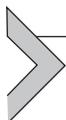




Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Vaccine Adjuvant Nanotechnologies

T. Barclay* and N. Petrovsky**†

*Future Industries Institute, University of South Australia, Mawson Lakes, SA, Australia

**Department of Endocrinology and Diabetes, Flinders University, Adelaide, SA, Australia

†Vaxine Pty Ltd, Adelaide, SA, Australia

Contents

- 7.1 Introduction 127
- 7.2 Emulsion Adjuvants 130
 - 7.2.1 Other organic nanoparticulate adjuvants 132
 - 7.2.2 Inorganic nanoparticulate adjuvants 132
 - 7.2.3 Self-assembling virus-like particles 132
 - 7.2.4 Polypeptide-based self-assembled adjuvants 133
 - 7.2.5 Liposome and virosome particles 133
 - 7.2.6 Electrostatic particle self-assembly 134
 - 7.2.7 Lipid-like self-assembling adjuvant particles 134
 - 7.2.8 QS21 and immune-stimulating complexes 135
- 7.3 Microparticulate Polysaccharide Adjuvants 135
- 7.4 Immune Targeting Strategies 136
- 7.5 Innate Immune Receptor Ligands 137
- 7.6 Template-Based Nanoparticle Manufacturing Methods 138
 - 7.6.1 Emulsions for template-based particle assembly 138
 - 7.6.2 PRINT-based adjuvant production 139
- 7.7 Conclusions 140
- References 140



7.1 INTRODUCTION

Reduced immunogenicity and thereby efficacy is an unfortunate downside to vaccines based on highly purified or synthetic antigens. This requires the use of vaccine adjuvants to improve vaccine immunogenicity. The term *adjuvant* has traditionally referred to as any substance that when added to a vaccine antigen increases its immunogenicity.¹ As such, the study of adjuvant action is made complicated by the wide diversity of agents including small molecule immune modulators, mineral salts, oil emulsions, and even whole viruses or bacteria. These agents all exhibit adjuvant action in vivo with no one common feature explaining this activity. Almost certainly, these compounds work through many and varied different mechanisms to enhance immune responses to coadministered antigens. Increasing use of small protein or peptide antigens allows greater

control over vaccine design and synthesis but has further exacerbated issues of poor immunogenicity. This necessitates closer examination of how best to design vaccine delivery and adjuvant systems to maximize antigen immunogenicity, assist targeted immune delivery, and provide potent adjuvant activity. Most importantly, this must all be done in a manner that does not compromise vaccine tolerability or safety.^{2,3}

The immune system has evolved to protect against invading pathogens. As pathogens present as nano- to micro-sized particles, it should not be surprising that the immune system has evolved strategies to specifically recognize and respond to particles of particular sizes and shapes.^{4,5} This helps explain the boost to immunogenicity when antigens are presented to the immune system as virus-like particles (VLPs) rather than as soluble proteins. Particle size was shown to be important for uptake by dendritic cells (DCs), with spherical particles less than 500 nm in diameter having better uptake than larger particles.⁶ Size is also relevant to T cell maturation whereby poly(lactide-co-glycolide) (PLGA) microparticles modified with recognition and costimulatory ligands with similar dimensions to DCs were better able to induce T cell activation when compared with nanosized particles⁷ that were rapidly phagocytosed.⁸ While the idea that size is important to vaccine action is now well accepted, it is also likely that shape, texture, and surface chemistry are equally highly relevant to adjuvant action,⁸ albeit less well-explored.

Shape is known to be important to particle uptake *in vivo*, half-life, and biodistribution.^{9,10} For example, cylindrical silicon particles exhibited strong liver accumulation, whereas discoidal particles accumulated away from the liver and spherical particles were distributed evenly across tissues.¹¹ Decreasing the diameter of spherical particles from 3 to 0.7 μm resulted in increased accumulation in reticuloendothelial tissues.¹¹ These tissues are rich in various immune cells, and consequently accumulation in the reticuloendothelial system is an advantage from a vaccine standpoint. Notably, high-axial-ratio nanoparticles were shown to better target draining lymph nodes and thereby enhanced antigen presentation and memory B cell responses.^{12,13}

Surface chemistry has also been shown to be important for adjuvant particle properties, with uptake of larger particles by DCs being enhanced by a positive surface charge.⁶ Furthermore, while 25 nm diameter polyhydroxylated and polymethoxylated poly(propylene sulfide) nanoparticles and polystyrene nanoparticles were all equally efficient in inducing DC uptake, only the polyhydroxylated particles induced high levels of DC maturation,¹⁴ an effect attributed to the polyhydroxylated surface activating complement thereby acting as an immune danger signal for DC activation.¹⁴ Use of hydrophilic polymeric coatings such as polyethylene glycol and polyzwitterions with an overall neutral surface charge that bind water to the surface has also been shown to change particle behavior,⁸ with the hydrophobic regions of amphiphilic systems considered to act as immune danger signals that provide adjuvant effects.¹⁵

Mechanical properties are yet another factor that need to be taken into account when assessing the immune effect of particles. Macrophages and DC have been shown to preferentially phagocytose rigid particles even if these have identical surface chemistry to softer particles.¹⁶ Hence making particles more flexible so that they mimic red

blood cell properties was shown to increase their half-life in vivo,^{11,17,18} whereas making them more rigid should improve their uptake by DCs and thereby their immune action.

Increasingly sophisticated particle manufacturing approaches have enabled ever-tighter control of the size, shape, external chemistry, and mechanical properties of adjuvant particles. This has provided unique opportunities to modify vaccine and adjuvant particles with the aim to maximize their biological action. The remainder of this chapter will describe a wide variety of nano- and microparticulate vaccine and adjuvant systems, their method of manufacture, and how size, shape, and surface chemistry are all important to adjuvant action (Fig. 7.1).

Size	Shape	Structure	Chemistry	Outcome
20–45 nm			Polyhydroxylated polypropylene sulfide	Efficient DC uptake Efficient DC activation ¹⁹
20–25 nm			Polymethoxylated polypropylene sulfide, polystyrene	Efficient DC uptake No DC activation ¹⁴
100 nm			Polyhydroxylated polypropylene sulfide	Less efficient DC uptake No DC activation ^{14, 19}
80 x 180 nm			PEG	Accumulation in draining lymph nodes ¹²
80 x 320 nm			PLGA + cationic additive + hemagglutinin	Enhanced vaccine performance ¹³
< 500 nm			Polystyrene	More efficient DC uptake than larger particles ⁶
1 μm			Polystyrene modified with positive charge	More efficient DC uptake than negative charge or neutral ⁶
1 x 1 μm			Silicon	Favors accumulation in the liver ¹¹
0.3 x 1.6 μm			Silicon	Favors accumulation in tissue other than liver ¹¹
1 x 1 μm			Silica	More even distribution than cylinder and disk ¹¹
> 1 μm			PLGA + external ligands	More efficient T cell activation than nanoparticles ⁷
1–3 μm		Nano-structured surface	Inulin	Modulation of DC function ²⁰
1–6 μm		More rigid Less rigid	Polyacrylamide	More efficient macrophage uptake ¹⁶ Less efficient macrophage uptake ¹⁶

Figure 7.1 Nanoadjuvant characteristics. Listed in the table are different types of nano- and microadjuvant particles, their physical characteristics, and immunological properties.



7.2 EMULSION ADJUVANTS

Emulsions are composed of a dispersion of droplets of one liquid in another immiscible liquid, commonly described as either water-in-oil or oil-in-water emulsions.²¹ In use as adjuvants, the droplets in an emulsion act as particles and are perceived by the immune system as such. There are two different forms of nanoscale emulsions, both of which are defined as having droplet radii from 1 to 100 nm, which is sufficiently small to generate transparent, isotropic liquid media. The first form of nanoscale emulsion has historically been termed a *microemulsion* but is better described as a nanodimensional lyotropic liquid crystalline phase.²² In a microemulsion the structural organization of the two liquids is supported by a relatively large concentration of surfactant. Formation is by spontaneous self-assembly with relatively gentle mixing, resulting in stable organization at thermodynamic equilibrium.^{22,23} By contrast, what is termed a *nanoemulsion* generally uses significantly less surfactant and hence requires high shear forces to make a metastable structure.^{22,24}

Emulsion adjuvants are thought to work through a depot effect, creating inflammation around the site of antigen injection and thereby enhancing humoral immune responses.²⁵ Early examples of emulsion adjuvants were water-in-mineral-oil emulsions.^{26,27} However, such adjuvants were problematic as mineral oil is not biodegradable, is proinflammatory, and has long-term persistence in the body, thereby leading to major inflammatory side effects including injection site granulomas, pyrexia, and long-term safety issues.²⁸ Care must also be taken with any emulsion adjuvant to avoid any oxidation of the oil component as this could otherwise lead to increases in vaccine reactogenicity. Generally, oil-in-water nanoemulsions using biodegradable oils have a better tolerability profile than water-in-oil formulations. A well-known example of an oil-in-water nanoemulsion adjuvant is MF59, which is composed of squalene oil in a citrate buffer using nonionic surfactants.^{29,30} The nanoemulsion is key to the adjuvant effect of MF59 as when tested individually the various components of MF59 failed to demonstrate adjuvant activity.³¹ MF59-based vaccine formulations have been used extensively in research,³² and recent work has included tests as a cationic nanoemulsion delivery system for delivery of a self-amplifying mRNA vaccine.³³ Nonetheless, the bulk of research has been for influenza vaccines,³⁴ and while MF59 is licensed for this purpose in elderly humans³¹ and has extensive safety data in the elderly population, more limited safety data are available for use in children. Of potential relevance, a 2009 pandemic influenza vaccine containing AS03, a squalene-based nanoemulsion that is similar to MF59 except for the addition of tocopherol, was associated with a rise in childhood cases of narcolepsy,^{35,36} an autoimmune sleep disorder. This has put the question of pediatric safety of squalene-based emulsion adjuvants back under the spotlight.

