WIREs NANOMEDICINE AND NANOBIOTECHNOLOGY

Article type: Advanced Review

Article title:

Particulate delivery systems for vaccination against bioterrorism agents and emerging infectious pathogens

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Conflict of interests: The authors declare no conflict of interests

Abstract

Bioterrorism agents that can be easily transmitted with high mortality rates and cause debilitating diseases pose major threats to national security and public health. The recent Ebola virus outbreak in West Africa and ongoing Zika virus outbreak in Brazil, now spreading throughout Latin America, are case examples of emerging infectious pathogens that have incited widespread fear and economic and social disruption on a global scale. Prophylactic vaccines would provide effective countermeasures against infectious pathogens and biological warfare agents. However, traditional approaches relying on attenuated or inactivated vaccines have been hampered by their unacceptable levels of reactogenicity and safety issues, whereas subunit antigen-based vaccines suffer from suboptimal immunogenicity and efficacy. In contrast, particulate vaccine delivery systems offer key advantages, including efficient and stable delivery of subunit antigens, co-delivery of adjuvant molecules to bolster immune responses, low reactogenicity due to the use of biocompatible biomaterials, and robust efficiency to elicit humoral and cellular immunity in

systemic and mucosal tissues. Thus, vaccine nanoparticles and microparticles are promising platforms for clinical development of biodefense vaccines. In this review, we summarize the current status of research efforts to develop particulate vaccine delivery systems against bioterrorism agents and emerging infectious pathogens.

Introduction

The United States Centers for Disease Control and Prevention (CDC) has classified bioterrorism agents into three categories.[1] Category A pathogens are associated with the highest risk to national security and public health due to their ease of dissemination and high mortality rates. Category B pathogens are of the second highest priority with moderate ease of dissemination and mortality rates while category C includes infectious pathogens that could be modified for widespread dissemination. Many of these emerging infectious pathogens are zoonotic without any available prophylactic vaccines or effective post-exposure treatments, and their natural outbreak or malicious dissemination can have grave consequences, as recently manifested during the outbreaks of Ebola virus in West Africa and Zika virus in Brazil that have caused widespread fear and economic and social disruption on a global scale. Therefore, there is an urgent need for vaccines that can elicit concerted cellular and humoral immune responses and establish protective immunity against pathogens with spared doses and short-term immunization regimens. In addition, it is critical to maintain robust public preparedness programs with sufficient vaccine stockpiles that can be distributed readily to protect the general public. Such organizational preparedness rooted in robust vaccine programs will also have the added benefits of deterring any terrorist organizations from developing and deploying biological weapons.

For decades, research efforts on countermeasures against emerging infectious pathogens and biological warfare agents have been focused on attenuated or inactivated whole-bacteria or whole-virus vaccines. Despite their strong immunostimulatory efficacy, pre-clinical and clinical studies performed with these traditional vaccines have raised serious concerns, as they have induced unacceptable levels of reactogenicity and caused inadvertent pathogenic infections with ill-prepared live-cell vaccines in the past.[2-4] In contrast, molecularly defined subunit antigens derived from whole pathogens offer safer alternatives. However, subunit antigens are usually far less immunogenic than live and attenuated vaccines and are also more susceptible to deactivation and degradation. Recent advances in particulate vaccine delivery systems have addressed these challenges faced by subunit antigen-based vaccines.[5-8] In particular, various biomaterial-based particulate carriers have been shown to protect encapsulated antigens from enzymatic degradation, co-deliver antigens together with adjuvants to lymphatic organs, and prolong the stability of vaccine products without the infrastructure for cold-chain.[9] Synthetic delivery systems such as poly(lactic-co-glycolic acid) (PLGA) microparticles, liposomes, and lipid-based

particles composed of FDA-approved materials have been intensely investigated by us and others for delivery of peptides, proteins, and DNA antigens.[10-13] Use of biocompatible polymers and lipids can alleviate safety concerns often associated with viral vectors. Display of antigens on particle surfaces and formation of antigenic depots *in situ* can promote humoral immunity, characterized by robust, long-term, and balanced Th1/Th2 antibody responses.[14, 15] Compared with soluble antigens which predominantly elicit humoral immune responses, particulate vaccines can enhance uptake and cross-presentation of antigens by dendritic cells and activate cytotoxic $CD8^+$ T lymphocyte responses that are critical to eradicate intracellular infections.[16-19] In addition, mucosal administration of nanoparticle vaccines can induce both mucosal and systemic immunity,[20-23] thereby fortifying mucosal surfaces as the frontline of immunological defense against aerosolized bioterrorism agents or contaminated food and water supplies.

This review is focused on particulate vaccine delivery systems developed against emerging infectious pathogens, including Category A (**Table 1**) and B agents (**Table 2**) as defined and classified by the U.S. CDC. Each agent/disease is introduced with basic pathological facts and a brief history of vaccination approaches, followed by an overview on research efforts to develop particulate vaccines.

PARTICULATE VACCINES AGAINST CATEGORY A BIOTERRORISM AGENTS

Category A agents include bacteria causing anthrax, plague, botulism, and tularemia as well as viruses leading to smallpox and viral hemorrhagic fevers. Although there are successful vaccines, perhaps best exemplified by the control and elimination of smallpox epidemic, vaccines for other causative pathogens in Category A are still in investigational status and are of the highest priority for biodefense research. To protect the general public from unpredictable events such as documented anthrax terror attacks and recent outbreaks of emerging infectious diseases, caused by Ebola virus and Zika virus, there is an urgent need to expedite pre-clinical development and clinical translation of promising vaccine candidates. Synthetic particulate systems for delivery of subunit antigens have been widely examined to potentiate both humoral and cellular immune responses. Particulate vaccines under investigation for Category A agents are summarized in **Table 1**.

Anthrax

Anthrax is an epizootic disease commonly affecting hoofed animals, and humans can also be infected upon contact with infected animals or their products. Anthrax is one of the most dangerous bioterror agents because spores of its pathogen, *Bacillus anthracis*, can survive for decades or even centuries under extreme temperature or chemical treatment and can be easily

aerosolized and disseminated.[24] The threat of anthrax was realized by the 2001 U.S. postal attack that left five people killed and thousands exposed to the pathogen.[25] To date, two prophylactic vaccines against anthrax have been developed. A live-spore vaccine was an effective formulation but often caused severe toxicity at injection sites; therefore, it has since been replaced by the current anthrax vaccine, AVA, registered as BioThrax®.[26] AVA is produced by adsorbing the formalin-treated culture filtrates of a toxigenic but avirulent anthrax strain onto aluminium hydroxide. Although AVA can elicit robust humoral immune responses, it requires at least three initial vaccinations and yearly boosts for generation of long-term memory immune responses.[24] To address the limitations of current anthrax vaccines, subunit vaccines delivered by various particulate systems including polymeric nano/microparticles,[27-30] nanoemulsions,[31] and liposomes[32] have been investigated and shown to improve the protective efficacy while reducing the number of administrations necessary to produce robust immune responses. In particular, the anthrax protective antigen (PA), which is a non-toxic, cell-binding component derived from the anthrax toxin, has been demonstrated to induce protection by elicitation of robust antigen-specific antibodies in both animal models and humans.[33] A pilot study demonstrated that encapsulation or attachment of PA to poly-L-lactide (PLA) microparticles enhanced immune responses.[27] When delivered through either the intramuscular or intranasal route, the vaccine particles protected all immunized mice against anthrax infection. In another approach, an intranasal vaccine was prepared by formulating PA with a water-in-oil nanoemulsion system, which elicited higher levels of mucosal anti-PA IgA and IgG than conventional adjuvants and conferred robust protection against an intranasal challenge with *B. anthracis* live spores (**Figure 1**).[31] Immunization of PA absorbed on aluminium hydroxide nanoparticles of ~0.1 μm in diameter increased the serum level of anti-PA IgG and reduced skin inflammation at the injection site, compared with vaccination with microparticles of ~10 μm.[34] In addition to serving as adjuvants, particulate carriers can mediate co-delivery of antigen and danger signals, therefore amplifying immune responses. Intranasal immunization with chitosan nanoparticles co-loaded with PA and the adjuvant compound 48/80 achieved both mucosal and systemic humoral immune responses with a lower dose of PA, compared with soluble vaccines.[29] Liposomes co-encapsulating PA and monophosphoryl lipid A (MPLA), a Toll-like receptor (TLR) 4 agonist, were shown to elicit higher levels of toxin-neutralizing antibodies, compared with PA adsorbed on Alhydrogel® or PA displayed on bacteriophages.[32] Interestingly, dextran microparticles co-encapsulating PA and resiquimod, a TLR 7/8 agonist, exhibited robust protective efficacy without raising high neutralizing antibody responses. Upon re-stimulation, splenocytes from immunized mice secreted high levels of IL-2 and IFN-^{3},[30] suggesting that cellular immune responses may contribute to a successful anthrax vaccine.

