycobacterial challenge.

In addition to be used as a vaccine vehicle, synthetic polymeric nanoparticles have also been applied as a carrier for immunomodulators that help to shape desirable immune responses. For example, coadministration of IL-12 encapsulated PLGA particles with conventional adjuvant-formulated mycobacterial antigens was shown to boost both stronger antigen-specific humoral and cellular responses compared to those without the IL-12 PLGA particles.[175] More importantly, the combination of the AS01B adjuvant and the PLGA particle entrapped IL-12 resulted in a more effective reduction of bacterial load following virulent *M. tuberculosis* challenge than BCG. As opposed to stimulating the innate immunity, the IL-12 particles adopt the role analogous to an activated antigen present cell. By releasing cytokines involved in the differentiation of naïve T cells into Th1 cells, the particles directly modulate the cytokine milieu that influences the outcome of the vaccination. With an efficacy superior to the conventional BCG vaccine, this subunit vaccine formulation can be of great clinical importance.

4.2. H. pylori

H. pylori is a Gram-negative, spiral-shaped, flagellated bacterium that has infected more than half the human population.[176,177,226] Most *H. pylori* organisms reside in the mucus layer, but some organisms attach to the apical surface of gastric epithelial cells and can elicit gastritis in the stomach mucosa.[178] *H. pylori* strongly interacts with gastric epithelial cells, and such interaction can lead to a variety of gastrointestinal disorders, such as chronic gastritis, duodenal ulcers, gastric ulcers, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, gastric adenocarcinoma, and gastric cancer.[179] These pathogens are classified as a class I carcinogen by the World Health Organization. In addition, they also contribute to gastric autoimmunity.[180]

Even though standard therapies that combine proton pump inhibitor and antibiotics are commonly administered to treat *H. pylori*, challenges including antibiotics resistance and strain-dependent efficacy variation make bacterial eradication difficult and often results in disease relapse.[177,181] Vaccination against *H. pylori* has thus been considered as a valid and cost-effective alternative to control the prevalence of *H. pylori*. Development of *H. pylori* vaccine began in the early 1990s. Several *H. pylori* proteins, such as urease subunits, VacA, catalase, superoxide dismutase, thiolperoxidase, flagellin, heat-shock proteins, *H. pylori* adhesin A, NAP, and CagA have been reported as vaccine candidates.[182] However, there is no licensed *H. pylori* vaccine on the market to this date.[183] One critical challenge in *H. pylori* vaccine design is that the mechanism of protection against *H. pylori* remains elusive. The pathogen has evolved multiple mechanisms to induce immune tolerance, and through its many virulence factors the pathogen has been shown to inhibit T cell activation, reduce T cell proliferation, induce T cell death, and impair Th1 response.[184] Encouragingly, several studies indicate that the presence of a Th1 response contribute to protective immunity against *H. pylori*,[185] offering a glimmer of hope toward an effective *H. pylori* vaccine.

Another major challenge in *H. pylori* vaccine development is the induction of mucosal immune response in the gastrointestinal area. As *H. pylori* colonizes the surface of epithelium and mucus layer in the stomach and areas of gastric metaplasia in the duodenum, immunity against *H. pylori* infection logically requires mucosal immune response, such as the presence of immunoglobulin A (IgA) and lymphocytes in the gastrointestinal track. Given the distinction between the mucosal immune system and the peripheral lymphoid system,[186] vaccines need to be delivered to the MALT to activate the mucosal immune system. To reach the gastric MALT, antigens and adjuvants would need to withstand the degradative environment of the digestive system. A delivery platform that can protect antigen cargos from acidic and enzymatic degradation would thus be required for *H. pylori* vaccination.