The immunogenicity of emulsion adjuvants can be increased further by coformulation with additional immune-stimulatory components. Interestingly, the original MF59

squalene emulsion adjuvant was initially designed as a delivery system for muramyl tripeptide phosphatidylethanolamine (MTP-PE), a modified mycobacterial peptidoglycan with potent adjuvant activity.³⁷ MTP-PE activates innate immune receptors including nucleotide-binding oligomerization domain-containing protein 2 (NOD2)³⁸ and various Toll-like receptors (TLR),³⁹ leading to nuclear factor kappa-B (NFκB) activation and induction of inflammatory cytokines.⁴⁰ However, the MTP-PE component was ultimately removed from MF59 because of excessive reactivity.⁴¹ A more recent version of a combined adjuvant emulsion and immunostimulator approach is the AS02 adjuvant, which is an oil-in-water emulsion combined with QS21 saponin and the TLR4 agonist, monophosphoryl lipid A (MPL).⁴²

Intranasal delivery of vaccines provides advantages in terms of convenience and induction of mucosal immunity, a major benefit when protecting against respiratory or mucosal pathogens.^{43,44} A nasal vaccine containing a soybean oil nanoemulsion adjuvant successfully enhanced humoral and cellular responses in mice,⁴³⁻⁴⁷ consistent with the utility of this approach. A library of more than 100 intranasal adjuvant candidate formulations consisting of oil-in-water nanoemulsions containing various cationic and nonionic surfactants were tested in mice and demonstrated that varying the physicochemical properties of the surfactant components (charge, surfactant polar head size, and hydrophobicity) and the surfactant blend ratio of the intranasal adjuvant formulations could modulate the strength and type of the immune response.⁴⁸ This indicates that there may be considerable scope for further optimizing nanoemulsion adjuvants for intranasal use. However, the tolerability and safety in humans of this approach have yet to be confirmed. It was recently shown that nanoemulsion adjuvants induce epithelial cell death via activation of caspases 1, 3, and 6–9.⁴⁹ Furthermore, the safety of nasal vaccine adjuvants has remained under a cloud since the experience of NasalFlu, an intranasal influenza vaccine containing a detoxified enterotoxin adjuvant, that was associated with increased cases of facial nerve palsy in clinical trials.⁴⁴

Multiple or double emulsions, most often water-in-oil-in-water formulations, have also been used in vaccine research.⁵⁰ However, water-in-oil-in-water multiple emulsion proved to be less stable and induced an excessive inflammatory response when compared with an oil-in-water microemulsion of isopropyl myristate, which successfully enhanced humoral responses to rabies vaccine in mice.⁵¹ These stability issues arise as there are two interfaces with high free energy to stabilize, generally requiring two surfactants that may interact, thereby interfering with stabilization. Also, for multiple emulsions there is the potential for an osmotic pressure to develop, placing further strain on the system.⁵² Even simple emulsions are not without issues. Nanoemulsions not at thermodynamic equilibrium have poor stability, and microemulsions suffer from the need for high concentrations of surfactants, which may cause toxicity *in vivo*. Furthermore, the stability of any microemulsion can be easily compromised by dilution, heating, or pH changes.

7.2.1 Other organic nanoparticulate adjuvants

Various nanoparticles constructed from disordered, precipitated polymers, also described as nanobeads, have been used for vaccine delivery. Poly- ϵ -caprolactone nanoparticles of two sizes were made, with mean diameters of 61 and 467 nm, by solvent evaporation. Only the smaller diameter particles were found to have an adjuvant effect, eliciting both cellular and humoral responses in mice.⁵³ Polystyrene nanobeads covalently conjugated to ovalbumin were found to best activate CD8 T cells when they were 40–49 nm diameter when beads ranging in size from 20 to 2000 nm diameter were compared.^{54,55} Another study found that larger beads with diameters from 93 to 123 nm were best at activating CD4 T cells.⁵⁵ Thus, it may be possible to specifically tune nanoparticle size to induce either cellular or humoral immunity. In another system poly(γ -glutamic acid) grafted with L-phenylalanine ethyl ester on 53% of the carboxylic acid groups self-assembled upon the addition of water from organic solution into nanoparticles with diameters of 150–200 nm.⁵⁶ In the presence of ovalbumin the nanoparticles encapsulated the antigen and targeted it to DCs, resulting in enhanced DC maturation and immunity.^{56,57}

7.2.2 Inorganic nanoparticulate adjuvants

Inorganic nanoparticles have been used as vaccine delivery vehicles; aluminum oxide nanoparticles having distinctly better performance compared with other metal oxide nanoparticles.⁵⁸ Such aluminum oxide nanoparticles of 113–355 nm diameter elicited adjuvant effect when peptomers derived from an HIV antigenic sequence and retaining the native α -helical conformation were covalently attached to the external surface.^{59,60} Aluminum oxide nanoparticles were also functionalized with ovalbumin and shown to enhance DC activation of T cells via an autophagy-dependent mechanism.⁵⁸ Particle size was also shown to be important as while both 60 and 200 nm nanoparticles were effective in vitro, the 60 nm aluminum oxide particles were much more effective in being transported to the draining lymph nodes in vivo.

7.2.3 Self-assembling virus-like particles

VLPs can be made using recombinant protein techniques whereby antigenic proteins such as HBsAg are synthesized in yeast, following which the antigen self-assembles into 22 nm VLPs.⁶¹ VLPs are safe and immunogenic, making them ideal vaccine antigens.^{62,63} However, cell-culture systems used for manufacture of VLPs can be low yielding and expensive, and hence synthetic methods have been developed for VLP production. For example, short linear synthetic proteins with coiled-coil domains were used for self-assembly of α -helical motif-containing nanoparticles able to present repetitive epitopes on their surface.^{64,65} These synthetic particles had a 25 nm diameter and elicited strong immune responses to surface-expressed epitopes.^{64–66} A related nanoparticulate vaccine delivery system was also assembled from peptide chains containing a coiled-coil sequence to drive helical assembly. In this

instance the peptide was modified at the N-terminus with a dual chain lipid and with synthetic epitopes at the C-terminus. Upon dispersion in aqueous solution, the construct aggregated into ordered synthetic particles having a diameter of 17–20 nm and presenting multiple copies of the antigen on the surface that induced a humoral immune response.⁶⁷

7.2.4 Polypeptide-based self-assembled adjuvants

In another synthetic system polypeptides designed to self-assemble into β -sheet-rich nanofibers with epitopes attached to the C-terminus generated strong antibody responses without inflammation.⁶⁸ Similarly to VLP's, the self-assembled structure was critical to the adjuvant effect, the unassembled sequence having no adjuvant properties.⁶⁹ It has been shown that the nanofibers are internalized by DCs and macrophages at the injection site, resulting in increased expression of the activation markers CD80 and CD86 and enhanced production of T follicular helper cells and germinal center B cells.⁶⁸ Nanostructuring of materials within tissue depots may also generate adjuvant activity. Peptides having alternating hydrophobic and hydrophilic amino acids can undergo a sol-gel transition upon exposure to physiological conditions whereby they form a hydrogel made up of interconnected nanofibers.⁷⁰ In this way a mixture of biphasic oligopeptide, antigen, and adjuvants can be injected with the peptides, subsequently undergoing postinjection self-assembly into hydrated nanofiber gel matrices, forming an antigen and adjuvant depot in the aqueous phase.⁷¹ A self-assembling nanofibrous hydrogel induced an antibody response when tested as a vaccine delivery platform, either alone or formulated with CpG adjuvant (TLR9 agonist) as a delivery system for recombinant hepatitis B surface antigen (HBsAg).⁷¹ Similarly, the self-assembling peptide Q11 conjugated to a CD8 T cell epitope of ovalbumin elicited a strong antigen-specific CD8 T-cell response.⁷²