Plague

Plague, caused by infection with *Yersinia pestis*, is an ancient zoonotic disease that is naturally carried in rodent reservoirs. The disease can be transmitted to humans by direct contact with infected rodents or bites from inflected fleas from these rodents that cause bubonic plague, or by pathogenic aerosols that cause pneumonic plague. Historically, plague had been one of the most devastating epidemic diseases, including the infamous Black Death pandemic in the 14th century that killed one third of the European population. In particular, aerosolized *Yersinia pestis* can result in deadly pneumonic diseases with a mortality rate of 50-90% without treatment.[35] Although an intensive antibiotic therapy can reduce the mortality rate associated with pneumonic plague down to ~15%, it must be given within 24-36 h after exposure.[35] In addition, there are reported cases of multidrug-resistant strains, raising concerns about antibiotic therapies.[36] Therefore, vaccine development against plague is an indispensable strategy for biodefense. Two types of plague vaccines have been used since the late 19th century: a killed whole-cell vaccine, which is only protective against bubonic plague, and a live whole-cell vaccine, which generates protective immunity against both bubonic and pneumonic plagues.[2] However, both vaccines have been discontinued due to local and systemic side effects, such as regional lymphadenopathy, anorexia, and mild fever, long-term booster doses required, and safety concerns with the live bacterial vectors. Current research efforts have been mainly focused on the development of safer subunit vaccines utilizing the capsular subunit protein F1 and low-calcium response V antigen (LcrV). F1 and V, screened from a panel of key virulent factors from *Yersinia pestis*, have been reported to be promising antigens against bubonic and pneumonic plagues in various animal models.[37] Polymeric microspheres have been tested as vaccine carriers to potentiate the efficacy of subunit plague antigens.[38-40] PLA microspheres co-encapsulating F1 and V were shown to elicit superior humoral immune responses, compared with soluble vaccines irrespective of administration routes.[38] A recent study on F1-loaded PLGA/PEG microspheres showed that a single vaccine dose was sufficient to protect mice from challenge.[39] Nanoparticle delivery systems composed of poly(anhydride),[41, 42] gold,[43] lipoproteins,[44] or a hybrid of lipids and biopolymers[23] have been developed for plague vaccines. A single intranasal dose of recombinant F1-V-loaded poly(anhydride) nanoparticles led to prolonged lung disposition[41] and generated high levels of antigen-specific antibody responses,[42] with the overall kinetics dictated by the chemical composition, hydrophobicity, and degradation rate of poly(anhydride) particles.[45] In another approach, F1 antigen was conjugated on the surfaces of gold nanoparticles via EDC/NHS chemistry, and the conjugates were resuspended in Alhydrogel®.[43] This vaccine system elicited higher titers of both anti-F1 IgG_1 and IgG_{2a} than those elicited by the vaccine

without Alhydrogel® as well as the soluble F1 mixed with the adjuvant. Lipid-based delivery systems have also shown promising results for delivery of subunit plague antigens.[23, 44] Nanolipoprotein particles were constructed with lipids, cholate, and apolipoprotein that self-assembled into nanostructures mimicking high density lipoproteins (**Figure 2A).**[44] V antigen was terminally modified with poly-histidine for complexation with nickel-modified lipids while MPLA or cholesterol-modified CpG was co-encapsulated via lipid insertion. The resulting vaccine particles significantly enhanced V-specific IgG titers in mice, compared with the physical mixture of V antigen and soluble or particulate adjuvants (**Figure 2B**). Recently, we have developed a lipid/biopolymer hybrid nanoparticle system, composed of cationic lipids and an anionic polymer hyaluronic acid, for intranasal delivery of F1-V (**Figure 2C**).[23] Shielding of cationic liposomes with hyaluronic acid significantly reduced cytotoxicity of cationic lipids by at least 20-fold in dendritic cells. When administered via intranasal route in mice, the hybrid nanoparticles co-loaded with F1-V and MPLA generated potent humoral immune responses with 11-, 23-, and 15-fold higher titers of anti-F1-V total IgG, IgG_1, and IgG_{2c}, respectively, and a more balanced Th1/Th2 response, compared with the soluble vaccine (**Figure 2D**). It remains to be seen how these lipid-based nanoparticles perform against pneumonic plague.

Hemorrhagic fever caused by filoviruses and flaviviruses

Filoviruses

Filoviruses, including Marburg virus and Ebola virus, are the main causative pathogens for hemorrhagic fever in humans, which is a deadly disease transmitted by direct contact with infected subjects. Since their discovery in 1970s, several outbreaks resulted in fatality rates ranging from 25% to 90%. At the time of this writing, no vaccine or specific antiviral drug is available for the disease. The recent Ebola outbreaks in Africa have intensified research efforts to develop Ebola vaccines, resulting in two vaccine candidates, rVSV-EBOV and ChAd3-ZEBOV, both of which have entered Phase III trials as of late 2015.[46] Historically, development of vaccines for Ebola began with inactivated whole viruses, and a whole virion inactivated by formalin was shown to provide better protection than the gamma-irradiation approach.[47] More recently, a replication-defective whole-virus vaccine showed complete protection in a pilot trial on non-human primates.[48] Given the variable potency of inactivated viruses and emergence of mutant strains, the primary vaccine approach has shifted from the direct use of whole virions to over-expression of genes encoding the Ebola glycoprotein (GP) and nucleoprotein (NP) in the host to elicit potent humoral and cellular immune responses. Specifically, immunity can be elicited by replication-deficient recombinant adenoviruses or plasmid vectors that transduce Ebola antigens or by attenuated recombinant viruses bearing Ebola GPs on their surfaces.[49]

However, booster immunizations are often required, and safety concerns remain for viral vectors.[49] For instance, although recombinant vesicular stomatitis virus (rVSV) expressing Ebola GPs achieved complete protection under a "ring vaccination" scheme in a recent clinical trial,[50] previous pre-clinical studies in non-human primates have reported cases of vector-induced viremia;[51, 52] thus, safety and compliance concerns need to be meticulously addressed in the ongoing clinical trials. As an alternative to the viral vector-based approaches described above, virus-like particles (VLPs) are the most promising vector-free vaccine platform in the pre-clinical pipeline for Ebola vaccines. Filovirus GPs along with a viral matrix protein VP40 have been produced from mammalian cell lines and self-assembled into VLPs (**Figure 3A**).[53] Immunization with Ebola VLPs completely protected mice and non-human primates from a viral challenge (**Figure 3B**).[54, 55] Follow-up studies revealed that the protection was dependent on type I interferons (IFNs),[56] and VLPs combined with polyinosinic-polycytidylic acid (polyI:C),[57] a TLR3 agonist capable of driving the production of type I IFNs, significantly augmented cellular and humoral immune responses (**Figure 3C and D**). In addition, a trimeric hybrid VLP was constructed to express GPs of the Marburg virus, Ebola Zaire and Sudan viruses.[58] Immunization with these VLPs induced protection rates higher than 70% against a Marburg challenge but varying rates from 20% to 70% against an Ebola challenge depending on subunits of the Ebola GP used for the VLPs. In a separate line of studies, a liposomal formulation encapsulating irradiated Ebola virions and lipid A as adjuvant has been shown to elicit cytotoxic T-lymphocyte responses and achieve a protection rate of ~100% in a murine model; however, this liposomal vaccine failed to protect non-human primates from lethal challenge.[59, 60] These results imply the daunting task of moving nanoparticle vaccines from small to large animal models, as various preclinical animal models exhibit different patterns of pathogenesis and susceptibility to a particular viral infection as well as varying degrees of immune responses elicited by vaccines. In addition, it remains to be seen how synthetic nanoparticles compare with widely explored VLPs in terms of safety profiles, reactogenicity, and immunogenicity against viral challenge in large animal models.

Flaviviruses

In contrast to filoviruses that primarily infect primates, flaviviruses are naturally hosted in arthropods and only occasionally transmitted to humans by bites from infected mosquitoes or ticks. The pathogen family includes more than 70 different viruses, of which six agents are mainly responsible for disease burdens in humans: yellow fever virus, dengue virus, Japanese encephalitis virus, West Nile virus (WNVs), tick-borne encephalitis virus, and Zika virus.[61] Diseases caused by some of these agents have been controlled by successful live-attenuated or

killed whole-virus vaccines.[62] In particular, the first tetravalent dengue vaccine that provides protection against all four dengue virus serotypes has been approved in Mexico, Philippines, and Brazil in December 2015.[63, 64] However, there are currently no effective therapeutic interventions or prophylactic vaccines against WNV. To address the shortage of available vaccines, particulate platforms have been developed for vaccine delivery of WNV antigens. One study has compared immune stimulatory effects elicited by gold nanoparticles of different sizes and shapes and coated with WNV envelope protein antigen on their surfaces.[65] Although rod shape particles were most favorable for antigen uptake by macrophages and dendritic cells, 40-nm spherical gold nanoparticles elicited the highest titer of antigen-specific antibodies, which were 2-fold greater than those induced by gold nanorods. In another approach, PLGA nanoparticles were used to encapsulate WNV envelope protein in the particle core and display CpG on particle surfaces.[66] Compared with Alhydrogel® which predominantly drove Th2 responses, the nanoparticle vaccine skewed humoral immune responses to the Th1 type, eliciting antigen-specific cellular immunity, and protecting 94% of animals against a viral challenge in mice. These studies highlight promising engineering approaches of delivery carriers to effective particulate vaccines against WNV.