4.2.1. Nanoparticulate Vaccines against H. pylori

Several nanoformulations have been designed as *H. pylori* vaccine candidates. For instance, Kim et al. prepared a PLGA-based nanoparticle vaccine that carries *H. pylori* lysates for oral delivery.[187] As compared to immunization with soluble lysates, which did not induce immunoglobulin upon oral delivery, the PLGA nanoparticles induced significant levels of antigen-specific mucosal IgA and serum IgG. The enhanced immune potentiation effect was attributed to the nanoparticles' ability to better transport antigens to M cells and antigen presenting cells in the Peyer's patch. Although the nanoparticle vaccine was shown to be inferior to a cholera toxin-based *H. pylori* vaccine formulation in the study, the improved safety could make the nanoformulations more applicable for human use. Similarly, a microsphere formulation comprising of poly (D,L-lactide)-polyethylene glycol copolymer (PELA) and *H.* pylori

lysates has been demonstrated to induce mucosal immunity.[188] Using miniature pigs as a model, the authors showed that the microspheres adhered to the gastrointestinal mucous membrane upon oral administration and subsequently arrived at the Peyer's patch. As a result, immunization with the microspheres increased the number of antibody-secreting cells in the intestine and induced a threefold enhancement in saliva and gut IgA as compared to soluble lysates.[188] These works serve as proof-of-concept studies that highlight the potential of particulate carriers in *H. pylori* vaccine development.

Protection from *H. pylori* infection using nanoparticle-based vaccine was more recently demonstrated using a mouse model of *H. pylori* challenge. In order to protect antigen from premature release in the stomach and degradation by gastric acid, Tan et al. prepared a nanoparticle vaccine by blending PLGA with HP-55, which is a special type of enteric coating polymer that only dissolves at pH > 5.5.[88] Incorporated into the nanoparticles were two adjuvants, a chimeric flagellum and cholera toxin B subunit, and the Th and B cell epitopes of *H. pylori* urease. Following oral vaccination with the acid-resistant nanoparticles, higher levels of *H. pylori*-specific IgG, IgA, and T cell infiltrate were observed as compared to immunizations with an alum-based vaccine and a PLGA only formulation. In particular, the expression of IFN-γ and IL-17 mRNA was significantly increased by the PLGA/HP-55 formulation, indicating potent induction of Th1 and Th17 immune responses. In a prophylactic setting, the nanoparticle vaccine resulted in 10–100-fold reduction in bacterial load. 43% of vaccinated mice were completely clear of *H. pylori* infection following the pathogen challenge. The study highlights the synthetic flexibility of nanoparticle-based formulation toward overcoming the physiological barrier in gastrointestinal delivery. By protecting and shuttling antigens and adjuvants past the gastric environment, nanoparticles offer a compelling platform toward modulating mucosal antimicrobial defense against *H. pylori*.

4.3. S. aureus

S. aureus infection presents an urgent public health threat that is compounded by the growing prevalence of methicillin-resistant *S. aureus* (MRSA). Also known as golden staph, *S. aureus* is a Gram-positive bacterium that can be found in the normal flora of the body.[227] The commensal bacterium resides primarily on the skin and in the nasopharynx. Pathogenic strains of the bacterium are associated with a high expression of proteins toxins can cause skin infection, osteomyelitis, infective endocarditis, pneumonia, bacteremia, and septic shock syndrome.[189] The bacterium is one of the most common causes of hospital-acquired infections, and contraction of the infection, frequently following surgery, is associated with a fivefold increased risk of in-hospital death.[190] A 2003 study highlighted the exorbitant economic burden incurred by *S. aureus* infections, estimating that *S. aureus*-related healthcare cost amounted to $14.5 billion for inpatient stays and $12.3 billion for surgical patient stays in the U.S. alone.[191] Prophylactic measures against the bacteria is thus of urgent need, particularly as antibiotic therapeutics are faced with increasing antidrug resistance.