7.2.5 Liposome and virosome particles

Liposome-based vaccines were an early form of particulate adjuvant formulation based on the use of cell-like spherical lipid bilayer vesicles containing antigens within the internal aqueous compartment, within the lipid bilayer, or attached externally.^{73,74} For example, 165 nm-diameter liposomes assembled from cationic lipid, cationic polymer, and plasmid DNA were shown to target antigen to draining lymph nodes, resulting in enhanced DC activation and immunity.⁷⁵ Coating of these liposomes with a cholesterol-modified mannan further enhanced the therapeutic anticancer action of an oncoprotein vaccine.⁷⁶ As liposomes themselves are generally poorly immunogenic, they may require combination with other immunostimulatory adjuvants to enhance their immunogenicity.⁴ For example, liposomal nanoparticles self-assembled from a mixture of phospholipids containing 5% pegylated lipid to prevent protein binding were used to target cyclic diguanylate as an agonist of stimulation of INF genes to draining lymph nodes, thereby enhancing vaccine potency.⁷⁷ Single bilayer liposomes have also been constructed using viral lipid envelopes, retaining the viral cell binding and fusion glycoproteins and resulting in 150 nm-diameter

virosomes that promote attachment to respiratory mucosal surface and subsequent fusion release of contents into the cytoplasm of endocytosing cells.⁷⁸ Further, the viral components enhance the efficiency of the interaction with antigen-presenting cells (APCs), making them an ideal vaccine delivery system.^{79,80} More recent virosome research has included the incorporation of additional components to improve the particle formation and stability characteristics as well as enhance immunogenicity.⁸¹ For example, an amphiphilic saponin derivative (GPI-0100) was incorporated into a virosome and provided potent immunogenicity to allow the use of very low antigen doses.⁸²

7.2.6 Electrostatic particle self-assembly

Electrostatic interactions are an important part of nanoparticle vaccine formulations, both between components of the formulation^{83,84} and between the formulation and the anionic cell membranes of APCs.⁸⁵ These charged interactions have demonstrated importance in various nanovaccines, including liposomal systems,⁸⁶ but perhaps are best exploited in polymeric systems. A mixture of an anionic poly(γ -glutamic acid) acid grafted with phenylalanine ethylester and a cationic ϵ -polylysine was self-assembled from aqueous solution into nanoparticles with 200–300 nm diameter.⁸³ When formed in the presence of ovalbumin, the particles were loaded with antigen and induced effective cellular and humoral immune responses in immunized mice.⁸⁷ Poly(γ -glutamic acid) has also been electrostatically attached to complexes of dendrigraft poly-L-lysine with plasmid DNA. In this instance the poly(γ -glutamic acid) moderates the cytotoxicity of the electrostatic complex to enable delivery and expression of genes in the spleen, suggesting application in vaccine formulations.⁸⁸ This is related to a similar gene delivery formulation in which electrostatic interactions between positively charged polyethyleneimine and negatively charged plasmid DNA and poly(γ -glutamic acid) were used to construct a nanoparticle DNA vaccine. This formulation conferred protection against malaria infection in mouse models.⁸⁴ Standard PLGA particles have negative charge, and electrostatic interactions have been identified as a critical aspect of the binding to protein antigens. Consequently, protein binding can be tuned through adjusting the pH of binding conditions to optimize the charge differential between components.⁸⁹ PLGA nanoparticles have also been constructed with positive charge using PRINT technology through the incorporation of cationic additives. These particles were specifically designed to electrostatically interact with commercial hemagglutinin antigens to generate an influenza vaccine with enhanced immune responses compared with the hemagglutinin alone.^{12,13}

7.2.7 Lipid-like self-assembling adjuvant particles

Lipid-like amphiphiles have been used in several self-assembling nanoparticulate-based self-adjuvanting antigen delivery systems because their structure mimics bacterial lipoproteins and as such can activate the immune system.⁹⁰ This type of vaccine formulation

includes a hydrophobic core covalently attached to a hydrophilic peptide antigen such that the antigen epitope is presented on the surface of the nanoparticle.⁹¹ A nontoxic, dendritic poly tert-butyl acrylate polymer core has been used and when conjugated to relevant peptide epitopes induced cellular responses able to kill human papillomavirus-infected cancer cells.⁹² Epitope packing on the particle surface was dense enough to maintain a native conformation and enable proper recognition by the immune system.^{91,92} In other examples the particle core was made up of either lipid-like peptides⁹³ or lipidated peptides.^{94,95} Such formulations include a peptide epitope from a hookworm protein flanked by coil-producing sequences attached to a lipid-like peptide core. The native alpha helix structure of the epitope presented on the surface of the nanoparticle was able to generate antibodies in mice.⁹⁶

7.2.8 QS21 and immune-stimulating complexes

As previously suggested by their use in emulsion and liposome adjuvants, saponins can provide potent immunogenicity to vaccine formulations. A commonly used adjuvant is the saponin QS21, which is an acylated saponin at the 4-hydroxyl position on fucose with two linked 3,5 dihydroxy-6-methyloctanoic acids.⁹⁷ QS21 induces inflammatory cytokines and imparts a Th1 bias in vaccine responses,^{98,99} but because of its ability to lyse cell membranes, hemolysis and injection site pain are major limiting factors in its use.¹⁰⁰ QS21's toxicity can be reduced by forming it into an immune-stimulating complex (ISCOM), which is a spherical particle of ~40 nm diameter that self-assembles from specific mixtures of cholesterol, phospholipids, and QS21.¹⁰¹⁻¹⁰³ An advantage of ISCOMs is that the toxic hemolytic effect of the saponin is moderated by its incorporation into the liposomal structure while its immune-stimulatory properties are retained. The liposomal particle also helps target the saponin adjuvant and antigen formulation to the draining lymph nodes.⁵ While initially thought important for immunogenicity that antigens were loaded inside the ISCOM structure, it is now recognized that adjuvant action can be achieved by simple admixture of the antigen with the ISCOMs, a major advantage as many antigens are difficult to load into the particles.¹⁰⁴



7.3 MICROPARTICULATE POLYSACCHARIDE ADJUVANTS

Many plant-based polysaccharides have been shown to have adjuvant activity, with the additional benefit that as sugar-based compounds polysaccharides are generally extremely safe and well tolerated.²⁰ While some polysaccharides (eg, mannan) may have adjuvant activity when in soluble form, most are only adjuvant active when present as insoluble particles. This is clearly seen with the polysaccharide inulin (β -D-[2-1] poly(fructo-furanosyl)-D-glucose), a natural plant-derived storage carbohydrate of plants of the *Compositae* family that has no immunological activity when in the usual soluble

form but has potent adjuvant activity when crystallized into the nanostructured delta inulin microparticles.^{105–108} Delta inulin particles have been shown to enhance humoral and cellular immune responses to a wide variety of viral, bacterial, and protozoan antigens as well as toxins and allergens. For example, enhanced vaccine protection was seen in models of influenza,^{109,110} Japanese encephalitis,^{111,112} West Nile virus,¹¹³ hepatitis B,¹¹⁴ human immunodeficiency virus,¹¹⁵ anthrax,¹¹⁶ SARS coronavirus,¹¹⁷ listeriosis,^{118,119} and African horse sickness.¹²⁰ Its adjuvant effects are seen across a broad range of animal species including mice, rats, guinea pigs, rabbits, chickens, dogs, sheep, monkeys, horses, and camels. It has proved effective in human studies when combined with a recombinant pandemic influenza vaccine,¹²¹ a hepatitis B vaccine,¹²² or a bee sting allergy vaccine.¹²³ An interesting feature of delta inulin is its ability to enhance adaptive immune responses even when injected a day prior to the antigen, a feature not shared by alum adjuvant.¹¹⁴ Delta inulin does not work like other polysaccharide adjuvants, as it has not been found to activate innate immune receptors such as TLRs, Dectin-1, or the inflammasome. Instead, delta inulin adjuvant appears to work by directly modulating DC function, thereby resulting in enhanced antigen presentation to memory T and B cells. This was recently shown in human subjects to translate into enhanced B cell receptor affinity maturation with upregulation of activation-induced cytidine deamidase in day 7 postimmunized plasmablasts.¹²⁴ Notably, delta inulin has proved safe and effective when administered to pregnant dams¹²⁵ or their 7-day-old mouse pups¹²⁶ and, in a large animal model, to either pregnant mares or their foals,¹²⁷ a feature that may be attributable to its noninflammatory mode of action. Interestingly, a powder particle formulation of delta inulin has recently been shown to be safe and effective when administered with influenza vaccine directly into the lungs of mice,¹²⁸ opening up a further novel vaccine application of this technology.

Other polysaccharide particles with adjuvant activity include dextran, β -glucan, lentinan, zymosan, mannan, and chitosan.²⁰ These all work through the action of specific innate immune receptors expressed on APCs known as lectins that specifically recognize and respond to sugars. These include the β -glucan receptor, the mannan receptor, Dectin 1, and TLR. Activation of these innate immune receptors results in NF κ B activation and production of proinflammatory cytokines that enhance adaptive immune responses. Hence delta inulin currently appears to be the only polysaccharide particulate adjuvant that works through an alternative noninflammatory pathway.