On the other hand, Zika virus first discovered in 1950s has received little attention[67] and remained as one of the neglected tropical diseases without any research effort devoted to the vaccine development until the recent outbreak in Brazil. As of this writing, the World Health Organization has declared an international public health emergency on the ongoing Zika virus outbreak that is now spreading throughout Latin America. Zika infection can easily spread through mosquito bites, and there is also a report of suspected transmission by sexual intercourse.[68] While Zika infection does not trigger perceivable symptoms in infected hosts, it may endanger fetus development in pregnant women, causing infant microcephaly birth defects.[69] This has prompted world leaders to devote more resources to research on vaccine development and diagnostics as well as disease control and public preparedness to combat against the global health threat.

Botulism

The botulinum toxin, produced by *Clostridium botulinum*, triggers neuroparalytic diseases and is regarded as one of the most lethal poisons.[70] The disease can occur due to accidental food poisoning or inhalation of maliciously dispersed bacterial spores.[71] Interestingly, the toxin can also be used to treat neurological disorders such as dystonia when locally injected at an appropriate dose.[70] Formalin-inactivated toxoid vaccines have been developed previously, including a pentavalent vaccine registered as an Investigational New Drug in the US.[3, 71]

However, this vaccine was discontinued in 2011 due to declining potency and increasing reactogenicity following annual boosts, and currently there is no botulism vaccine available.[3] For vaccination with a non-toxic subunit of the botulinum toxin, an adjuvant-free nanogel system has been prepared by self-assembly of polysaccharide pullulan modified with cholesteryl and amino groups (**Figure 4A**).[72] Compared with a soluble vaccine, the nanogel significantly increased the residence time of antigen in nasal epithelium upon intranasal vaccination (**Figure 4B**) and elicited robust antigen-specific mucosal IgA and systemic IgG responses (**Figure 4C**), achieving complete protection of animals against an intraperitoneal or intranasal challenge with *Clostridium botulinum* neurotoxin (**Figure 4D**). Apart from these studies, there have been only limited research efforts for vaccine development against botulinum toxin. Given the promising results presented here, nanoparticle-based vaccine approaches warrant further investigations.

Tularemia

Tularemia is caused by *Francisellar tularensis* which infects both animals and humans. There are various pathogenic strains that can cause ulceroglandular, gastroinstestinal, oropharyngeal, or pneumonic tularemia.[4] Similar to plague, the pneumonic form of tularemia poses the highest public health risk and is the major concern for bioterrorism due to its low lethal dose, high virulence, and ease of aerosol dissemination. Although an attenuated live vaccine strain (LVS) was developed for vaccination, it has been terminated due to potential safety issues associated with pathogenic mutations of the vaccine strain and unsatisfactory efficacy.[4] Particulate delivery approaches for tularemia vaccines have been mostly focused on lipid-based nanoparticles and have offered some initial success. In one example, vaccination with synthetic liposomes incorporating the membrane proteins of LVS and alum plus IL-12 protected mice against a LVS challenge but not against the virulent strain.[73] In another study, bacterial lysates were loaded into catanionic vesicles formed by surfactants with opposite charges.[74] Immunized mice were completely and partially protected from challenge with LVS and a virulent strain, respectively. Transferring the immune sera into naïve mice also protected recipients against a LVS challenge, indicating the importance of humoral immunity. In addition, archaeal lipids have been tested as an adjuvant for LVS lysates.[75] Intranasal immunization with this vaccine elicited high antigen-specific antibody titers in serum and bronchial lavage fluids as well as cellular immune responses, characterized by the proliferation of antigen-specific splenocytes and IL-17 secretion. In fact, cellular immunity directed against intracellular *Francisellar tularensis* may play a vital role in vaccination against tularemia as the bacteria can evade phagocytic degradation and reside within macrophages.[76, 77] As demonstrated by these studies, it is relatively easy to show the protective efficacy of vaccines against LVS. Future studies should be directed to enhance

immunogenicity of particulate vaccines and assess their potency in more stringent animal models utilizing virulent strains of *Francisellar tularensis*.

PARTICULATE VACCINES AGAINST CATEGORY B BIOTERRORISM AGENTS

Category B agents are of the second highest priority due to their moderate ease of dissemination, morbidity, and mortality. Several pathogens of this category, including salmonellosis, shigellosis, and cholera, are mainly transmitted by the fecal-oral route. Although their natural outbreaks can be prevented by improving hygiene resources in epidemic areas, prophylactic vaccines are needed for protection against malicious dissemination by aerosolized pathogens or contaminated food and water supplies. Particulate vaccines investigated for Category B agents are summarized in **Table 2**.

Brucellosis

Brucellosis is a zoonosis that mainly infects livestock, such as cattle, swine, and goats. Brucellosis is caused by gram-negative *Brucella* species, among which *B. melitensis* causes the most severe disease with frequent debilitating relapses.[78] *Brucella* is transmitted to humans by fluid discharges from infected animals, especially dairy products, or by aerosol dissemination.[79] Although Brucellosis is currently controlled by vaccination of susceptible livestock, these veterinary vaccines are pathogenic to humans.[80] Several attenuated *Brucella* strains and extracted bacterial fractions have been used as vaccines in humans in the last century. However, none of these are in use nowadays due to suboptimal efficacy.[81] To remedy these issues, many particulate delivery systems have been examined. In one study, a hydrophobic portion of the bacterial extract was encapsulated in microparticles composed of poly (ε-caprolactone), β-cyclodextrin, and Pluronic F68.[82] This vaccine protected mice against *B. melitensis* challenge with a similar efficacy as the live attenuated vaccine. In another study, subcellular bacterial extracts were loaded in poly(anhydride) nanoparticles modified with mannose and delivered via the ocular route to target the mucosal immune system.[83] A single vaccination dose achieved higher IgA titers and improved protection, compared with the live attenuated vaccine. Since *Brucella* species predominantly reside in macrophages and monocytes, cellular immunity, supported by the killing of infected cells by cytotoxic $CD8^+$ T lymphocytes and secretion of Th1 cytokines by $CD4^+$ T cells, was shown to be vital for elimination of the bacteria.[78, 84] In order to amplify cellular immunity against *Brucella*, a subunit nucleoprotein or T-cell epitopes derived from the bacteria have been encapsulated in PLGA microparticles. Although these particles offered protection against virulent *Brucella* infection, their efficacy was suboptimal compared with the live attenuated vaccine.[85, 86] Hence, future studies should be devoted to developing particulate

systems capable of eliciting potent, concerted humoral and cellular immune responses against Brucellosis.

Food safety threats

Salmonellosis

The gram-negative bacterium *Salmonella*, especially *S. enterica,* is an enteric pathogen and a common cause of food-borne diarrheal illness. Four serovars of *S. enterica*, namely *Typhi*, *Paratyphi A*, *Typhimurium*, and *Enteritidis*, are responsible for severe infections in humans, with the former two types causing enteric fever and the latter two types causing the invasive nontyphoidal *Salmonella* (iNTS) disease.[87] Every year there are 21.7 million cases of infection and 200,000 fatalities due to typhoid fever from *Typhi* infection.[88] Current vaccines against *Salmonella* are limited to the *Typhi* serotype, and they include an inactivated whole-cell vaccine, a live-attenuated vaccine made of a mutant strain Ty21a, and a subunit vaccine made of Vi polysaccharide.[89] Among these, the whole-cell vaccine has achieved the highest three-year cumulative efficacy of 73%; however, high reactogenicity limits its general use.[87] Ty21a and Vi polysaccharide vaccines have protection rates of ~50% with reduced adverse reactions, but neither is suitable for infants, who suffer most from the disease.[87, 90] In addition, the emerging antibiotic-resistant strains provide further motivation for the development of new vaccine products.[91] Particulate delivery systems have been examined to improve the efficacy of subunit *Salmonella* vaccines. Subcellular extracts from the *Enteritidis* serovar were encapsulated into nanoparticles composed of copolymer poly(methyl vinyl ester/maleic acid) (PVM/MA).[92] The particulate formulation elicited robust IFN-³ release from splenocytes in immunized mice and completely protected animals against a lethal challenge. In another study, Vi polysaccharide delivered by PLA nanoparticles or microparticles induced higher levels of antigen-specific IgG and memory antibody responses than a soluble vaccine.[93] Furthermore, high density of antigens displayed on the surfaces of PLA particles correlated with robust humoral immune responses. These results suggest that engineering of the interface between antigen-displaying vaccine particles and B-cells is crucial to prime strong humoral immunity. In addition, particulate delivery of compiled subunit antigens for individual serotypes or a common subunit, e.g. a conserved region across all four pathogenic serotypes, may lead to a successful *Salmonella* vaccine.

Shigellosis

The enteric pathogen *Shigella*, which is usually transmitted by the fecal-oral route, is another major cause of diarrhea. Four *Shigella* species have been identified to date: *S. dysenteriae*, *S.*

flexneri, *S. sonnei,* and *S. boydii*, among which the first three are more common for human enteric diseases.[94] Specifically, the invasive *S. dysenteriae* Type 1 causes dysentery and life-threatening kidney damage by releasing Shiga toxin.[95] Currently, there is no vaccine available for Shigellosis, but several vaccine candidates are undergoing clinical trials.[95] Due to their favorable safety profiles, subunit vaccines are ideal alternatives to live-attenuated vaccines, as vaccine safety is of the utmost importance for *Shigella* vaccines as children under 5 years old are most vulnerable to Shigellosis.[96] In one approach, outer membrane vesicles (OMVs) from *S. flexneri* have been encapsulated into nanoparticles made of copolymer PVM/MA as a subunit vaccine.[97, 98] After intradermal, oral, or intranasal immunization, the vaccine nanoparticles completely protected mice against a pathogen challenge. Notably, future studies should be directed to optimize particulate delivery systems for mucosal vaccination against *Shigella* as such strategy may offer a key advantage to effectively stop its transmission at mucosal surfaces.