There are significant research and commercial interests in the development of staph vaccine, and a variety *S. aureus* antigens—i.e., clumping factor A (ClfA), manganese transporter MntC, capsular polysaccharides, collagen binding adhesion protein (CNA), and iron-regulated surface determinant B (IsdB)—and secreted virulence factors—i.e., α-hemolysin (Hla), toxic shock syndrome toxin (TSST), and staphylococcal enterotoxin B (SEB)—have been applied for vaccine development.[192] Despite varying degrees of success with these vaccine formulations, there is no clinically approved vaccine to date. Past studies have shown a lack of definitive correlation between humoral responses to *S. aureus* antigens and immunity against the bacterial pathogen. For instance, antibodies mounted against ClfA and IsdB have failed to offer protection from the bacterial infection,[193,194] and this lack of efficacy by antibodies has been attributed, at least in part, to the bacteria's surface expression of protein A, which is an immunoglobulin-sequestering protein that can block antibody-mediated phagocytosis by binding to the Fc domain of immunoglobulin.[195] On the other hand, Th1 and Th17-mediated immunity has been shown to play a key role in antimicrobial defense against *S. aureus*. In a study that examines the efficacy of a subunit vaccine, CD4+ lymphocyte-derived IFN-γ and IL-17A were found to recruit functional phagocytes and mediate superior killing of *S. aureus*.[196] Notably, Th17 also prompts the development of antimicrobial peptide in skin and mucosal cells, which may further contribute to *S. aureus* immunity.[197]

4.3.1. Nanoparticle Vaccines against S. aureus Antigens

With the aim to enhance the immunogenicity of *S. aureus* antigens, several nanoparticles have been formulated to deliver *S. aureus* antigens as potential vaccine candidates. Genta et al., for instance, applied PLGA nanoparticles to encapsulate a purified recombinant CNA fragment of *S. aureus*.[198] The extracellular-matrix-binding, cell wall-anchored protein has been implicated as a major mediator of *S. aureus* pathogenesis by enabling bacterial adherence to susceptible tissues. Several complications of *S. aureus* infection, including infective endocarditis, sceptic arthritis, and osteomyelitis are linked to the bacteria's collagen-binding phenotype.[199] Using a w/o/w double emulsion process, the authors prepared CNA nanoparticle vaccines and demonstrated enhanced antigen immunogenicity in a mouse model. Compared to free CNA fragment formulated with alum, the nanoparticles elicited 3 times the anti-CNA antibodies upon subcutaneous administration.[200] The authors also combined the nanoformulations with a thermos-responsive hydrogel comprised of chitosan-glycerolphosphate for intranasal administration. Both subcutaneous and intranasal vaccinations induced significant levels of anti-CNA IgG and IgM titers. The study also showed that serum from vaccinated mice was able to abrogate the collagen-binding activity of *S. aureus*, offering a promising formulation toward intercepting one of the bacteria's many pathogenic factors.

Efforts to improve systemic and mucosal immunity against MRSA have also inspired Sun et al. to develop an nanoemulsion-based vaccine consisting of staphylococcal cell-wall anchored IsdB, Hla, isopropyl myristate, Cremophor EL-35,

and propylene glycol.[201] The 30 nm nanoemulsion was shown to enhance serum IgG1, IgG2a, and IgG2b levels as compared to the free antigen formulation following a prime-boost-boost vaccination regime via the intramuscular route. Notably, upon intranasal vaccination, the nanoemulsion elevated the level of IL-17.[194] In addition, IFN-γ level was increased upon the nanoemulsion vaccination, indicating the induction of a Th1 response. In a mouse model of MRSA infection, immunization with the nanoemulsion showed significant survival benefit and reduced bacterial burden in the lung as compared to the free protein formulation. The improved protective effect was attributed to the improved humoral response as well as the Th1/Th17-mediated immune responses elicited by the nanoformulations.