7.4 IMMUNE TARGETING STRATEGIES

Given the high expression of lectins on APCs, decorating the surface of vaccine or adjuvant particles with sugar groups can assist vaccine particle targeting to APCs. For example, mannose has been used to target plasmid DNA-containing liposomes to macrophages.¹²⁹ Coating of cationic liposomes with mannan significantly enhanced the ability

of a DNA vaccine to induce HIV-specific cellular immunity and also enhanced the activity of a DNA vaccine against melanoma.^{130,131} Mannosylated niosomes composed of span 60, cholesterol, and stearylamine (all coated with the modified polysaccharide O-palmitoyl mannan) have been used as orally administered DNA vaccine carriers with a demonstration of enhanced mucosal immunity in mice.¹³² A similar approach using O-palmitoyl mannan coating was used to target niosomes to Langerhan's cells in the skin after topical delivery.¹³³ Chitin, a linear β -1-4-linked polymer of D-glucosamine and N-acetyl-D-glucosamine extracted from shrimp, and chitosan, obtained by partial deacetylation of chitin, act by binding innate receptors including Dectin-1, macrophage mannose receptor, and TLR-2.¹³⁴ Chitosan particles produced by cross-linking with a counter ion were used to entrap antigen and enhance its immunogenicity in mice.¹³⁵ By virtue of their mucoadhesive qualities, particles coated with chitin and its derivatives have been extensively used as nasal adjuvants for delivery of inactivated¹³⁶ or even live viral vectors. Hence mucosal delivery of adenoviral vectors microencapsulated in a chitosan microparticle not only helped to protect and improve the viability of the viral vector but also made its release dependent on cell surface contact.¹³⁷



7.5 INNATE IMMUNE RECEPTOR LIGANDS

Given the ability of specific nanoparticles to target APCs, as discussed previously, such particles can also be loaded with immune stimulators to further increase their adjuvant potency. MDP (*N*-acetyl muramyl-*L*-alanine-*D*-isoglutamine), a mycobacterial peptidoglycan with potent adjuvant activity,³⁷ binds and activates NOD2³⁸ and TLR receptors,³⁹ leading to NF κ B activation, inflammatory cytokine production, and DC maturation. A modified form, MTP-PE, was the key immune stimulator for which the original MF59 emulsion adjuvant was designed as a delivery system, given the highly hydrophobic nature of MTP-PE.⁴⁰ Ironically, the combined MTP-PE in MF59 formulation was abandoned because of excess reactogenicity,⁴¹ but the MF59 vehicle was found to have some adjuvant activity in its own right, and hence its use in influenza vaccines was continued. In a similar fashion, liposomes formulated with DTP-GDP (*N*-acetylglucosaminyl-*N*-acetylmuramyl-*L*-Ala-*D*-isoGlu-*L*-Ala-glyceroldipalmitate) had potent adjuvant activity and induced remission in human metastatic colorectal cancer, although reactogenicity with fever, chills, and hypotension was a problem at higher doses.^{138,139} The combination of chitosan microparticles with the mucosal toxin-based adjuvant LTK63 significantly enhanced the immunogenicity of an intranasal group C meningococcal polysaccharide vaccine in mice.¹³⁶ Similarly, intranasal administration of alginate-coated chitosan nanoparticles loaded with antigen and CpG adjuvant enhanced antibody and cellular responses in mice.¹⁴⁰ CpG has also been incorporated as the hydrophilic head group into a lipid-like adjuvant macromolecule, the hydrophobic tail composed of cholesterol, simple monoacyl, or diacyl lipidic groups. This formulation

was used to successfully target CpG adjuvant and the antigen to lymph nodes, thereby helping decrease systemic CpG toxicity while enhancing vaccine immunogenicity.¹⁴¹

7.6 TEMPLATE-BASED NANOPARTICLE MANUFACTURING METHODS

7.6.1 Emulsions for template-based particle assembly

Emulsions are not only useful as adjuvants, as discussed earlier, but they can also be used as templates to produce controlled-size solid adjuvant particles (Fig. 7.2). Using this method, the polysaccharide inulin was cosolubilized with an ovalbumin antigen into a water-in-oil emulsion with the inulin then precipitated by the slow addition of acetone. This resulted in formation of spherical 1.5 μm -diameter inulin particles encapsulating

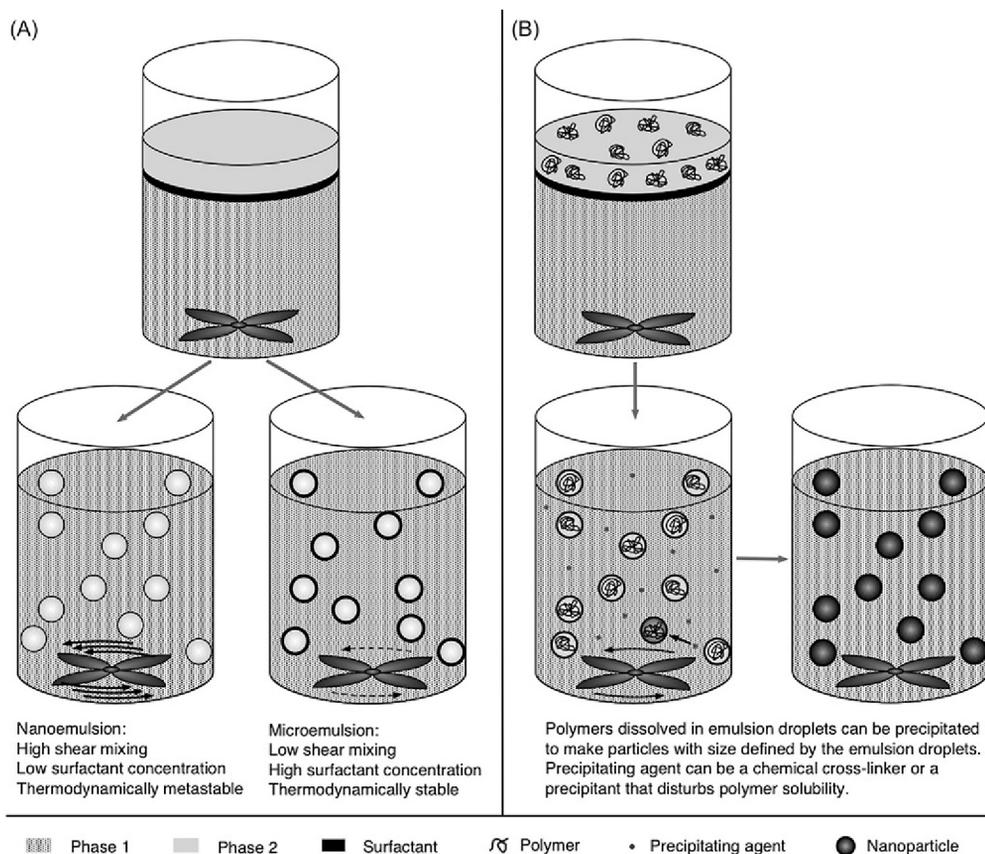


Figure 7.2 Formation and use of nanoemulsions as templates for formation of nanoadjuvant particles. (A) shows method of formation and characteristics of nanoemulsions versus microemulsions. (B) shows use of microemulsion as templates for formation of solid nanoadjuvant particles.

the ovalbumin with high efficiency.¹⁴² These antigen-loaded inulin microparticles improved APC uptake of ovalbumin and enhanced antiovalbumin antibody responses in mice. Emulsions have similarly been used as templates to precipitate many other biodegradable polymeric nanoparticles for use in vaccine delivery, including the formation of PLGA nanoparticles from a nanoemulsion. The PLGA nanoparticles acted as an antigen delivery system although, unlike inulin particles, the PLGA particles themselves appeared to have no intrinsic adjuvant properties with an immunomodulator being required to create a strong immune response.^{15,143} By contrast, poly(γ -glutamic acid)-based nanoparticles generated an immune response without the requirement of other adjuvants.⁵⁷

Emulsion polymerization is another method whereby polymeric particles of size defined by the emulsion can be generated.¹⁴⁴ In this method the soluble polymer droplets within the emulsion are chemically cross-linked such that the droplet-sized particles remain solid when the emulsion is removed. Poly(ortho ester) microparticles of 5 μm diameter produced in this way were used to deliver plasmid DNA antigen to APCs. The acidic conditions in the APC lysosome degraded the orthoester bonds holding the particles together, thereby releasing the DNA. The DNA was then transcribed into protein that was presented to T and B cells, resulting in a cellular and humoral memory response.¹⁴⁴ In another example, size-tuneable cross-linked poly(propylene sulfide) nanoparticles with sizes ranging from 25 to 250 nm were prepared from emulsions, with monomers in the water phase stabilized by Pluronic F-127.¹⁴⁵ Particle size was then controlled by varying the concentrations of the monomer and surfactants in the emulsion. These particles presented pluronic hydroxyl groups at the surface, resulting in complement activation, DC maturation, and a strong adjuvant effect.¹⁴ Such particles are unstable and release any attached drug under oxidative conditions, such as those found in the lysosome.^{14,145} The ability to produce particles of different sizes enabled the effect of particle size on antigen delivery to be directly studied, revealing that small 20–25 nm nanoparticles delivered attached antigen to draining lymph nodes with 10-fold higher efficiency than 100 nm particles.^{14,19} Other studies using these particles have shown that particle surface chemistry is important to the action of nanoadjuvants with, for example, polyhydroxylated surfaces performing much better than polymethoxylated surfaces¹⁴ despite both particles being targeted to the DCs.

