Protein and Peptide Nanocluster Vaccines

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Contents

Abstract Recombinant protein- and peptide-based vaccines can deliver large amounts of specific antigens for tailored immune responses. One class of these are protein and peptide nanoclusters (PNCs), which are made entirely from the cross-linked antigen. PNCs leverage the inherent immunogenicity of nanoparticulate

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antigens while minimizing the use of excipients normally used to create them. In this chapter, we discuss PNC fabrication methods, immunostimulatory properties of nanoclusters observed in vitro and in vivo, and protective benefits of PNC vaccines against influenza and cancer mouse models. We conclude with an outlook on future studies of PNCs and PNC design strategies, as well as their use in future vaccine formulations.

1 Introduction

Generating a successful immune response involves not only delivering antigen to the immune system but presenting that antigen in an immunostimulatory context. To accomplish this, the subunit vaccine nanoparticle design generally follows two strategies: (i) internal encapsulation of antigen and/or (ii) native antigen display on a particle surface. In the first strategy, the antigen is blended into the nanoparticulate polymer matrix. Interaction of the nanoparticle with the immune cells, together with the controlled release of the antigen from the nanoparticle, result in a stronger stimulation of the immune system compared to the soluble antigen (Gregory et al. 2013; Singh 2007; Liu et al. 2016). In the second strategy, the antigen is attached to the nanoparticle surface instead of being blended into the polymer matrix. This enables interaction of the particle with immune cells in a manner that leads to a superior immune response. For example, virus-like particles display multimeric epitopes in native conformations, enhancing the quality and quantity of the humoral immune response (Jegerlehner et al. 2002; Roldão et al. 2010). Protein and peptide nanocluster (PNC) vaccines capture the advantages of both these strategies. PNCs are composed almost entirely of antigen protein or peptide. These nanoclusters deliver large quantities of the antigen to antigen-presenting cells (APCs) and also display epitopes on their surface, which results in a better immune response. Unlike other vaccine nanoparticles, PNCs are made entirely of biodegradable, crosslinked antigen, minimizing the possibility of off-target immune responses. Furthermore, the peptide degradation products of protein nanoclusters are capable of contributing to an immune response, through their presentation on the major histocompatibility (MHC) proteins (Tsoras and Champion 2018). The lack of a lipid bilayer membrane in PNCs, such as that found in VLP-based vaccines, reduces manufacturing complexity and also enhances the resilience of PNCs to osmotic stresses brought on by cold chain-independent storage, a desirable property for vaccine transportation to the developing world (Chang et al. 2018).

Similar protein-based nanoparticle vaccines have been developed that use self-assembly motifs to create geometrically well-defined repeats of antigen. Kanekiyo et al. fused influenza hemagglutinin (HA) to a 24-mer self-assembling ferritin core, which resulted in 20 nm nanoparticles that could protect against an influenza challenge (Kanekiyo et al. 2013). The self-assembling protein nanoparticles (SAPNs) developed by the Burkhard group also display between 20 and 60 copies of fused protein antigens (Karch et al. 2018). One concern with these designs

is the immunogenicity of the self-assembly tags. Kanekiyo et al. showed that immunization with HA-ferritin nanoparticles did not induce an immune response to endogenous host ferritin, but antibodies against the core ferritin were generated. Immune responses against the self-assembly motif in these particles could preclude strong antigen-specific responses because of original antigenic sin. This phenomenon, in which the immune system preferentially generates antibodies to epitopes previously encountered instead of new ones, needs to be addressed specifically in the case of immunizations requiring multiple boosts (Murphy et al. 2012).

Since PNC vaccines can direct nanoparticle formation without the use of an extra self-assembly tag on the antigen, PNC vaccines possess reduced chances of stimulating an off-target immune response (Willett et al. 2004). However, a robust and high-yield method for nanocluster formation must be established in the absence of engineered self-assembly. Our lab has found that desolvation is a simple, yet efficient method for generating protein and PNC. Based on an assortment of results obtained from the use of model and disease-specific proteins and peptides as antigens, PNCs have proven to be effective vaccines, inducing robust humoral and cellular responses in mice and protecting against lethal viral challenges.

2 Nanoclusters by Desolvation

Desolvated PNCs are formed entirely from protein or peptide by the solvent-directed assembly (Weber et al. 2000). In desolvation, an unfavorable solvent is introduced into a protein solution to increase solute-solute interactions, causing proteins or peptides in solution to coalesce into nanoparticles (Fig. 1).