To highlight the role of Th1 and Th17 responses against *S. aureus* infection in the absence of humoral response, Misstear et al. prepared polystyrene particles surfaced functionalized with ClfA for intranasal vaccination.[202] The particulate vaccine was coated with *Ulex europaeus* agglutinin I (UEA-1), a fructose binding lectin, to mediate binding and transcytosis across antigen-sampling microfold cells in the mucus. Following a prime-boost-boost intranasal vaccination schedule on day 0, 14, and 28, the particulate vaccine induced a potent cellular response but curiously no humoral response. Vaccinated mice possessed an increased level of IFN-γ and IL-17 positive CD4 and CD8 T cell, yet they had no detectable IgG or IgA titer against the bacterial antigen. Despite the lack of neutralizing antibodies, the particulate vaccine was effective in protecting the mice against an acute *S. aureus* systemic infection, demonstrating that the cellular immunity alone is sufficient for the protection. Mice receiving the particulate vaccines showed reduced bacterial burdens in the peritoneal exudate, kidneys, and spleen 72 h following the bacterial challenge, and increased numbers of macrophages and neutrophils were observed on the sites of infection. This study highlights the role of cellular immunity in *S. aureus* vaccine development.

4.3.2. Antivirulence Nanoparticle Vaccines against Staphylococcal Toxins

An alternative strategy to vaccinate against *S. aureus* is through the use toxoid vaccines, which elicit immune responses against bacterial toxins rather than the microbes themselves. The virulent strains of *S. aureus* are frequently associated with a high expression of toxin factors, which can damage cellular targets for immune evasion, nutrient derivation, and tissue colonization. A high antitoxin humoral response may thus disarm the pathogens and render them less pathogenic. One major toxin factor expressed by *S. aureus* is Hla, which is a pore-forming toxin that oligomerizes to form a membrane-spanning pore for cell disruption. Therapeutic intervention against the protein toxin using monoclonal antibodies has demonstrated reduction in disease severity,[203] suggesting immunity against the toxin may improve disease symptoms. With the advancement of nanotechnology, innovative strategies have also been introduced for the preparation of toxoid vaccines. Several toxoid nanoformulations have been shown in preclinical animal models to reduce the severity of *S. aureus* infections.[204]

A fundamental challenge in preparing toxoid vaccine lies in the toxin inactivation process, the disruptive nature of which can compromise the protein's antigenicity and negatively influence the toxoid vaccine's potency. Many early attempts in developing anti-Hla vaccines were undermined by poor quality control as chemical- and heat-mediated protein denaturation methods were difficult to fine-tune.[205] To overcome the inherent tradeoff between toxoid safety and potency, a nanoparticle detainment strategy was introduced to trap Hla and preclude them from inducing cellular damages without compromising the toxin's immunogenicity.[206,207] To facilitate toxin–particle association, a biomimetic nanoparticle was constructed via surface cloaking with erythrocyte membranes, which are a target substrate of Hla. The resulting nanoparticles readily interacted with Hla, and stabilization by the polymeric nanoparticle core served to detain the toxins, preventing them from interacting with other cellular targets. The nanoparticle-detained Hla, or nanotoxoid(Hla), did not induce any tissue damage upon subcutaneous administration and was capable of eliciting enhanced anti-Hla antibodies as compared to heat-inactivated Hla. Nanotoxoid(Hla)-vaccinated mice also showed extraordinary immunity against the necrotizing damage induced by a high-dose Hla challenge. In a later study by Wang et al., immunization with the nanotoxoid(Hla) was demonstrated to reduce lesion formation and bacteria invasiveness in a mouse model of skin MRSA infection.[204] Immunized animals showed lower skin *S. aureus* count and decreased bacteria dissemination to internal organs such as the spleen, heart, kidneys, and heart.