7.6.2 PRINT-based adjuvant production

Controlled production of defined-size adjuvant nanoparticles can also be achieved through particle replication in nonwetting template (PRINT) technology.^{9,13} PRINT techniques use photolithography to create a template that is then transferred to a perfluoropolyether elastomer mold. The mold is filled with the desired material under pressure and then allowed to set. Release of the material from the mold produces particles in the nano- or microscale that can have complex and highly conserved morphology.⁹ This technology has been used for a range of medical applications, including production

of stimuli-responsive targeted drug delivery vehicles^{9,146} and vaccine adjuvants.¹³ The size, shape, and chemistry of PRINT nanoparticles can be optimized to maximize their transport to lymph nodes, enabling their use as an antigen delivery vehicle.¹² This work found that anionic 80 x 180 nm cylinders covalently bound to ovalbumin through a 500-dalton polyethylene glycol spacer optimized delivery of antigen to lymph nodes and enhanced vaccine responses.¹²



7.7 CONCLUSIONS

This chapter highlighted the increasing sophistication of vaccine adjuvant design driven by the convergence of improved immunological understanding of the importance of nanofeatures to adjuvant function together with advanced nanomanufacturing techniques that allow very precise control of particle size, shape, texture, and surface chemistry. Principles from antigen design (eg, particle self-assembly) are increasingly being incorporated into adjuvant nanoparticle design. These individual concepts are now being applied to the design of a single integrated vaccine particle, incorporating antigen, adjuvant, and APC-targeting strategies. In the process, the concept of what is an adjuvant needs to be broadened to not only include specific immune-stimulatory substances but also to include any vaccine design features that enhance immune responses against a relevant antigen. Thus, the definition of an adjuvant could potentially now include aspects of antigen formulation such as nanoparticle incorporation; aspects of particle size, shape, and surface chemistry that enhance immunogenicity; or even purely physical processes such as texturing of the particle surface to maximize immunogenicity. Looking forward, adjuvants will increasingly not be seen as separate add-on items but as wholly integrated elements of a complete vaccine delivery package. Vaccine systems are steadily approaching the complexity and sophistication of the pathogens they mimic, with particle properties and behaviors designed to maximize long-term immune protection while maintaining a high safety profile.

REFERENCES

1. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. *Immunol Cell Biol* 2004;82:488–96.
2. Petrovsky N, Heinzel S, Honda Y, Lyons AB. New-age vaccine adjuvants: friend or foe? *BioPharm Int* 2007;20:24–33.
3. Petrovsky N. Freeing vaccine adjuvants from dangerous immunological dogma. *Exp Rev Vaccin* 2008;7:7–10.
4. Liang MT, Davies NM, Blanchfield JT, Toth I. Particulate systems as adjuvants and carriers for peptide and protein antigens. *Curr Drug Del* 2006;3:379–88.
5. Singh M, O'Hagan D. Advances in vaccine adjuvants. *Nat Biotechnol* 1999;17:1075–81.
6. Foged C, Brodin B, Frokjaer S, Sundblad A. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. *Int J Pharm* 2005;298:315–22.
7. Steenblock ER, Fahmy TM. A comprehensive platform for ex vivo T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells. *Mol Ther* 2008;16:765–72.

8. Moon JJ, Huang B, Irvine DJ. Engineering nano- and microparticles to tune immunity. *Adv Mater* 2012;24:3724–46.
9. Perry JL, Herlihy KP, Napier ME, DeSimone JM. PRINT: a novel platform toward shape and size specific nanoparticle theranostics. *Acc Chem Res* 2011;44:990–8.
10. Gratten SEA, Pohlhaus PD, Lee J, Guo I, Cho MJ, DeSimone JM. Nanofabricated particles for engineered drug therapies: a preliminary biodistribution study of PRINT (TM) nanoparticles. *J Control Rel* 2007;121:10–8.
11. Decuzzi P, Godin B, Tanaka T, Lee SY, Chiappini C, Liu X, Ferrari M. Size and shape effects in the biodistribution of intravascularly injected particles. *J Control Rel* 2010;141:320–7.
12. Mueller SN, Tian S, DeSimone JM. Rapid and persistent delivery of antigen by lymph node targeting PRINT nanoparticle vaccine carrier to promote humoral immunity. *Mol Pharm* 2015;12:1356–65.
13. Galloway AL, Murphy A, DeSimone JM, Di J, Herrmann JP, Hunter ME, Kindig JP, Malinoski FJ, Rumley MA, Stoltz D, Templeman T, Hubby B. Development of a nanoparticle-based influenza vaccine using the PRINT® technology. *Nanomed Nanotechnol Biol Med* 2013;9:523–31.
14. Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O’Neil CP, Lee LK, Swartz MA, Hubbell JA. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. *Nat Biotechnol* 2007;25:1159–64.
15. Skwarczynski M, Toth I. Peptide-based subunit nanovaccines. *Curr Drug Del* 2011;8:282–9.
16. Beningo KA, Wang Y-I. Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target. *J Cell Sci* 2002;115:849–56.
17. Hu CMJ, Zhang L, Aryal S, Cheung C. Erythrocyte membrane-camouflaged polymeric nanoparticles as a biomimetic delivery platform. *Proc Natl Acad Sci USA* 2011;108:10980–5.
18. Doshi N, Zahr AS, Bhaskar S, Lahann J, Mitragotri S. Red blood cell-mimicking synthetic biomaterial particles. *Proc Natl Acad Sci USA* 2009;106:21495–9.
19. Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA. In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. *J Control Rel* 2006;112:26–34.
20. Petrovsky N, Cooper PD. Carbohydrate-based immune adjuvants. *Expert Rev Vaccin* 2011;10:523–37.
21. Lopez-Quintela MA. Synthesis of nanomaterials in microemulsions: formation mechanisms and growth control. *Curr Opin Colloid Interface Sci* 2003;8:137–44.
22. Mason TG, Wilking JN, Meleson K, Chang CB, Graves SM. Nanoemulsions: formation, structure, and physical properties. *J Phys Condens Matter* 2006;18:R635–66.
23. Capek I. Preparation of metal nanoparticles in water-in-oil (w/o) microemulsions. *Adv Colloid Interface Sci* 2004;110:49–74.
24. Tadros T, Izquierdo P, Esquena J, Solans C. Formation and stability of nano-emulsions. *Adv Colloid Interface Sci* 2004;108–109:303–18.
25. Aucouturier J, Dupuis L, Ganne V. Adjuvants designed for veterinary and Hum Vaccin. *Vaccine* 2001;19:2666–72.
26. Freund J, Casals J, Hosmer EP. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. *Proc Soc Exp Biol Med* 1937;37:509–13.
27. Herbert WJ. The mode of action of mineral-oil emulsion adjuvants on antibody production in mice. *Immunology* 1968;14:301–18.
28. Tefit JN, Serra V. Outlining novel cellular adjuvant products for therapeutic vaccines against cancer. *Expert Rev Vaccin* 2011;10:1207–20.
29. Ott G, Barchfeld GL, Chernoff D, Radhakrishnan R, van Hoogevest P, Van Nest G. MF59. Design and evaluation of a safe and potent adjuvant for Hum Vaccin. *Pharm Biotechnol* 1995;6:277–96.
30. Kahn JO, Sinangil F, Baenziger J, Murcar N, Wynne D, Coleman RL, Steimer KS, Dekker CL, Chernoff D. Clinical and immunologic responses to human immunodeficiency virus (HIV) type 1SF2 gpl20 subunit vaccine combined with MF59 adjuvant with or without muramyl tripeptide dipalmitoyl phosphatidylethanolamine in non-HIV-infected human volunteers. *J Infect Dis* 1994;170:1288–91.
31. Calabro S, Tritto E, Pezzotti A, Taccone M, Muzzi A, Bertholet S, De Gregorio E, O’Hagan DT, Baudner B, Seubert A. The adjuvant effect of MF59 is due to the oil-in-water emulsion formulation, none of the individual components induce a comparable adjuvant effect. *Vaccine* 2013;31:3363–9.