Both proteins and small peptides can be desolvated into PNCs, depending on the specific antigens of interest. In some instances, immune responses to only particular domains of a protein are desired. In the case of the influenza hemagglutinin (HA) protein, the variable head region is immunodominant, while the conserved stalk region is harder to raise antibodies (Zhang et al. 2019). Antibodies against the HA stalk are cross-protective against multiple influenza strains (Kallewaard et al. 2016; Krammer and Palese 2013), and vaccine nanoparticles containing only the stalk region of HA are one strategy for eliciting those antibodies specifically (Deng et al. 2018a, b). In cancer vaccines, the antigen is usually of host origin and contains many non-immunogenic epitopes. In these cases, designing an immunogenic antigen requires enriching for the immunodominant epitopes (Li et al. 2014). Combined with the fact that T cell responses to cancer vaccines are essential, minimal peptide epitopes are an ideal antigen for cancer PNC vaccines (Tsoras and Champion 2018). In general, MHC I-restricted presentation of peptides to T cells requires least eight amino acids in length, while MHC II-restricted presentation requires peptides of at least 13 amino acids (Murphy et al. 2012). Peptides smaller than this length are not recommended as antigens.

Following antigen identification, a solvent must be selected. For large protein antigens, this is typically phosphate-buffered saline (PBS), as the goal is to keep the

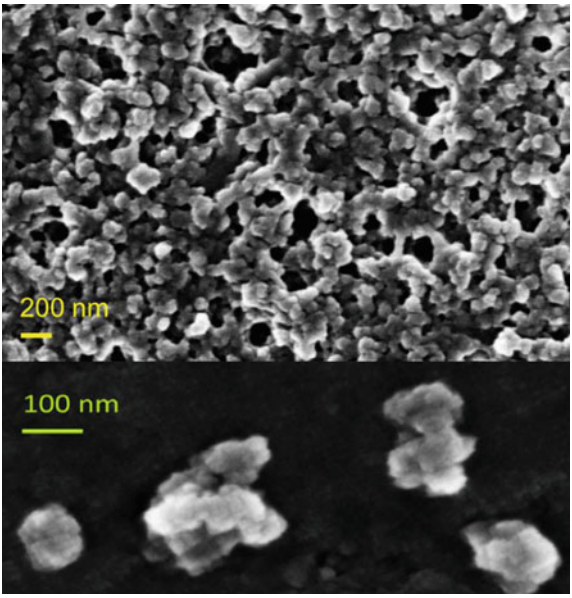

Fig. 1 Scanning electron micrographs of ovalbumin protein nanoclusters (Chang et al. 2016) Reproduced from Chang et al. (2016) with permission from the Royal Society of Chemistry

protein folded and soluble in the solvent and, ultimately, preserve folding during the nanocluster formation process. The solvent pH should avoid the isoelectric point of the protein, as this could cause premature aggregation. Solution pH is also important for antigens that undergo conformational changes in the endosome. Many viral coat proteins responsible for facilitating endosomal escape undergo drastic, pH-dependent conformational changes (Russell et al. 2018; Kirchdoerfer et al. 2018). As a result, low pH can produce conformational antigens not normally found on extracellular viruses, reducing the quality of the induced immune response. For small peptide antigens, there are more choices for solvent as peptide solubility varies widely based on the sequence and often peptide antigens have little or no secondary structure. Hexafluoroisopropanol (HFIP) is most commonly used for peptides as it is used in solid-state peptide synthesis due to its ability to solubilize most peptides, regardless of sequence. This solvent is quite dangerous to work with, however, and extreme care must be taken.

The soluble protein or peptide antigen is then desolvated into nanoclusters by the slow addition of desolvent to antigen under stirring. The thermodynamically unfavorable interaction between the desolvent and the proteins or peptides forces them to cluster together into nanoaggregates of hundreds of nanometers in size (Fig. 2a). The desolvent needs to be miscible with the solvent to allow solvent–desolvent interactions to overcome solvent-protein interactions (Fig. 2b). A similar principle underlies "salting out" of proteins in protein purification, and sodium chloride was