In addition to Hla, *S. aureus* also secrete a number of other membrane-damaging toxins, including phantom-valentine leucocidin (PVL), bicomponent leukocidins, and γ-toxin.[208] The redundancy of pore-forming virulence factors is conducive to the bacteria's survival and may limit the efficacy of single-antigen toxoid formulations. To combat the plethora of staphylococcal toxins, Wei et al. demonstrated a multitoxin laden nanotoxoid formulation using the aforementioned cell membrane cloaked nanoparticles.[209] By incubating the biomimetic nanoparticles with the hemolytic secreted protein (hSP) fraction derived from *S. aureus* culture supernatant, particles carrying detained Hla, PVL, and γ-toxin were prepared (**Figure 6**A–C). The nanotoxoid(hSP) was nonhemolytic and showed no observable toxicity upon subcutaneous administration in mice. Immunization with the nanoformulations induced germinal center formation in the lymph node and increased antibody titers against the three staphylococcal virulence factors. The nanotoxoid vaccine was shown to be effective in reducing skin lesion formation and bacteria count in a mouse model of *S. aureus* skin infection (Figure 6D–F). These results highlight the nanoparticle-mediated toxin detainment strategy as a promising approach toward combating the various virulence factors of *S. aureus*.

4.4. C. trachomatis

C. trachomatis is the most common sexually transmitted bacterial infection, which is estimated to cause 131 million new infections each year globally.[210,228] *C. trachomatis* infects