32. El Sahly H. MF59TM as a vaccine adjuvant: a review of safety and immunogenicity. *Expert Rev Vaccin* 2010;9:1135–41.
33. Brito LA, Chan M, Shaw CA, Hekele A, Carsillo T, Schaefer M, Archer J, Seubert A, Otten GR, Beard CW, Dey AK, Lilja A, Valiante NM, Mason PW, Mandl CW, Barnett SW, Dormitzer PR, Ulmer JB, Singh M, O'Hagan DT, Geall AJ. A cationic nanoemulsion for the delivery of next-generation RNA vaccines. *Mol Ther* 2014;22:2118–29.
34. O'Hagan DT, Rappuoli R, De Gregorio E, Tsai T, Del Giudice G. MF59 adjuvant: the best insurance against influenza strain diversity. *Expert Rev Vaccin* 2011;10:447–62.
35. Nohynek H, Jokinen J, Partinen M, Vaarala O, Kirjavainen T, Sundman J, Himanen SL, Hublin C, Julkunen I, Olsén P, Saarenpää-Heikkilä O, Kilpi T. AS03 Adjuvanted AH1N1 vaccine associated with an abrupt increase in the incidence of childhood narcolepsy in Finland. *PLoS One* 2012;7:e33536.
36. Partinen M, Saarenpää-Heikkilä O, Ilveskloski I. Increased incidence and clinical picture of childhood narcolepsy following the 2009 H1N1 Pandemic Vaccination Campaign in Finland. *PLoS One* 2012;7:e33723.
37. Yamamura Y, Azuma I, Sugimura K, Yamawaki M, Uemiya M. Adjuvant activity of 6-0-mycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine. *Gann* 1976;67:867–77.
38. Uehara A, Yang S, Fujimoto Y, Fukase K, Kusumoto S, Shibata K, Sugawara S, Takada H. Muramyl-dipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively in human monocytic cells in culture. *Cell Microbiol* 2005;7:53–61.
39. Takada H, Uehara A. Enhancement of TLR-mediated innate immune responses by peptidoglycans through NOD signaling. *Curr Pharm Design* 2006;12:4163–72.
40. Valensi JP, Carlson JR, Van Nest GA. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. *J Immunol* 1994;153:4029–39.
41. Ott G, Barchfeld GL, Van Nest G. Enhancement of humoral response against human influenza vaccine with the simple submicron oil/water emulsion adjuvant MF59. *Vaccine* 1995;13:1557–62.
42. Leroux-Roels G, Leroux-Roels I, Clement F, Ofori-Anyinam O, Lievens M, Jongert E, Moris P, Ballou WR, Cohen J. Evaluation of the immune response to RTS,S/AS01 and RTS,S/AS02 adjuvanted vaccines: randomized, double-blind study in malaria-naïve adults. *Hum Vaccin Immunotherap* 2014;10:2211–9.
43. Myc A, Kukowska-Latallo JF, Bielinska AU, Cao P, Myc PP, Janczak K, Sturm TR, Grabinski MS, Landers JJ, Young KS, Chang J, Hamouda T, Olszewski MA, Baker JJR. Development of immune response that protects mice from viral pneumonitis after a single intranasal immunization with influenza A virus and nanoemulsion. *Vaccine* 2003;21:3801–14.
44. Hamouda T, Chepurinov A, Mank N, Knowlton J, Chepurnova T, Myc A, Sutcliffe J, Baker JJ. Efficacy, immunogenicity and stability of a novel intranasal nanoemulsion-adjuvanted influenza vaccine in a murine model. *Hum Vaccin* 2010;6:585–94.
45. Myc A, Kukowska-Latallo JF, Smith DM, Passmore C, Pham T, Wong P, Bielinska AU, Baker JJR. Nanoemulsion nasal adjuvant W805EC induces dendritic cell engulfment of antigen-primed epithelial cells. *Vaccine* 2013;31:1072–9.
46. Wong PT, Wang SH, Ciotti S, Makidon PE, Smith DM, Fan Y, Schuler IVCF, Baker JJR. Formulation and characterization of nanoemulsion intranasal adjuvants: effects of surfactant composition on mucosal adhesion and immunogenicity. *Mol Pharm* 2014;11:531–44.
47. Stanberry LR, Simon JK, Johnson C, Robinson PL, Morry J, Flack MR, Gracon S, Myc A, Hamouda T, Baker JJR. Safety and immunogenicity of a novel nanoemulsion mucosal adjuvant W805EC combined with approved seasonal influenza antigens. *Vaccine* 2012;30:307–16.
48. Wong PT, Leroueil PR, Smith DM, Ciotti S, Bielinska AU, Janczak KW, Mullen CH, Groom JV 2nd, Taylor EM, Passmore C, Makidon PE, O'Konek JJ, Myc A, Hamouda T, Baker JR Jr. Formulation, high throughput in vitro screening and in vivo functional characterization of nanoemulsion-based intranasal vaccine adjuvants. *PLoS One* 2015;10:e0126120.
49. Orzechowska BU, Kukowska-Latallo JF, Coulter AD, Szabo Z, Gamian A, Myc A. Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells. *Vaccine* 2015;33:2289–96.

50. Gresham PA, Barnett M, Smith SV, Schneider R. Use of a sustained-release multiple emulsion to extend the period of radio protection conferred by cysteamine. *Nature* 1971;234:149–50.
51. Leclercq SY, dos Santos RMM, Macedo LB, Campos PC, Ferreira TC, de Almeida JG, Seniuk JGT, Serakides R, Silva-Cunha A, Fialho SL. Evaluation of water-in-oil-in-water multiple emulsion and microemulsion as potential adjuvants for immunization with rabies antigen. *Eur J Pharm Sci* 2011;43:378–85.
52. Jiao J, Burgess DJ. . In: Aserin A, editor. Multiple emulsions: technology and applications. John Wiley & Sons, Inc; 2008. p. 1–27.
53. Prashant CK, Bhat M, Srivastava SK, Saxena A, Kumar M, Singh A, Samim M, Ahmad FJ, Dinda AK. Fabrication of nanoadjuvant with poly- ϵ -caprolactone (PCL) for developing a single-shot vaccine providing prolonged immunity. *Int J Nanomed* 2014;9:937–50.
54. Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, McKenzie IFC, Plebanski M. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J Immunol* 2004;173:3148–54.
55. Mottram PL, Leong D, Crimeen-Irwin B, Gloster S, Xiang SD, Meanger J, Ghildyal R, Vardaxis N, Plebanski M. Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus. *Mol Pharm* 2007;4:73–84.
56. Akagi T, Kaneko T, Kida T, Akashi M. Preparation and characterization of biodegradable nanoparticles based on poly(γ -glutamic acid) with 1-phenylalanine as a protein carrier. *J Control Rel* 2005;108:226–36.
57. Uto T, Wang X, Sato K, Haraguchi M, Akagi T, Akashi M, Baba M. Targeting of antigen to dendritic cells with poly(γ -glutamic acid) nanoparticles induces antigen-specific humoral and cellular immunity. *J Immunol* 2007;178:2979–86.
58. Li H, Li Y, Jiao J, Hu HM. Alpha-alumina nanoparticles induce efficient autophagy-dependent cross-presentation and potent antitumour response. *Nature Nanotechnol* 2011;6:645–50.
59. Frey A, Neutra MR, Robey FA. Peptomer aluminum oxide nanoparticle conjugates as systemic and mucosal vaccine candidates: synthesis and characterization of a conjugate derived from the C4 domain of HIV-1 MNGp120. *Bioconjug Chem* 1997;8:424–33.
60. Frey A, Mantis N, Kozlowski PA, Quayle AJ, Bajardi A, Perdomo JJ, Robey FA, Neutra MR. Immunization of mice with peptomers covalently coupled to aluminum oxide nanoparticles. *Vaccine* 1999;17:3007–19.
61. Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 1982;298:347–50.
62. Schirmbeck R, Deml L, Melber K, Wolf H, Wagner R, Reimann J. Priming of class I-restricted cytotoxic T lymphocytes by vaccination with recombinant protein antigens. *Vaccine* 1995;13:857–65.
63. Martin SJ, Vyakarnam A, Cheingsong-Popov R, Callow D, Jones KL, Senior JM, Adams SE, Kingsman AJ, Matear P, Gotch FM. Immunization of human HIV-seronegative volunteers with recombinant p17/p24:Ty virus-like particles elicits HIV-1 p24-specific cellular and humoral immune responses. *AIDS* 1993;7:1315–23.
64. Pimentel T, Yan Z, Jeffers SA, Peptide. Nanoparticles as novel immunogens: design and analysis of a prototypic severe acute respiratory syndrome vaccine. *Chem Biol Drug Design* 2009;73:53–61.
65. Kaba SA, McCoy ME, Doll TAPF, Brando C, Guo Q, Dasgupta D, Yang Y, Mittelholzer C, Spaccapelo R, Crisanti A, Burkhard P, Lanar DE. Protective antibody and CD8+ T-cell responses to the *Plasmodium falciparum* circumsporozoite protein induced by a nanoparticle vaccine. *PLoS One* 2012;7:e48304.
66. Kaba SA, Brando C, Guo Q, Mittelholzer C, Raman S, Tropel D, Aebi U, Burkhard P, Lanar DE. A non-adjuvanted polypeptide nanoparticle vaccine confers long-lasting protection against rodent malaria. *J Immunol* 2009;183:7268–77.
67. Boato F, Thomas RM, Ghasparian A, Freund Renard A, Moehle K, Robinson JA. Synthetic virus-like particles from self-assembling coiled-coil lipopeptides and their use in antigen display to the immune system. *Angew Chem* 2007;119:9173–6.
68. Chen J, Pompano RR, Santiago FW, Maillat L, Sciammas R, Sun T, Han H, Topham DJ, Chong AS, Collier JH. The use of self-adjuvanting nanofiber vaccines to elicit high-affinity B cell responses to peptide antigens without inflammation. *Biomaterials* 2013;34:8776–85.