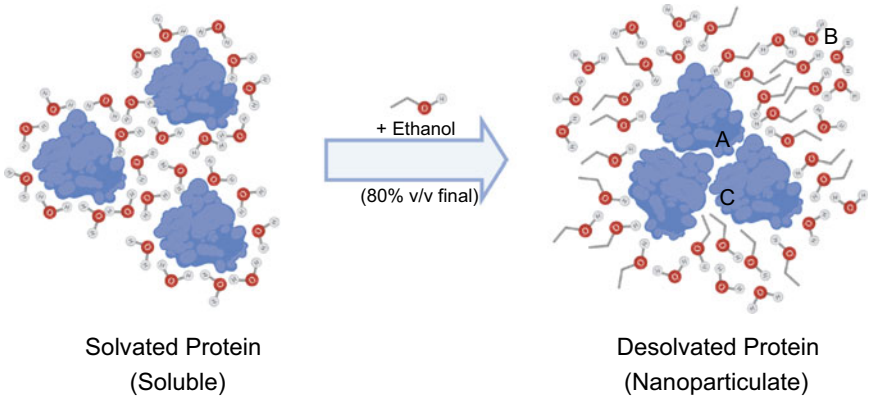

Fig. 2 Desolvation of protein into nanoclusters involves the addition of ethanol, or another water-miscible solvent, to a protein solution. Nanocluster formation relies in part on (**a**) stabilizing protein-protein interactions induced by unfavorable protein-desolvent interactions, and (**b**) solvent sequestration or "salting out" by the desolvent. The desolvent can also stabilize hydrophobic protein domains (**c**), which can lead to the denaturation of surface-exposed epitopes. Figure created with Biorender.com

also used as an early desolvent (Kreuter 1991). For large protein antigens, ethanol is typically used as the desolvent for PBS, while diethyl ether is used with HFIP for peptide antigens. Other desolvents for proteins in PBS include acetone (Langer et al. 2003), acetonitrile (Mohammad-Beigi et al. 2016), methanol (Mohammad-Beigi et al. 2016; Doan and Ghosh 2019), and ethanol/methanol blends (Storp et al. 2012). Desolvent choice has a significant effect on protein nanocluster size (Mohammad-Beigi et al. 2016; Storp et al. 2012). While proteins generally undergo desolvation to a final concentration of 80% ethanol by volume, peptides require a much higher ratio of desolvent to solvent (Tsoras and Champion 2018), possibly owing to their higher solvent accessibility.

Following desolvation, a crosslinker is added to covalently stabilize the nascent nanoparticles and prevent aggregation or disintegration. Glutaraldehyde is a commonly used crosslinker to link primary amines, such as those found in lysines and the N termini of proteins and peptides. While glutaraldehyde has been used in the past to crosslink protein nanoparticles (Estrada et al. 2014; Wang et al. 2014), our work also uses the crosslinker 3,3′-dithiobis[sulfosuccinimidylpropionate], or DTSSP. DTSSP is also an amine-reactive crosslinker but contains a central disulfide bond that has the potential to be reduced once the particle is inside the cell. DTSSP crosslinking of PNCs in the presence of desolvent or pure solvent modestly affects PNC size (Chang et al. 2016), while glutaraldehyde concentration and crosslinking time strongly influence PNC size (Baseer et al. 2019). Though bifunctional crosslinkers are sufficient for stabilizing protein nanoclusters, trifunctional crosslinkers are needed for PNC. This is likely due to the much smaller size and number of reactive groups in peptides compared to larger proteins. Similar to bifunctional crosslinkers, both non-reducible and reducible trifunctional

crosslinkers are available that react with amine or thiols in the peptides. Genipin, a compound derived from the *Genipa americana* fruit, has also been used to crosslink PNCs (Dong et al. 2019). To couple carboxyl groups to amines, EDC/NHS (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide) can also be used to crosslink PNCs. However, the unstable reaction intermediate and incompatibility with phosphate-based buffers, combined with the instability of some proteins at the optimal pH 6 for this reaction make this a less attractive option.

The delivery of properly folded protein antigen is especially desirable in protein nanocluster vaccine design. Desolvation of proteins can lead to denaturation of protein on the surface of the nanocluster (Fig. 2c), which could diminish antigen recognition. In addition to less efficient antigen presentation, exposure of denatured epitopes can alter the protein corona or layer of host proteins that coat the surface of any nanoparticle administered in vivo (Lindman et al. 2007; Fleischer and Payne 2014; Ezzat et al. 2019). To avoid a sub-optimal immune response, we have found that coating protein nanoclusters with an additional layer of protein, either antigen (Wang et al. 2017) or adjuvant (Chang et al. 2017), enhances the immune response. While coating may not be necessary for vaccine nanoclusters made of small antigens or peptides (Tsoras and Champion 2018; Wang et al. 2014), it is useful for presenting conformational antigens to the humoral immune system (Bergtold et al. 2005). Surface antigen display on vaccines is emerging as a trend in other vaccine designs (Gregory et al. 2013; Xiang et al. 2013), as opposed to viewing particulates as mere antigen depots. This shift has been driven by two insights: (1) an immunological understanding that surface receptor engagement on antigen-presenting cells (APC) is essential for optimal interfacing with the innate and adaptive immune systems (Zhao et al. 2014), and (2) the discovery that APC engagement with nanoparticles themselves triggers inflammatory responses (Li et al. 2008; Hornung et al. 2008; Sharp et al. 2009).