Escherichia coli O157:H7

Although the majority of *E. coli* strains are benign inhabitants in the human gastrointestinal tract, enterohemorrhagic *E. coli* strains, which produce the Shiga toxin (Stx), can cause diarrheal illness, even hemorrhagic colitis, and hemolytic uremic syndrome.[99] *E. coli* O157:H7 is the predominant serotype responsible for frequent outbreaks.[100] Cattle are the natural host of *E. coli* O157:H7, and humans can be infected by consumption of contaminated meat.[101] Although vaccines for cattle are used to control the disease transmission,[102] it is difficult to eliminate the human disease burden due to other animal and environmental reservoirs of the pathogen.[101] To develop vaccines for human use, liposomes have been investigated as a vaccine carrier for either the whole bacteria or subunit bacterial proteins. Killed bacteria along with adjuvant MPLA have been co-incorporated into liposomes to produce an oral vaccine, which elicited both systemic and mucosal IgA and IgG antibodies specific to the pathogen.[103] Alternatively, Stx was conjugated to amine-modified liposomal surfaces via glutaraldehyde-mediated reaction, which also inactivated the toxin during the coupling process.[104] This liposomal vaccine induced robust antigen-specific serum IgG titers and conferred protection against an intravenous toxin challenge in both murine and non-human primate models.[104, 105] Recently, a novel anti-bacterial vaccine approach has been developed by coating OMVs on nanoparticle templates.[106] OMVs derived from a model pathogen *Escherichia coli* were stably coated on the surfaces of gold nanoparticles which enhanced activation of dendritic cells, elicited higher serum titers of antigen-specific IgG, and robust cytokine secretion from splenocytes after a single subcutaneous injection in mice, compared with vaccination with native OMVs.

Staphylococcal toxins

Staphylococcal toxins, especially staphylococcal enterotoxin B (SEB) secreted by the gram-positive bacterium *Staphylococcus aureus*, are a common cause of food poisoning,[107] while inhaled SEB provokes more serious syndromes. SEB is a "superantigen" that can lead to hyper T-cell activation and "cytokine storm", characterized by massive secretion of TNF-±, IFN-γ, IL-1, IL-2, and IL-6.[108] Initial attempts to develop SEB vaccines were focused on the formalin-inactivated toxin and recombinant mutant strains, but there are still no agents available to protect against SEB or treat SEB intoxication.[109] To address these challenges, PLGA particles have been used to deliver inactivated SEB.[110, 111] When multiple routes of vaccine delivery were tested with PLGA microparticles, an intratracheal booster dose offered the highest rate of protection in monkeys against a challenge with aerosolized SEB, compared with the intramuscular or oral route of vaccination.[110] Inactivated SEB delivered by PLGA nanoparticles also elicited humoral immune responses comparable to those elicited by a vaccine formulation with alum.[111] In an alternative approach, staphylococcal α-haemolysin was trapped by erythrocyte membranes coated on the surfaces of PLGA nanoparticles (**Figure 5A**).[112] Compared with heat-inactivated toxin, this nanotoxoid vaccine alleviated toxicity at injection sites while enhancing antigen-specific serum IgG titers and protecting mice against a toxin challenge (**Figure 5B**).

Cholera

The gram-negative bacterium *Vibrio cholerae* is transmitted to humans most often by contaminated drinking water and causes the acute diarrheal disease cholera.[113] *V. cholerae* has been classified into more than 200 serogroups according to different O antigens of the bacterial lipopolysaccharides (LPS), and only O1 and O139 strains are known to cause epidemic cholera.[114] Currently there are three oral vaccine products available: Dukoral®, mORC-Vax™, and Shanchol™.[115] However, these vaccines require a booster dose, provide only limited and short-term protection, and are not suitable for children under two years old.[114, 115] Therefore, their use is mainly restricted to travelers rather than the general population in endemic areas.[116] Polymeric particles have been intensely investigated as a delivery system for cholera vaccines.[117] A mutant *V. cholerae* strain was efficiently loaded into PLGA microparticles with encapsulation efficiency of ~98%.[118] The particulate vaccine administered orally elicited higher humoral immune responses than a soluble vaccine, and the addition of an anti-fungal drug, amphotericin B, as an adjuvant further enhanced the immune responses. Another study compared the efficacy of whole-bacterium vaccine formulated into polymeric microparticles composed of PLGA (50:50), PLGA (75:25), or PLA/PEG copolymer.[119] Those particulate vaccines showed similar

size distribution, encapsulation efficiency, and *in vitro* release profiles of the encapsulated antigen. Upon oral immunization in mice, the PLA/PEG formulation elicited the highest antigen-specific IgG, IgA, and IgM titers and a superior survival rate post-challenge. In an alternative approach, an oral cholera vaccine was produced by encapsulating inactivated *V. cholerae* into microparticles composed of the enteric excipient Eudragit® and mucoadhesive agents alginate or Carbopol®.[120, 121] Following oral vaccination, the formulation incorporating alginate elicited higher vibriocidal titers than a soluble vaccine, demonstrating the promise of mucoadhesive particles for oral vaccination against cholera.

Melioidosis and glanders

Burkholderia pseudomallei and *Burkholderia mallei* are the causative agents of melioidosis and glanders, respectively. Both pathogens are gram-negative bacteria that reside in host immune cells and establish infection after oral ingestion, aerosol inhalation, or skin contact with cutaneous wounds.[122] Melioidosis is a severe human endemic disease in Southeast Asia and Northern Australia, with mortality rates of 50% and 20%, respectively.[123] In contrast, glanders mainly infects solipeds, such as horses, mules, and donkeys, but rarely infects humans.[124] Although it is reported that glanders has been eradicated in the developed world, it still poses a threat to public health, and *B. mallei* has a documented history as a bio-warfare agent.[125] In addition, both pathogens are resistant to most antibiotics, and there is no vaccine product available.[125, 126] Early vaccine approaches include live-attenuated or inactivated bacteria and subunit vaccines for both diseases; however, none have achieved complete protection.[122] Since an intracellular cycle is indispensable for the infectivity of these pathogens, induction of cellular immune responses by specific adjuvants are of particular interest. For instance, pre-treatment with CpG, a TLR9 agonist, has been shown to promote protection against *B. pseudomallei*.[127] Furthermore, CpG complexed with cationic DOTAP liposomes showed better protection than CpG complexed with zwitterionic DOPC liposomes or soluble CpG.[128] The CpG/DOTAP vaccine delivered intranasally in mice conferred partial protection against a lethal challenge of *B. mallei* or *B. pseudomallei*, and the protection relied on an elevated level of IFN-³ and activation of NK cells, suggesting the important roles of adaptive and innate immune responses in disease control.[129] However, mice protected from the acute challenge still developed chronic infections in spleen and liver. Recently, a subunit particulate vaccine has been developed for *B. mallei* by conjugating LPS from a non-virulent strain to the surfaces of gold nanoparticles.[130, 131] Vaccinated mice were later challenged with aerosolized *B. mallei*. Compared with the soluble LPS, the nanoparticle vaccine elicited higher anti-LPS IgG titers, reduced bacterial burdens in the spleen, and enhanced protection.[130] However, when tested in non-human primates, the

vaccine failed to induce favorable protection compared with non-immunized controls, despite elicitation of anti-LPS IgG.[131]

Ricin toxin

Ricin toxin is derived from the common plant *Ricinus communis*, or castor beans, and can be a byproduct during the production of castor oil. Despite multiple routes that can induce poisoning, ricin toxin is rarely transmitted to humans, and interpersonal transmission is negligible.[132] Nevertheless, ease of production, storage, and dissemination, as well as the lack of effective protective and therapeutic options still make ricin toxin a potential bioterror threat.[133] Indeed, it has been utilized in espionage incidents and malicious mailing attacks.[134, 135] Ricin toxin is a heterodimeric glycoprotein consisting of an A chain (RTA) which enzymatically inactivates ribosomes, and a B chain (RTB) which facilitates entry of the toxin into target cells.[136] Since RTB is a poor inducer of humoral immune responses, inactivated forms of the toxin and RTA become the main antigen sources tested for vaccine development.[109, 133] In one approach, a single subcutaneous immunization of PLGA microparticles encapsulating the inactivated toxin induced similar serum levels of anti-ricin IgG and protection rates as three doses of a soluble vaccine.[137] Notably, humoral immune responses elicited by the particulate vaccine lasted as long as one year after immunization. In a follow-up study with single or dual doses delivered through the intranasal route, the microparticle vaccine elicited higher serum anti-ricin IgG and IgA titers and achieved a higher protection rate against an aerosol challenge, compared with the soluble vaccine.[138] The enhanced protection was observed when the challenge was performed at six weeks or one year after immunization. As an alternative strategy, liposomes encapsulating the inactivated toxin have been examined for intratracheal delivery.[139, 140] The liposomal vaccine elicited similar humoral immune responses and slightly better short-term protection against an aerosol challenge, compared with soluble vaccines with or without the Alhydrogel® adjuvant.[140] Further pathological analyses post-challenge revealed that the liposomal vaccine reduced infiltration of neutrophils into lungs and decreased pulmonary edema.[141]

Viral encephalitis caused by alphaviruses

Encephalitic alphaviruses, including eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV), infect both horses and humans in an epidemic area restricted to the Americas.[142] The mortality rates are 3-7% for WEEV and 50-75% for EEEV, and there are no available therapeutics or vaccines.[143, 144] Both attenuated and formalin-inactivated vaccines have been tested for three encephalitic alphaviruses, but none of the candidates were viable due to high reactogenicity of the attenuated vaccines and

poor efficacy of the inactivated vaccines.[144] To improve the efficacy, inactivated VEEV was encapsulated into PLGA microparticles and delivered via multiple routes.[145] Mice were immunized through the subcutaneous or intratracheal route, followed by a booster dose through the oral, subcutaneous, or intratracheal route. The intratracheal route elicited mucosal anti-VEEV IgG and IgA with higher activity and also enhanced protection, compared with the other two routes tested for the booster dose.