69. Rudra JS, Tian YF, Jung JP, Collier JH. A self-assembling peptide acting as an immune adjuvant. *Proc Natl Acad Sci USA* 2010;107:622–7.
70. Nishimura A, Hayakawa T, Yamamoto Y, Hamori M, Tabata K, Seto K, Shibata N. Controlled release of insulin from self-assembling nanofiber hydrogel, PuraMatrix (TM): application for the subcutaneous injection in rats. *Eur J Pharm Sci* 2012;45:1–7.
71. Grenfell RFQ, Shollenberger LM, Samli EF, Harn DA. Vaccine self-assembling immune matrix is a new delivery platform that enhances immune responses to recombinant HBsAg in mice. *Clin Vaccine Immunol* 2015;22:336–43.
72. Chesson CB, Huelsmann EJ, Lacey AT, Kohlhapp FJ, Webb MF, Nabatiyan A, Zloza A, Rudra JS. Antigenic peptide nanofibers elicit adjuvant-free CD8(+) T cell responses. *Vaccine* 2014;32:1174–80.
73. Allison AC, Gregoriadis G. Liposomes as immunological adjuvants. *Nature* 1974;252:252–1252.
74. Ambrosch F, Wiedermann G, Jonas S, Althaus B, Finkel B, Glick R, Herzog C. Immunogenicity and protectivity of a new liposomal hepatitis A vaccine. *Vaccine* 1997;15:1209–13.
75. Zhengrong C, Su-Ji H, Dileep Padinjarae Vangasseri a, Leaf H. Immunostimulation mechanism of LPD nanoparticle as a vaccine carrier. *Mol Pharm* 2004;2:22–8.
76. Cui Z, Han S-J, Huang L. Coating of mannan on LPD particles containing HPV E7 peptide significantly enhances immunity against HPV-positive tumor. *Pharm Res* 2004;21:1018–25.
77. Hanson MC, Crespo MP, Abraham W, Moynihan KD, Szeto GL, Chen SH, Melo MB, Mueller S, Irvine DJ. Nanoparticulate STING agonists are potent lymph node-targeted vaccine adjuvants. *J Clin Invest* 2015;125:2532–46.
78. Gluck R. Adjuvant activity of immunopotentiating reconstituted influenza virosomes (IRIVs). *Vaccine* 1999;17:1782–7.
79. Bungener L, Serre K, Bijl L, Leserman L, Wilschut J, Daemen T, Machy P. Virosome-mediated delivery of protein antigens to dendritic cells. *Vaccine* 2002;20:2287–95.
80. Sharma S, Mukkur TK, Benson HA, Chen Y. Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems. *J Pharm Sci* 2009;98:812–43.
81. Moser C, Muller M, Kaeser MD, Weydemann U, Amacker M. Influenza virosomes as vaccine adjuvant and carrier system. *Expert Rev Vaccin* 2013;12:779–91.
82. Liu H, de Vries-Idema J, Ter Veer W, Wilschut J, Huckriede A. Influenza virosomes supplemented with GPI-0100 adjuvant: a potent vaccine formulation for antigen dose sparing. *Med Microbiol Immunol* 2014;203:47–55.
83. Akagi T, Watanabe K, Kim H, Akashi M. Stabilization of polyion complex nanoparticles composed of poly(amino acid) using hydrophobic interactions. *Langmuir* 2010;26:2406–13.
84. Cherif MS, Shuaibu MN, Kurosaki T, Helegbe GK, Kikuchi M, Yanagi T, Tsuboi T, Sasaki H, Hirayama K. Immunogenicity of novel nanoparticle-coated MSP-1 C-terminus malaria DNA vaccine using different routes of administration. *Vaccine* 2011;29:9038–50.
85. Zhao L, Seth A, Wibowo N, Zhao CX, Mitter N, Yu C, Middelberg AP. Nanoparticle vaccines. *Vaccine* 2014;32:327–37.
86. Henriksen-Lacey M, Christensen D, Bramwell VW, Lindenstrom T, Agger EM, Andersen P, Perrie Y. Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. *J Control Rel* 2010;145:102–8.
87. Uto T, Akagi T, Akashi M, Baba M. Induction of potent adaptive immunity by the novel polyion complex nanoparticles. *Clin Vaccine Immunol* 2015;22:578–85.
88. Kodama Y, Nakamura T, Kurosaki T, Egashira K, Mine T, Nakagawa H, Muro T, Kitahara T, Higuchi N, Sasaki H. Biodegradable nanoparticles composed of dendrigraft poly-L-lysine for gene delivery. *Eur J Pharm Biopharm* 2014;87:472–9.
89. Chesko J, Kazzaz J, Ugozzoli M, O'Hagan DT, Singh M. An investigation of the factors controlling the adsorption of protein antigens to anionic PLG microparticles. *J Pharm Sci* 2005;94:2510–9.
90. Skwarczynski M, Zaman M, Toth I. . In: Kastin AJ, editor. *Handbook of biologically active peptides*. Elsevier Inc; 2013. p. 571–9.

91. Skwarczynski M, Zaman M, Urbani CN, Lin IC, Jia Z, Batzloff MR, Good MF, Monteiro MJ, Toth I. Polyacrylate dendrimer nanoparticles: a self-adjuvanting vaccine delivery system. *Angew Chem Int Ed* 2010;49:5742–5.
92. Liu T-Y, Hussein WM, Jia Z, Ziora ZM, McMillan NAJ, Monteiro MJ, Toth I, Skwarczynski M. Self-adjuvanting polymer-peptide conjugates as therapeutic vaccine candidates against cervical cancer. *Bio-macromolecules* 2013;14:2798–806.
93. Zhong W, Skwarczynski M, Toth I. Lipid core peptide system for gene, drug, and vaccine delivery. *Aust J Chem* 2009;62:956–67.
94. Abdel-Aal A-BM, Batzloff MR, Fujita Y, Barozzi N, Faria A, Simerska P, Moyle PM, Good MF, Toth I. Structure-activity relationship of a series of synthetic lipopeptide self-adjuvanting group A streptococcal vaccine candidates. *J Med Chem* 2008;51:167–72.
95. Phillipps KSM, Wykes MN, Liu XQ, Brown M, Blanchfield J, Toth I. A novel synthetic adjuvant enhances dendritic cell function. *Immunology* 2009;128:e582–8.
96. Skwarczynski M, Dougall AM, Khoshnejad M, Chandrudu S, Pearson MS, Loukas A, Toth I. Peptide-based subunit vaccine against hookworm infection. *PLoS One* 2012;7:e46870.
97. Kensil CR, Soltysik S, Wheeler DA, Wu JY. Structure/function studies on QS-21, a unique immunological adjuvant from *Quillaja saponaria*. *Adv Exp Med Biol* 1996;404:165–72.
98. Kensil CR, Wu JY, Soltysik S. Structural and immunological characterization of the vaccine adjuvant QS-21. *Pharm Biotechnol* 1995;6:525–41.
99. Meraldi V, Romero JF, Kensil C, Corradin G. A strong CD8+ T cell response is elicited using the synthetic polypeptide from the C-terminus of the circumsporozoite protein of *Plasmodium berghei* together with the adjuvant QS-21: quantitative and phenotypic comparison with the vaccine model of irradiated sporozoites. *Vaccine* 2005;23:2801–12.
100. Sun HX, Xie Y, Ye YP. Advances in saponin-based adjuvants. *Vaccine* 2009;27:1787–96.
101. Morein B, Lövgren K, Höglund S, Sundquist B. The ISCOM: an immunostimulating complex. *Immunol Today* 1987;8:333–8.
102. Cox JC, Sjolander A, Barr IG. ISCOMs and other saponin based adjuvants. *Adv Drug Del. Rev.* 1998;32:247–71.
103. Morein B, Bengtsson KL. Functional aspects of iscoms. *Immunol Cell Biol* 1998;76:295–9.
104. Morein B, Villacres-Eriksson M, Lovgren-Bengtsson K. Iscom, a delivery system for parenteral and mucosal vaccination. *Dev Biol Stand* 1998;92:33–9.
105. Cooper PD, Barclay TG, Ginic-Markovic M, Gerson AR, Petrovsky N. Inulin isoforms differ by repeated additions of one crystal unit cell. *Carbohydr Polym* 2014;103:392–7.
106. Cooper PD, Barclay TG, Ginic-Markovic M, Petrovsky N. The polysaccharide inulin is characterized by an extensive series of periodic isoforms with varying biological actions. *Glycobiology* 2013;23:1164–74.
107. Cooper PD, Petrovsky N. Delta inulin: a novel, immunologically active, stable packing structure comprising beta-D-[2->1] poly(fructo-furanosyl) alpha-D-glucose polymers. *Glycobiology* 2011;21:595–606.
108. Cooper PD, Rajapaksha KH, Barclay TG, Ginic-Markovic M, Gerson AR, Petrovsky N. Inulin crystal initiation via a glucose-fructose cross-link of adjacent polymer chains: atomic force microscopy and static molecular modelling. *Carbohydr Polym* 2015;117:964–72.
109. Honda-Okubo Y, Saade F, Petrovsky N. Advax, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses. *Vaccine* 2012;30:5373–81.
110. Layton RC, Petrovsky N, Gigliotti AP, Pollock Z, Knight J, Donart N, Pyles J, Harrod KS, Gao P, Koster F. Delta inulin polysaccharide adjuvant enhances the ability of split-virion H5N1 vaccine to protect against lethal challenge in ferrets. *Vaccine* 2011;29:6242–51.
111. Lobigs M, Pavy M, Hall RA, Lobigs P, Cooper P, Komiyama T, Toriniwa H, Petrovsky N. An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. *J Gen Virol* 2010;91:1407–17.