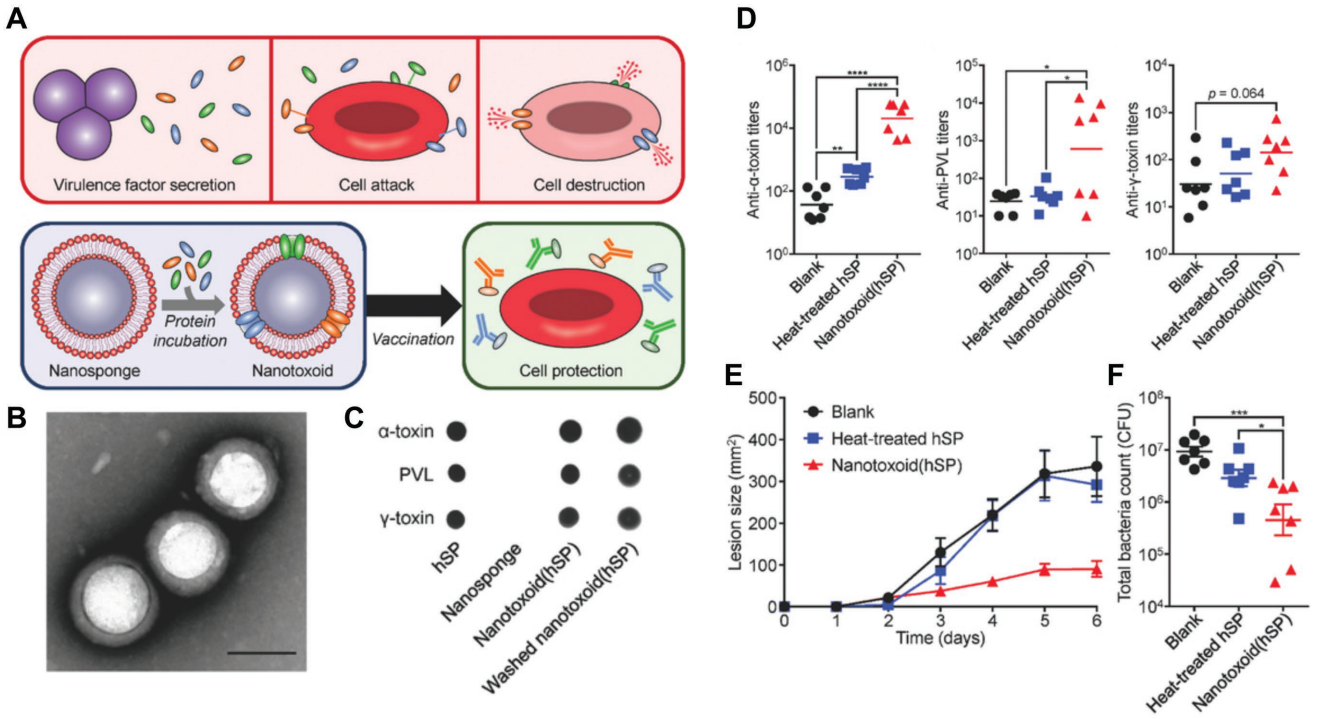

Figure 6. A) Schematic representation of capturing multiple staphylococcal virulence factors using a cell membrane cloaked nanoparticle platform (nanotoxoid(hSP)). B) A transmission electron microscope image of nanoparticles with detained virulence factors. C) Successful detainment of staphylococcal virulence factors, including Hla, PVL, and γ-toxin, was validated through Western blot. D) Immunization with nanotoxoid(hSP) induced antitoxin antibodies against multiple virulence factors. E) Immunization with nanotoxoid(hSP) reduced the skin lesion size and F) bacteria count following a bacterial challenge. Reproduced with permission.[209] 2017, John Wiley and Sons.

genital tracts, ocular and lung epithelium, and leads to distinct disease symptoms. Lower genital infection of *C. trachomatis* is often asymptomatic but can cause chronic infection and inflammation of the uterus, fallopian tubes, ovaries, and pelvic peritoneum.[211] Consequently, long-term sequelae including tubal factor infertility, ectopic pregnancy, and chronic pelvic pain will develop.[210,212,213]*C. trachomatis* is a Gram-negative intracellular bacterium, and it is able to evade immune surveillance by multiple mechanisms. For example, *C. trachomatis* can interfere with auto-lysosome fusion, thus avoiding autophagy-mediated lysis.[214] Also, the chlamydial protease-like activity factor (CPAF) degrades NF-κB and other transcription factors to impair the expression of proinflammatory cytokines as well as MHC class I and class II molecules, which are crucial for immune recognition.[215] The development of chlamydia vaccine has advanced over last few decades with the understanding of the required immunity against *C. trachomatis*. In spite of the general belief that CD8+ T cells play a critical role in controlling intracellular pathogens, the protection against *C. trachomatis* is primarily mediated by CD4+ T cells.[213] Studies have shown that mice lacking CD4+ T cells or IFN-γ expression failed to control chlamydial infection.[216] In particular, Su and Caldwell reported that adoptive transfer of CD4+ T cells obtained from immunized mice was able to protect naïve mice against C. trachomatis infection, whereas CD8+ T cells cannot.[217] It was also found that polyfunctional CD4+ T cells that expressed multiple Th1-associated cytokines including IFN-γ, TNF-α, and IL-2 correlated with optimal clearance of *C. trachomatis* infection.[218]

Currently, there is no vaccine available for *C. trachomatis*. Among different vaccination approaches, mucosal vaccines consisting of chlamydial antigens present the most promising candidates to elicit optimal protective responses. Of the different chlamydial proteins, the most widely used candidate is the MOMP, which is an immunodominant antigen that contains multiple T cell and B cell epitopes.[219] Recombinant MOMP formulated with various adjuvant delivery systems have been evaluated in animal models. For example, subcutaneous immunization with the cationic liposome formulation 1 (CAF01) system, which is based on DDA and Trehalose-6,6-dibehenate (TDB), was shown to induce robust CD4+ T cell responses and Th1-associated IgG2b antibodies that facilitate the clearance of bacteria following intravaginal challenge.[220] A lipid-based delivery system was also exploited for oral immunization, and better protection than a widely used adjuvant combination, cholera toxin plus CpG, was observed in genitally infected mice.[221] Nonetheless, sterile immunity through vaccination with MOMP was not achieved as bacteria were occasionally found in chlamydia challenged mice, thereby prompting development of more effective vaccination strategies.