Conclusion

Most particulate delivery systems examined in the past for biodefense vaccines are composed of biodegradable materials, such as PLGA and phospholipids, which are already approved for pharmaceutical products and would therefore facilitate clinical translation of promising vaccine candidates. Particulate vaccine vehicles can stably encapsulate or surface-display antigens while also serving additional roles as adjuvants or carriers for adjuvant molecules. As shown in studies discussed above, successful seroconversion usually correlates with high protection rates against lethal challenges, while cellular immunity is vital for elimination of intracellular reservoirs of pathogens.

It is the authors' opinion that more resources and research efforts should be devoted to the development of mucosal vaccine delivery systems for biodefense vaccines. Mucosal vaccination would preferentially elicit T- and B-cell immune responses in local and distal mucosal surfaces, including the respiratory tract, thereby establishing immunity in the frontline of protection against aerosol transmission of bioterrorism agents.[20-23] Compared with soluble antigens that are subjected to fast clearance or degradation, particulate vaccines could improve colloidal stability, increase residence time in mucosal tissues, and promote induction of antigen-specific IgA titers and peripheral tissue-resident effector $CD4^+$ and $CD8^+$ T-cells. Among various mucosal delivery strategies, oral vaccines may be most amenable to mass vaccination campaigns in the event of biological terror attacks and also most efficacious for immunization against enteric pathogens.[146] Indeed, oral vaccine delivery has been exploited for currently licensed and candidate cholera vaccines. However, one of the major challenges in oral vaccination lies in rapid denaturation and degradation of antigens in response to acidic pH and abundant proteases in the gastrointestinal tract. Thus, it would be valuable to investigate particulate carriers composed of biopolymers that can endure the harsh gastric environment or liposomes stabilized by bile salts or archaeobacterial lipids that can maintain antigenicity and immunogenicity of antigens.[147] In addition, vaccine carriers targeted to M cells and/or intestinal dendritic cells should be examined to further improve the efficiency of oral vaccination. Another mucosal route of vaccine delivery that warrants further investigation is transcutaneous vaccination. Notably, microneedle patches have

recently garnered much attention as a promising vaccine delivery tool that could offer good patient compliance and facile self-administration approaches, while achieving dose sparing by targeting of antigens and adjuvants to skin-resident antigen-presenting cells.[148-150] As in the case with oral dosage forms, pre-formulated microneedle patches could be easily stored for long-term preparedness and rapid distribution among susceptible populations in response to bioterror attacks. New microneedle technologies permit long-term storage of vaccines and allow precise tuning of drug release profiles by employing dissolvable polymeric patches,[151] layer-by-layer coatings on needles,[152] or therapeutic depots responsive to external tensile strength.[153] Future research efforts should be directed to exploit these technologies to achieve efficient and stable loading of antigens, to attract skin-resident DCs to vaccination sites with the antigen depot deployed during administration, and to augment mucosal immune responses. These advances will allow an unprecedented control over dosing and immunization schemes for transcutaneous vaccination.

There are numerous challenges that need to be overcome for clinical translation of particulate vaccines against biological warfare agents. It is crucial to streamline the manufacturing processes for industrial scale-up of particulate vaccines with minimal batch-to-batch variability. In addition, since large vaccine stockpiles and rapid and facile distribution of vaccines are vital components of countermeasures against outbreaks and bioterror attacks, it is critical to develop vaccine products that are stable for a long term without the cold-chain. Furthermore, as illustrated in this article, the majority of particulate vaccine candidates is still in the early stages of pre-clinical development and tested only in murine models. However, many vaccine candidates that may be adequate in small animals fail to exhibit strong immune responses in large animals or humans. Murine species cannot be naturally infected by many emergent pathogens introduced above. The pathogenesis, dosing for immunization and challenge, and types and durability of immune responses demonstrated in murine models may not correlate with those in natural hosts, including humans. Furthermore, following the disease challenge, small animals may clear pathogens by one-wave immune responses or easily succumb to death, making them inappropriate models to evaluate vaccine efficacy against latent intracellular infections or recurring and debilitating diseases.[154, 155] Therefore, more stringent screening process should be implemented in the early cycles of product development; this may involve direct comparison of particulate vaccine candidates against other strong benchmarks in vaccine research, including gold standard adjuvants, VLPs, or live vectors. In parallel, evaluation of vaccine candidates in more rigorous animal models, including non-human primates, should be considered early in the vaccine design and development.

In conclusion, particulate delivery systems have shown great promise for addressing current limitations in vaccine technologies against bioterrorism and emerging infectious agents, and they should be strongly considered for public health preparedness and countermeasures against potential outbreaks and bioterror threats.

ACKNOWLEDGMENT

This work is supported in part by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number UL1TR000433, the John S. and Suzanne C. Munn Cancer Fund of the University of Michigan Comprehensive Cancer Center, and National Science Foundation CAREER award. J.J.M. is supported by the Melanoma Research Alliance Young Investigator Award. Y.F. is supported by the Broomfield International Student Fellowship. We thank Joseph Bazzill and Cameron Louttit for critical review of the manuscript.

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Figure captions

Figure 1. A nanoemulsion (NE) system formulated with the anthrax protective antigen (PA) enhanced mucosal humoral immune responses and improved protection against bacterial spore challenges. Compared with conventional adjuvants, such as MPLA, CpG, and aluminium hydroxide, the NE vaccine administered via the intranasal route elicited higher titers of anti-PA IgA (**A**) and IgG (**B**) in bronchial alveolar lavage fluids from immunized mice. Vaccination with the NE vaccine also protected guinea pigs against an intranasal challenge with a 10-fold (**C**) or 100-fold (**D**) LD_{50} dose of bacterial spores. Reproduced with permission from ref. [31].

Figure 2. Lipid-based nanoparticles for delivery of subunit plague antigens. (**A**) A nanolipoprotein particle loaded with adjuvants and LcrV modified with poly-histidine. (**B**) Co-delivery of LcrV and adjuvants via the intraperitoneal route elicited higher anti-LcrV IgG titers than LcrV admixed with or without soluble or particulate adjuvants. (**C**) A cationic lipid/hyaluronic acid (HA) hybrid nanoparticle formed by cross-linking of thiolated HA and thiolated polyethylene glycol (PEG). (**D**) Intranasal vaccination with the hybrid particles co-loaded with F1-V and MPLA elicited significantly higher serum titers of anti-F1-V IgG, compared with the soluble mixture of F1-V and MPLA. (**A**) and (**B**) reproduced with permission from ref. [44]; (**C**) and (**D**) reproduced with permission from ref. [23].

Figure 3. Virus-like particles (VLPs) as an Ebola vaccine candidate. (**A**) Transmission electron microscope images of Ebola viruses (left) and VLPs (right). (**B**) Mice were immunized three

times with Ebola VLPs (eVLPs), inactivated Ebola viruses (iEBOV) or Marburg viruses (iMARV), or PBS, followed by a challenge with the mouse-adapted Ebola virus. (**C**) and (**D**) Humoral and cellular immune responses elicited by Ebola VLPs were augmented by polyI:C. (**C**) A low dose of VLPs along with 100 ng-100 μg polyI:C elicited high serum titers of antigen-specific IgG. (**D**) Splenocytes from immunized mice were cultured with Ebola GP, followed by stimulation with an Ebola GP peptide *in vitro*. Robust effector T cells were induced by immunization with 10 μg VLPs and polyI:C. (**A**) and (**B**) reproduced with permission from ref. [54]; (**C**) and (**D**) reproduced with permission from ref. [57].

Figure 4. A cationic nanogel developed for intranasal delivery of a subunit botulism neurotoxin. (**A**) The nanogel was self-assembled by the polysaccharide pullulan modified with cholesteryl and amino groups. Intranasal immunization with antigen-loaded nanogels significantly enhanced nasal residence of the antigen (**B**), antigen-specific antibody titers (**C**), and protection against challenge with the neurotoxin (**D**), compared with the soluble antigen. Reproduced with permission from ref. [72].