112. Larena M, Prow NA, Hall RA, Petrovsky N, Lobigs M. JE-ADVAX vaccine protection against Japanese encephalitis virus mediated by memory B Cells in the absence of CD8+ T cells and pre-exposure neutralizing antibody. *J Virol* 2013;87:4395–402.
113. Petrovsky N, Larena M, Siddharthan V, Prow NA, Hall RA, Lobigs M, Morrey J. An inactivated cell culture Japanese encephalitis vaccine (JE-ADVAX) formulated with delta inulin adjuvant provides robust heterologous protection against West Nile encephalitis via cross-protective memory B cells and neutralizing antibody. *J Virol* 2013;87:10324–33.
114. Saade F, Honda-Okubo Y, Trec S, Petrovsky N. A novel hepatitis B vaccine containing Advax, a polysaccharide adjuvant derived from delta inulin, induces robust humoral and cellular immunity with minimal reactogenicity in preclinical testing. *Vaccine* 2013;31:1999–2007.
115. Cristillo AD, Ferrari MG, Hudacik L, Lewis B, Galmin L, Bowen B, Thompson D, Petrovsky N, Markham P, Pal R. Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. *J Gen Virol* 2011;92:128–40.
116. Feinen B, Petrovsky N, Verma A, Merkel TJ. Advax-adjuvanted recombinant protective antigen provides protection against inhalational anthrax that is further enhanced by addition of murabutide adjuvant. *Clin Vaccine Immunol* 2014;21:580–6.
117. Honda-Okubo Y, Barnard D, Ong CH, Peng BH, Tseng CT, Petrovsky N. Severe acute respiratory syndrome-associated coronavirus vaccines formulated with delta inulin adjuvants provide enhanced protection while ameliorating lung eosinophilic immunopathology. *J Virol* 2015;89:2995–3007.
118. Calderon-Gonzalez R, Marradi M, Garcia I, Petrovsky N, Alvarez-Dominguez C. Novel nanoparticle vaccines for listeriosis. *Hum Vaccin Immunotherap* 2015;. doi.org/10.1080/21645515.2015.1063756.
119. Rodriguez-Del Rio E, Marradi M, Calderon-Gonzalez R, Frande-Cabanes E, Penades S, Petrovsky N, Alvarez-Dominguez C. A gold glyco-nanoparticle carrying a listeriolysin O peptide and formulated with Advax delta inulin adjuvant induces robust T-cell protection against listeria infection. *Vaccine* 2015;33:1465–73.
120. Eckersley AM, Petrovsky N, Kinne J, Wernery R, Wernery U. Improving the dromedary antibody response: the hunt for the ideal camel adjuvant. *J Camel Practice Res* 2011;18:35–46.
121. Gordon DL, Sajkov D, Woodman RJ, Honda-Okubo Y, Cox MM, Heinzl S, Petrovsky N. Randomized clinical trial of immunogenicity and safety of arecombinantH1N1/2009 pandemic influenza vaccine containing Advax polysaccharide adjuvant. *Vaccine* 2012;30:5407–16.
122. Gordon D, Kelley P, Heinzl S, Cooper P, Petrovsky N. Immunogenicity and safety of Advax, a novel polysaccharide adjuvant based on delta inulin, when formulated with hepatitis B surface antigen: a randomized controlled Phase 1 study. *Vaccine* 2014;32:6469–77.
123. Heddle R, Russo P, Petrovsky N, Hanna R, Smith A. Immunotherapy-2076. A controlled study of delta inulin-adjuvanted honey bee venom immunotherapy. *World Allergy Organ J* 2013;6:P158–P1158.
124. Li L, Honda-Okubo Y, Li C, Sajkov D, Petrovsky N. Delta inulin adjuvant enhances plasmablast generation, expression of activation-induced cytidine deaminase and B-cell affinity maturation in human subjects receiving seasonal influenza vaccine. *PLoS One* 2015;10:e0132003.
125. Honda-Okubo Y, Kolpe A, Li L, Petrovsky N. A single immunization with inactivated H1N1 influenza vaccine formulated with delta inulin adjuvant (Advax) overcomes pregnancy-associated immune suppression and enhances passive neonatal protection. *Vaccine* 2014;32:4651–9.
126. Honda-Okubo Y, Ong CH, Petrovsky N. Advax delta inulin adjuvant overcomes immune immaturity in neonatal mice thereby allowing single-dose influenza vaccine protection. *Vaccine* 2015;33:4892–900.
127. Bielefeldt-Ohmann H, Prow NA, Wang W, Tan CS, Coyle M, Douma A, Hobson-Peters J, Kidd L, Hall RA, Petrovsky N. Safety and immunogenicity of a delta inulin-adjuvanted inactivated Japanese encephalitis virus vaccine in pregnant mares and foals. *Vet Res* 2014;45:130–9.
128. Murugappan S, Frijlink HW, Petrovsky N, Hinrichs WL. Enhanced pulmonary immunization with aerosolized inactivated influenza vaccine containing delta inulin adjuvant. *Eur J Pharm Sci* 2014;66:118–22.
129. Kawakami S, Sato A, Nishikawa M, Yamashita F, Hashida M. Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. *Gene Ther* 2000;7:292–9.

130. Toda S, Ishii N, Okada E, Kusakabe KI, Arai H, Hamajima K, Gorai I, Nishioka K, Okuda K. HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-gamma antibody. *Immunology* 1997;92:111–7.
131. Lu Y, Kawakami S, Yamashita F, Hashida M. Development of an antigen-presenting cell-targeted DNA vaccine against melanoma by mannosylated liposomes. *Biomaterials* 2007;28:3255–62.
132. Jain S, Singh P, Mishra V, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B. *Immunol Lett* 2005;101:41–9.
133. Jain S, Vyas SP. Mannosylated niosomes as carrier adjuvant system for topical immunization. *J Pharm Pharmacol* 2005;57:1177–84.
134. Arca HC, Gunbeyaz M, Senel S. Chitosan-based systems for the delivery of vaccine antigens. *Expert Rev Vaccin* 2009;8:937–53.
135. Prego C, Paolicelli P, Diaz B, Vicente S, Sanchez A, Gonzalez-Fernandez A, Alonso MJ. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. *Vaccine* 2010;28:2607–14.
136. Baudner BC, Giuliani MM, Verhoef JC, Rappuoli R, Junginger HE, Giudice GD. The concomitant use of the LTK63 mucosal adjuvant and of chitosan-based delivery system enhances the immunogenicity and efficacy of intranasally administered vaccines. *Vaccine* 2003;21:3837–44.
137. Lameiro MH, Malpique R, Silva AC, Alves PM, Melo E. Encapsulation of adenoviral vectors into chitosan-bile salt microparticles for mucosal vaccination. *J Biotechnol* 2006;126:152–62.
138. Vosika GJ, Cornelius DA, Gilbert CW, Sadlik JR, Bennek JA, Doyle A, Hertsgaard D. Phase I trial of ImmTher, a new liposome-incorporated lipophilic disaccharide tripeptide. *J Immunother* 1991;10:256–66.
139. Vosika GJ, Cornelius DA, Bennek JA, Sadlik JR, Gilbert CW. Immunologic and toxicologic study of disaccharide tripeptide glycerol dipalmitoyl: a new lipophilic immunomodulator. *Mol Biother* 1990;2:50–6.
140. Borges O, Cordeiro-da-Silva A, Tavares J, Santarem N, de Sousa A, Borchard G, Junginger HE. Immune response by nasal delivery of hepatitis B surface antigen and codelivery of a CpG ODN in alginate coated chitosan nanoparticles. *Eur J Pharm Biopharm* 2008;69:405–16.
141. Liu H, Moynihan KD, Zheng Y, Szeto GL, Li AV, Huang B, Van Egeren DS, Park C, Irvine DJ. Structure-based programming of lymph-node targeting in molecular vaccines. *Nature* 2014;507:519–22.
142. Kumar S, Tummala H. Development of soluble inulin microparticles as a potent and safe vaccine adjuvant and delivery system. *Mol Pharm* 2013;10:1845–53.
143. Lutsiak ME, Kwon GS, Samuel J. Biodegradable nanoparticle delivery of a Th2-biased peptide for induction of Th1 immune responses. *J Pharm Pharmacol* 2006;58:739–47.
144. Wang C, Ge Q, Ting D, Nguyen D, Shen H-R, Chen J, Eisen HN, Heller J, Langer R, Putnam D. Molecularly engineered poly(ortho ester) microspheres for enhanced delivery of DNA vaccines. *Nature Mater* 2004;3:190–6.
145. Rehor A, Hubbell JA, Tirelli N. Oxidation-sensitive polymeric nanoparticles. *Langmuir* 2005;21:411–7.
146. Parrott MC, Finniss M, Luft JC, Pandya A, Gullapalli A, Napier ME, DeSimone JM. Incorporation controlled release of silyl ether prodrugs from PRINT nanoparticles. *J Am Chem Soc* 2012;134:7978–82.