4.4.1. Nanoparticle Vaccines against C. trachomatis

The self-assembled vault protein has been exploited as a vaccine carrier to encapsulate chlamydial MOMP or antigens derived from polymorphic membrane protein G-1 (PmpG).[36,37]

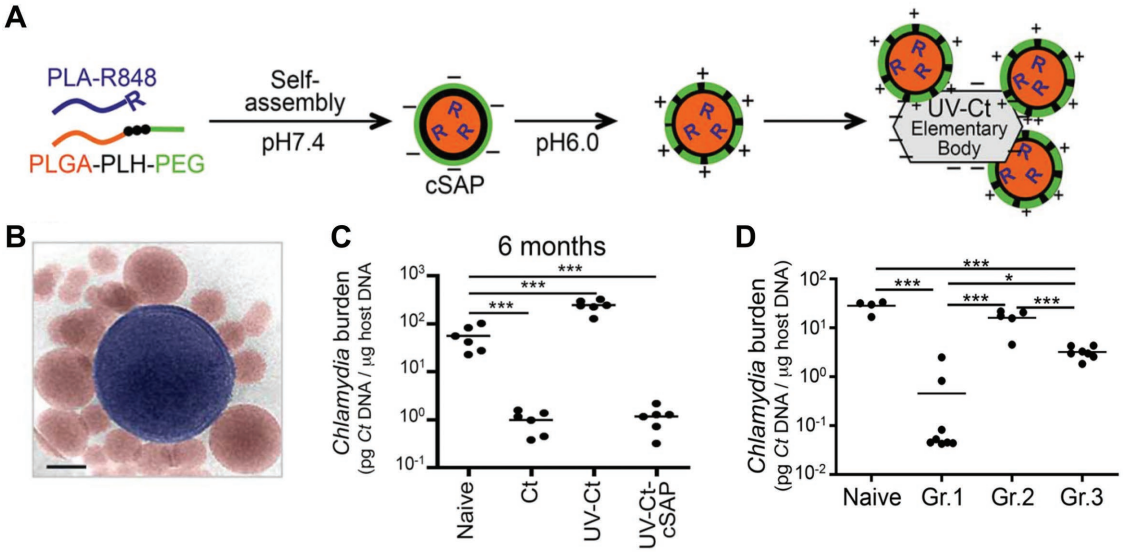

Figure 7. Schematic representation of surface of a charge-switching synthetic adjuvant particle (cSAP) and its conjugation with UV-treated *C. trachomatis* (UV-Ct). B) A representative cryogenic transmission electron microscope image of a UV-Ct-cSAP cluster showing cSAP in red and UV-Ct in blue (scale bar = 100 nm). C) Ct burden after i.u. Ct challenge 6 months after i.u. immunization (n = 4–10 mice per group; ***$P < 0.001$). Statistical differences were assessed using one-way ANOVA followed by Bonferroni posttest. D) Mice receiving adoptively transferred NR1 cells were intranasally immunized with UV-Ct-cSAP followed by treatment with control rat IgG and antiintegrin $\alpha 4$ blocking antibody at different stages to prevent homing of Ct-specific T cells to the uterus. Uterine Ct burden 3 d after i.u. Ct challenge was shown. Statistical differences were assessed using one-way ANOVA followed by Bonferroni posttest. Reproduced with permission.[126] 2015, American Association for the Advancement of Science.

The vault nanocapsules are under 100 nm in size and can be readily uptaken by dendritic cells. Intranasal vaccination with the chlamydial antigen-loaded vaults was shown to effectively induce antigen-specific T cell and antibody responses, and it protected mice from genital infection of *C. trachomatis*. Recently, PLA nanoparticles encapsulating an MOMP peptide (M278) was investigated for the use as a chlamydial vaccine. Following subcutaneous immunization, M278-loaded PLA nanoparticles elicited both higher peptide-specific T cell responses, including IFN-γ, IL-2, and IL-17, as well as antibodies compared to M278 peptide alone.[222] The adjuvanticity of PLGA nanoparticles in augmenting immunogenicity of another MOMP peptide or full protein has also been explored.[223,224] The data showed that in contrast to the PLGA vaccine vehicle, MOMP-encapsulated PLGA nanoparticles promoted the production of Th1-cytokines, such as IL-6 and IL-12p40, in J774 macrophages, as well as T cell expansion and MOMP-specific antibodies in immunized mice. However, the efficacy of these MOMP-containing polymeric vaccines against chlamydial infection was yet to be investigated in vivo and warrants further studies.