Figure 5. Erythrocyte membrane-coated PLGA nanoparticles for vaccine delivery of staphylococcal α-haemolysin (Hla). (**A**) Schematic illustration and an image by transmission electron microscope of the nanotoxoid. Scale bar, 80 nm. (B) The nanotoxoid vaccine enhanced humoral immune responses and protected mice against toxin challenge. Empty triangles, vaccine particles without the antigen; solid triangles, unvaccinated control; blue squares, single dose of the heat-inactivated Hla; blue spheres, single dose of the nanotoxoid; red squares, three doses of the heat-inactivated Hla; red spheres, three doses of the nanotoxoid. Reproduced with permission from ref. [112].

Table 1. Particulate Vaccine Delivery Systems Investigated for Category A Bioterrorism Agents.

Disease	Antigen	Delivery System	Adjuvants	Animal Model	Dosing Scheme	Major Results	Ref.
Anthrax	PA	PLA microparticle	None	Mice	i.m. or i.n., two doses	Complete protection mediated by robust levels of anti-PA IgG	[27]
	A subunit of PA	PLGA nanoparticle	None	Mice	i.p., single dose	Balanced Th1/Th2 humoral immune responses; prolonged survival compared with a soluble vaccine	[28]
	PA	Nanoemulsion	None	Mice, guinea pigs	i.n., two doses	More than 3-fold higher anti-PA IgG and IgA titers than vaccines composed of other adjuvants including MPLA, CpG or Alum; protection rates of 70% and 40% following a low and high dose of pathogen challenge, respectively	[31]
	PA	Chitosan nanoparticle	Compound 48/80	Mice	i.n., three doses	Enhanced mucosal and systemic humoral immune responses compared with soluble vaccines	[29]
	PA	Liposome	MPLA	Rabbits	i.m., two or three doses	Complete protection mediated by robust levels of neutralizing antibodies	[32]
	PA	Dextran microparticle	Resiquimod	Mice	s.c., two doses	Complete protection possibly by cellular immune responses	[30]
	PA	Aluminum nanoparticle	None	Mice	s.c., two doses	Durable anti-PA IgG titer for a month after the booster dose; enhanced antigen uptake, and milder inflammation at the injection site compared with the microparticle counterpart	[34]
Plague	F1 and LcrV	PLA microparticle	None	Mice	i.t., i.m., or i.n., two doses	Successful elicitation of antigen-specific antibodies	[38]
	F1	PLGA/PEG microparticle	None	Mice	s.c., single dose	Complete protection mediated by robust anti-F1 IgG titers	[39]

	B- and T-cell epitopes of LcrV	PLGA microparticle	None	Mice	i.n., single dose	Balanced Th1/Th2 responses; protection rates varied between 0-90% depending on epitope sequences	[40]
	F1-V	Poly(anhydride) nanoparticle	None	Mice	i.n., single dose	Complete protection mediated by high avidity, antigen-specific IgG	[42]

Table 1 (Continued)

Disease	Antigen	Delivery System	Adjuvants	Animal Model	Dosing Scheme	Major Results	Ref.
Plague	F1	Gold nanoparticle	Alhydrogel®	Mice	s.c., single dose	Two- to four-fold higher titers and avidity of anti-F1 IgG than those elicited by a soluble vaccine or the particulate vaccine without adjuvant	[43]
	LcrV	Lipoprotein nanoparticle	MPLA or CpG	Mice	i.p., single dose	Four-fold higher anti-V IgG titer than that elicited by soluble vaccines	[44]
	F1-V	Cationic lipid/hyaluronic acid hybrid nanoparticle	MPLA	Mice	i.n., three doses	Enhanced biocompatibility and 11-fold increase in serum titers of F1-V-specific IgG with balanced Th1/Th2 IgG subtypes	[23]
Hemorrhagic fever caused by filoviruses	Irradiated whole Ebola virus	Liposome	MPLA	Mice, NHP	i.v. or i.m., two doses	Elicitation of cytotoxic T-lymphocyte responses; complete protection was achieved in the murine but not the NHP model	[59, 60]
	Ebola GP and VP40	VLP	None	Mice	i.m. or i.p., three doses	Activation of dendritic cells in vitro; elicitation of cellular and humoral immune responses in vivo; complete protection	[54]
	Ebola GP, NP, and VP40	VLP	Ribi adjuvant	NHP	i.m., three doses	Elicitation of humoral and cellular immune responses; complete protection	[55]
	Ebola GP,	VLP	Poly I:C	Guinea pigs	Unknown route,	The protection rate against a Marburg viral challenge was	[58]

	Marburg GP, and VP40				two doses	higher than 70%; protection rates against an Ebola viral challenge varied between 20%-70% for Ebola GP subunits with different immunogenicity	
Hemorrhagic fever caused by flaviviruses	WNV envelope protein	Gold nanoparticles of different sizes and shapes	None	Mice	i.p., two doses	Rod-like particles facilitated antigen uptake by antigen-presenting cells, whereas 40-nm nanospheres elicited the highest levels of antigen-specific antibodies and inflammatory cytokines *in vivo*	[65]
	WNV envelope protein	PLGA nanoparticle	CpG	Mice	s.c., two doses	Th1-skewed humoral immune responses; a protection rate of 94%.	[66]

Table 1 (Continued)

Disease	Antigen	Delivery System	Adjuvants	Animal Model	Dosing Scheme	Major Results	Ref.
Botulism	Subunit of the botulinum toxin	Pullulan nanogel	None	Mice	i.n., single dose	Prolonged nasal residence of the antigen within 12 h post immunization; robust titers of antigen-specific mucosal IgA and systemic IgG; complete protection	[72]
Tularemia	Membrane proteins of LVS	Liposome	IL-12 and Alum	Mice	s.c, i.p., or i.n., three doses	Complete protection against LVS but not a virulent strain	[73]
	LVS lysates	Catanionic vesicle	None	Mice	s.c, i.p., or i.n., two to four doses	Protection rates were 100% and < 25% against LVS and a virulent strain, respectively	[74]

Abbreviations: PA, the protective antigen of *Bacillus anthracis*; PLA, poly-L-lactide; PLGA, poly(lactic-co-glycolic acid); PEG, polyethylene glycol; LcrV: low-calcium response V antigen of *Yersinia pestis*; F1-V, recombinant protein of capsular portion F1 and LcrV of *Yersinia pestis*; MPLA, monophosphoryl lipid A; LVS, live vaccine strain of attenuated *Francisellar tularensis*; GP, glycoprotein; NP, nucleoprotein; VP40, viral matrix protein 40 of filoviruses; VLP, virus-like particle; poly I:C, polyinosinic-polycytidylic acid; NHP, non-human primate; WNV, West Nile virus; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; s.c., subcutaneous, i.t., intratracheal; i.v., intravenous.

Table 2. Particulate Vaccine Delivery Systems Investigated for Category B Bioterrorism Agents

Disease	Antigen	Delivery system	Adjuvants	Animal Model	Dosing Scheme	Major Results	Ref.
Brucellosis	Subcellular extraction	PCL microparticle	None	Mice	s.c., single dose	Similar protection efficacy as a live attenuated vaccine	[82]
	Subcellular extraction	Mannosylated poly(anhydride) nanoparticle	None	Mice	Eye drop, single dose	Two-fold higher mucosal IgA titers and increased protective efficacy than those elicited by a live attenuated vaccine	[83]
	Subunit bacterial nucleoprotein	PLGA microparticle	None	Mice	i.p., two doses	High level of IFN-^{3} secreted by splenocytes from immunized mice; suboptimal protection compared with a live attenuated vaccine	[85]
	Bacterial T-cell	PLGA microparticle	None	Mice	s.c., two doses	Suboptimal protection compared with a	[86]

	epitopes					live-attenuated vaccine	
Salmonellosis	Subcellular extraction	PVM/MA nanoparticle	None	Mice	i.p., single dose	Release of IFN-^{3} from splenocytes; complete protection	[92]
	Vi polysaccharide	PLA nanoparticle or microparticle	Alhydrogel®	Mice	i.m., two doses (boosted with a low dose of soluble Vi)	Two-fold higher memory antibody responses compared with a soluble vaccine; humoral immune responses were further enhanced by display of antigens on the particle surface	[93]
Shigellosis	OMVs	PVM/MA nanoparticle	None	Mice	i.d., i.n., oral, or ocular delivery, single dose	Complete protection through four vaccination routes	[97, 98]
Enterohemorrhage caused by *E. coli* O157:H7	Killed whole bacteria	Liposome	MPLA	Mice	Oral, three doses	Elicitation of systemic and mucosal antigen-specific IgG and IgA	[103]
	Stx	Liposome	None	Mice, NHP	i.p. single dose for mice, i.m. four doses for NHP	Complete protection against a challenge by Stx due to robust antigen-specific serum IgG levels	[104, 105]

Table 2 (Continued)