A very effective approach in inducing protective immunity against *C. trachomatis* was recently demonstrated by Stary et al. by combining UV-inactivated *C. trachomatis* with synthetic nanoparticles composed of PLGA and PLA-coupled TLR7/8 agonist (R848) (**Figure 7**A,B).[126] The nanoparticles were surface functionalized with poly(L-histidine), the positive charge of which at pH 6.5 facilitated adherence to the negatively charged bacterial surface. In striking contrast to UV-inactivated chlamydia, which are low in immunogenicity and unable to reduce the bacterial burden following infection, intrauterine immunization with synthetic adjuvant particle-conjugated UV-treated *C. trachomatis* (UV-Ct-cSAP) conferred a robust immunity that is comparable to immunization with live bacteria (Figure 7C). Moreover, pathological changes caused by chlamydial infection such as increased serous exudate in fallopian tubes (hydrosalpinx) were significantly improved in UV-Ct-cSAP vaccinated mice as well. By using gene knock-out mice or antibody-mediated depletion, the authors also confirmed the UV-Ct-cSAP induced protection can be solely attributed to CD4+ T cells, but not CD8+ T cells or B cells. More importantly, mucosal immunization with UV-Ct-cSAP was able to induce the clustering of *C. trachomatis*-specific T_{RM} in the uterus that further contributed to the protection against intrauterine chlamydial challenge together with circulating effector memory T cells (Figure 7D). This study not only demonstrates the immunological mechanisms needed for *C. trachomatis* prevention, but also highlights the potential of nanoformulation for boosting the immunogenicity of whole bacteria cells. The charge-switching, bacteria-binding nanoparticle adjuvant may be applicable to other vaccine preparations via particle/pathogen complexation.

5. Conclusion

Vaccines are the most effective measure for disease management and prevention, and there is an urgent need to accelerate development of antibacterial vaccines amidst the rising threat of antibiotic resistance. Vaccinations against several major microbial pathogens—such as *M. tuberculosis*, *H. pylori*, *S. aureus*, and *C. trachomatis*—have proven to be challenging as bacteria can adopt complex immunoevasive mechanisms against pre-existing immunity. Against these difficult pathogens, novel strategies are needed to shape the adaptive immune response for more effective antimicrobial defense. The introduction

of nanotechnology has opened up new avenues for vaccine development, with nanoscale carriers capable of overcoming physiological barriers and coordinately delivering antigens and immunologic adjuvants. Many types of nanoparticles and nanomaterials have been adopted for antibacterial vaccine preparations, including OMVs, gold nanoparticles, polymeric nanoparticles, and other self-assembling nanomaterials. In addition, nanoparticle-based immunologic adjuvants have shown superior ability in enhancing immune potentiation by triggering specific innate immune pathways. Several nanoformulations have demonstrated promising results in preclinical studies, effectively reducing bacterial loads in animal models. As compared to conventional vaccines that typically consist of mixtures of free antigens and adjuvants or attenuated pathogens, the nanoparticle-based formulations offer unmatched synthetic flexibility for future improvements. With increasing knowledge in immunology that uncovers the profound immune responses needed for effective antimicrobial defense, nanoparticles are poised to attract growing attention in antibacterial vaccine development, serving as a robust platform for rational vaccine design.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

antibacterial vaccine, antibiotic resistance, immune modulation, nanoparticle vaccine, toxoid vaccine

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