Disease	Antigen	Delivery system	Adjuvants	Animal Model	Dosing Scheme	Major Results	Ref.
Poisoning by staphylococcal toxins	Inactivated SEB	PLGA microparticle	None	Monkey	i.m., i.t., or oral, two doses with different routes	Booster dose through the i.t. route achieved the highest antibody titers and best protection	[110]
	Inactivated SEB	PLGA nanoparticle	None	Rabbit	s.c., single dose	Humoral immune responses were comparable to those elicited by a soluble vaccine using Alum as	[111]

						adjuvant	
	α-haemolysin	Erythrocyte membrane-coated PLGA nanoparticle	None	Mice	s.c., single or three doses	Decreased skin toxicity, 10-fold higher antigen-specific IgG titers after booster doses, and increased protection rates compared with heat-inactivated toxin	[112]
Cholera	Inactivated whole bacteria	PLGA microparticle	Amphotericin B	Mice	Oral, single dose	Antigen-specific serum IgG and IgM levels were 10- and 4-fold higher, compared with the soluble vaccine and were further enhanced by the adjuvant	[118]
	Inactivated whole bacteria	PLGA or PLA/PEG microparticle	None	Mice	Oral, single dose	PLA/PEG particulate vaccine achieved the strongest humoral immune responses and the highest protection rate compared with PLGA counterparts	[119]
	Inactivated whole bacteria	Eudragit® plus alginate microparticle	None	Rat	Oral, two doses	Slightly higher vibriocidal titers than those induced by a soluble vaccine	[121]
Melioidosis and Glanders	None	Cationic liposomes	CpG	Mice	i.n., single dose	Animals were 100% protected from an aerosol challenge with *B. pseudomallei* or *B. mallei* by enhanced IFN-^{3} secretion and activation of NK cells, but chronic diseases still occurred	[129]
	Bacterial LPS	Gold nanoparticle	Alhydrogel®	Mice, NHP	i.n. for mice, s.c. for NHP, three doses	Protection was observed in the murine but not the NHP model	[130, 131]

Table 2 (Continued)

Disease	Antigen	Delivery system	Adjuvants	Animal Model	Dosing Scheme	Major Results	Ref.

Intoxication with ricin toxin	Inactivated toxin	PLGA microparticle	None	Mice	s.c. for a single dose; i.n. for two doses	A single s.c. dose elicited systemic humoral immune responses, lasting for 1 year post immunization; two i.n. doses achieved 100% protection	[137, 138]
	Inactivated toxin	Liposome	None	Rat	i.t., two doses	Similar humoral immune responses and protection rates compared with soluble vaccines; absence of lung inflammation post an aerosol challenge	[140, 141]
Viral encephalitis caused by alphaviruses	Inactivated VEEV	PLGA microparticle	None	Mice	s.c. or i.t. for the prime dose, s.c., i.t., or oral delivery for one booster dose	Higher activity of antigen-specific IgG and IgA, and 100% protection by the i.t. route than other routes for the booster dose	[145]

Abbreviations: PCL, poly(ε-caprolactone); PVM/MA, poly(methyl vinyl ester/maleic acid); PLA, poly-L-lactide; PLGA, poly(lactic-co-glycolic acid); OMVs, outer membrane vesicles; Stx, Shiga toxin; SEB, staphylococcal enterotoxin B; PEG, polyethylene glycol; LPS, lipopolysaccharides; NHP, non-human primate; VEEV, Venezuelan equine encephalitis virus; s.c., subcutaneous; i.p., intraperitoneal; i.m., intramuscular; i.d., intradermal; i.n., intranasal; i.t., intratracheal.

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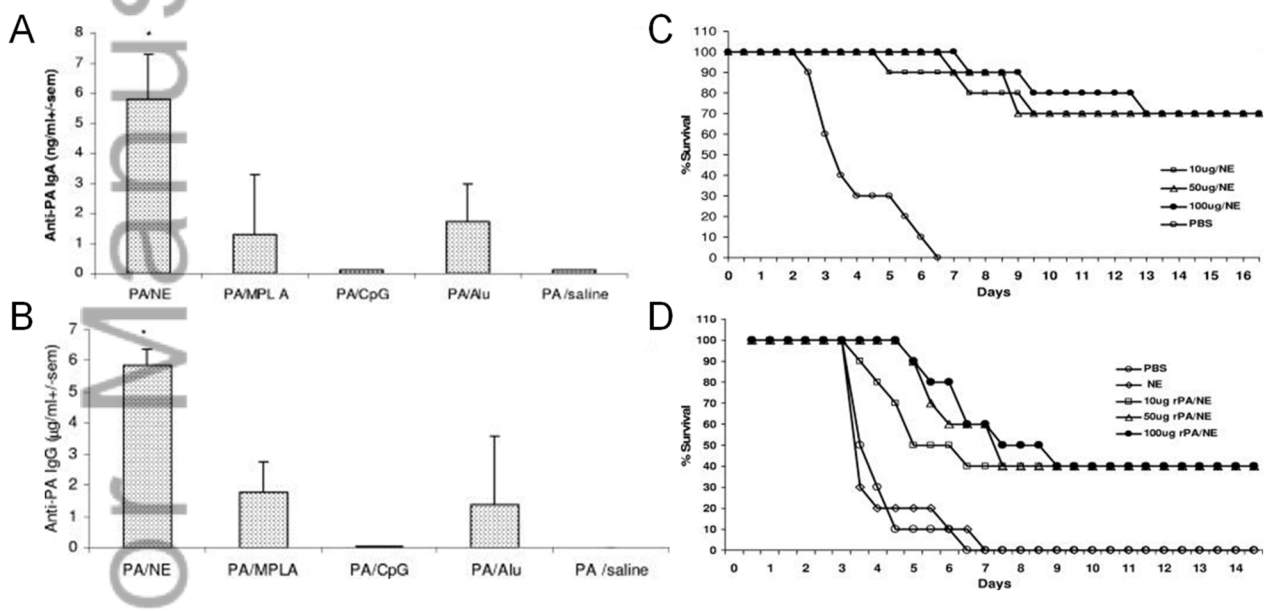

Figure 1.tif

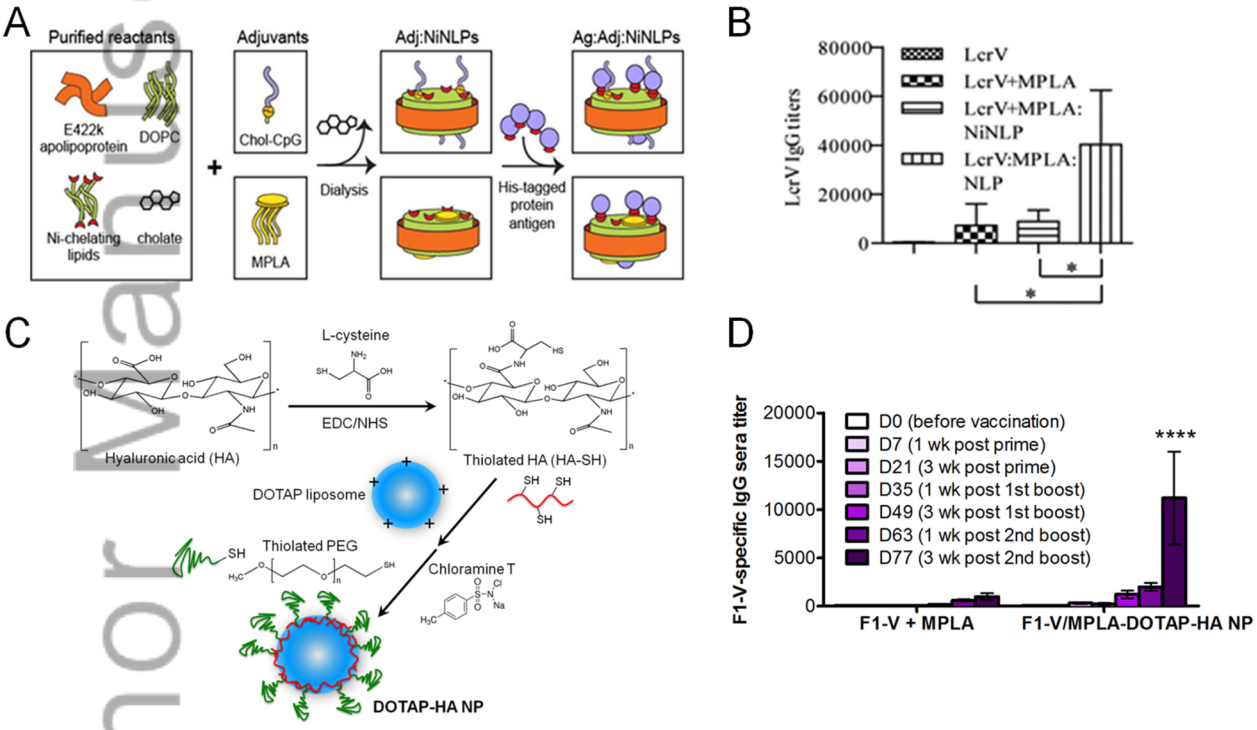

Figure 2.tif

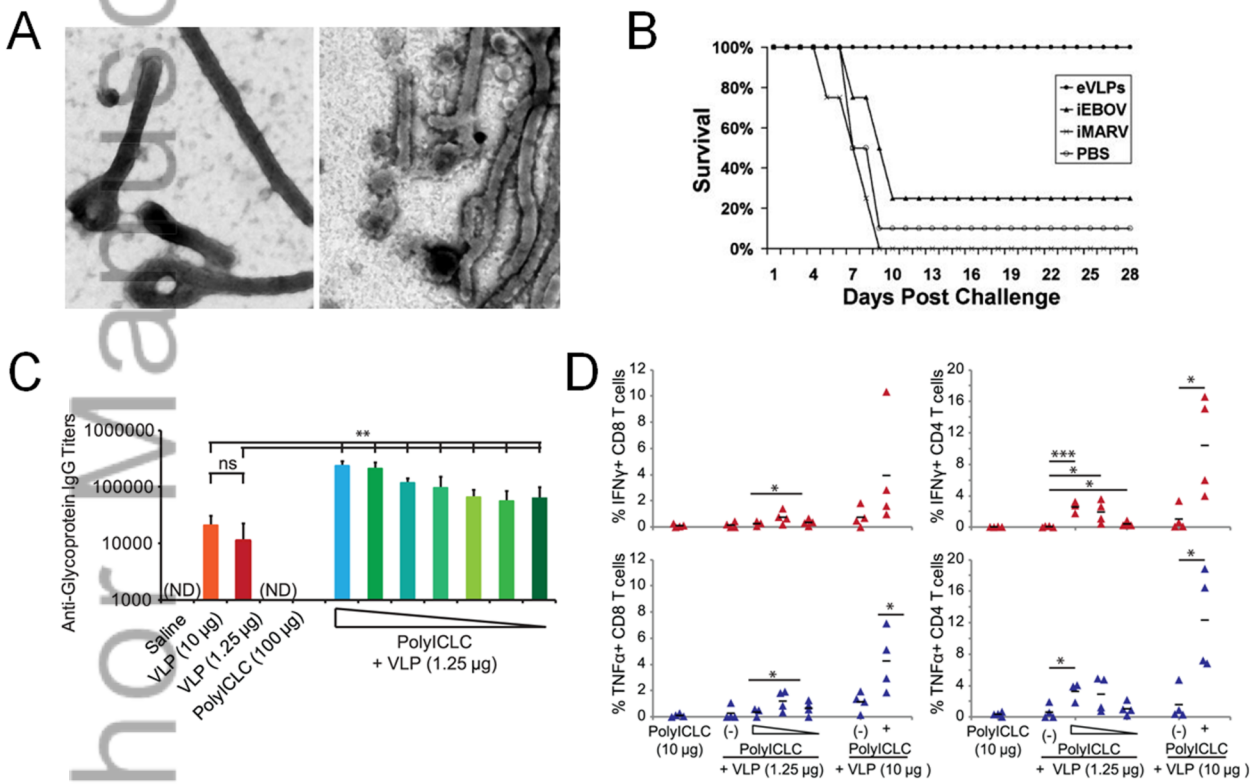

Figure 3.tif

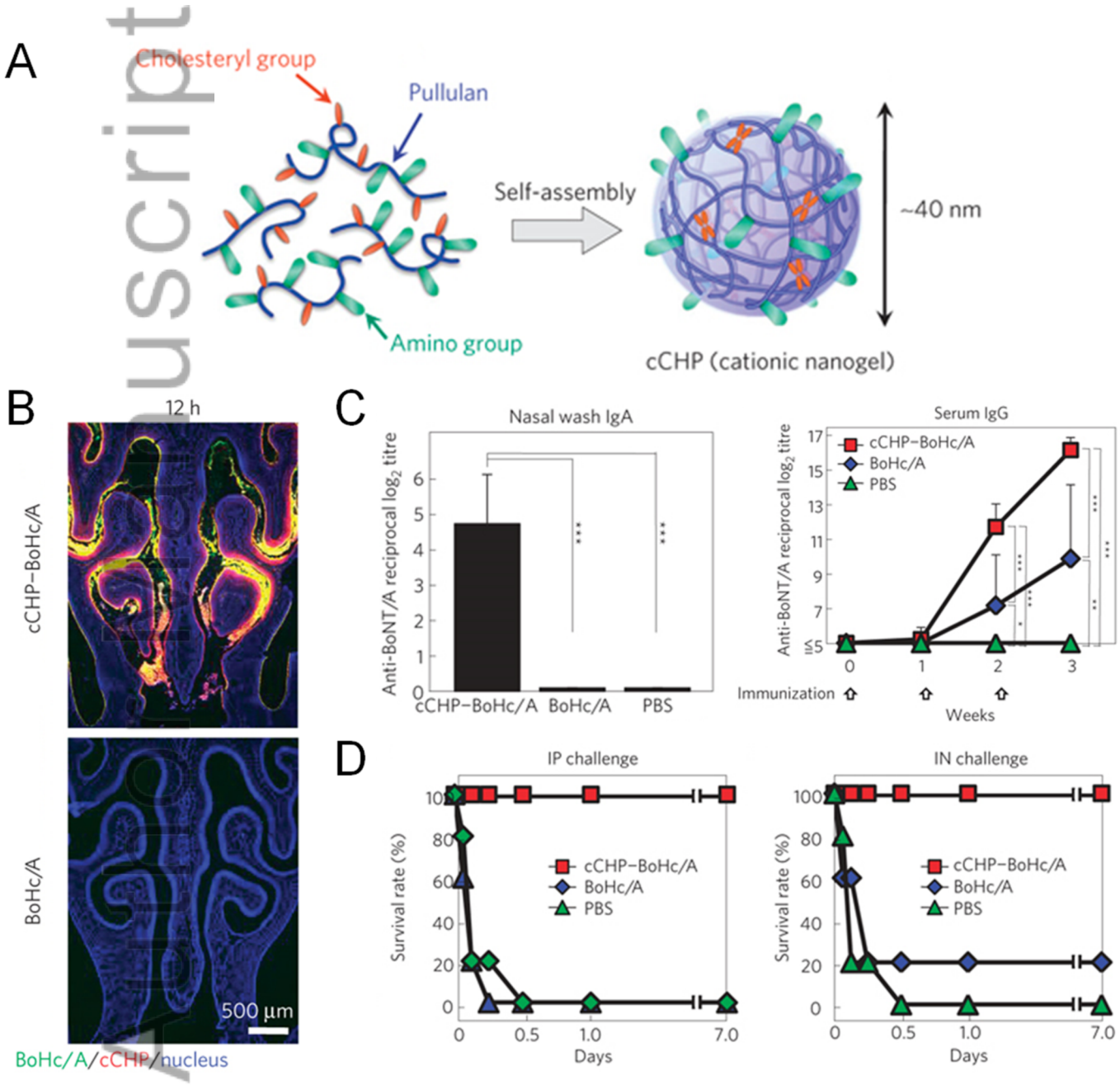

Figure 4.tif

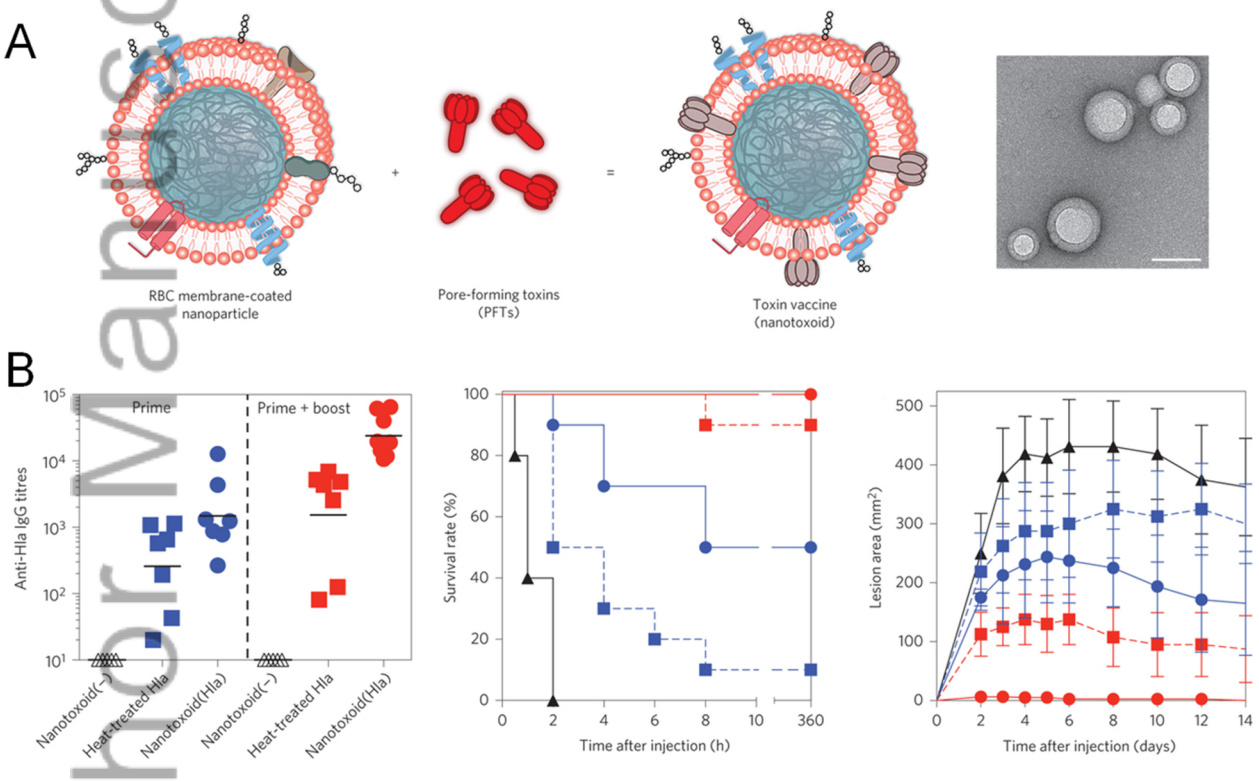

Figure 5.tif