Protein nanoclusters have been made from abundant proteins such as human and bovine serum albumin and gelatin since as early as the 1970s (Kreuter 1991; Marty et al. 1978). Even today, desolvated protein nanoclusters are made from albumin (Weber et al. 2000; Langer et al. 2003; Amighi et al. 2020) and gelatin (Subara et al. 2017; Jahanshahi 2008), have the most extensively characterized synthesis parameters of any protein nanoclusters. However, our lab has demonstrated that protein nanoclusters can also deliver active therapeutic enzymes to cells (Estrada et al. 2014; Herrera Estrada et al. 2017). Because of albumin's high affinity for hydrophobic small molecules (Al-Husseini et al. 2019), and its low antigenicity, albumin nanoclusters are an attractive drug delivery vehicle for enhancing small molecule half-life and biodistribution (Keuth et al. 2020; Luebbert et al. 2017). These nanoclusters are readily taken up by macrophages (Langer et al. 2003) and this can be used to target small molecule drugs to these cells in particular (Markovsky et al. 2007).

The same properties that make desolvated protein nanoclusters attractive to macrophages make them ideal vehicles for vaccine delivery. Nanoparticulate antigen is more immunogenic than soluble antigen (Gregory et al. 2013; Singh 2007; Wang et al. 2014), and targeting it to phagocytic APCs enhances

immunogenicity. In our studies of protein nanocluster vaccines, we demonstrate robust in vitro dendritic cell responses to PNCs, enhanced retention in draining lymphatic organs, and successful immunization with a variety of protein and peptide antigens.

3 Functional Benefits of Protein Nanocluster Vaccines

The particulate formulation of protein nanoclusters provides distinct advantages over soluble antigens, as summarized in Fig. 3. Some of these advantages are general to all nanoparticle vaccines and some are specific to crosslinked protein nanoclusters.

3.1 Delivery

All nanoparticles, including nanoclusters, benefit from improved delivery due to their relatively large size compared to soluble antigen. Upon intradermal (i.d.) or intramuscular (i.m.) administration, nanoclusters are retained at the injection site significantly longer than soluble antigen (Tsoras and Champion 2018; Deng et al.

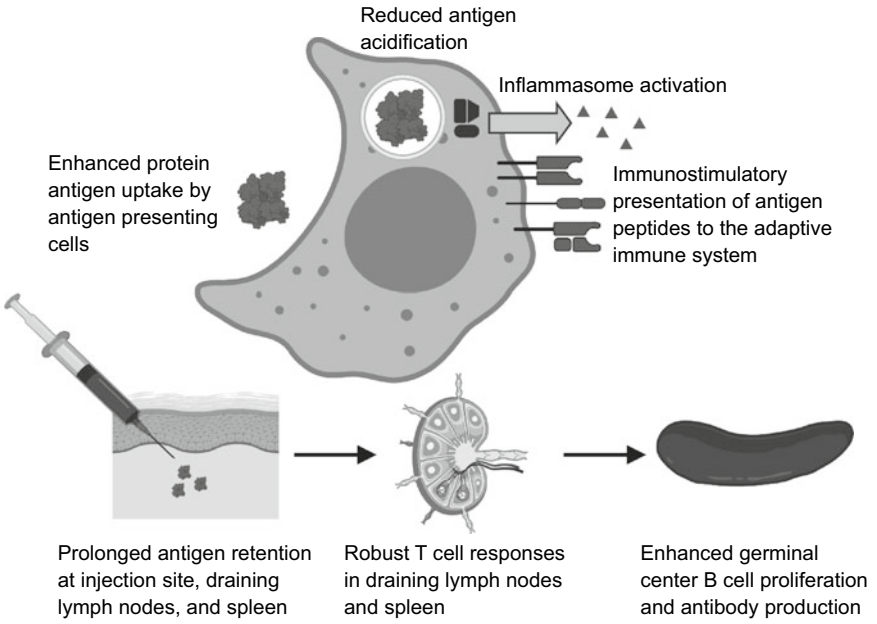

Fig. 3 Summary of benefits of protein and PNC vaccines observed at cellular and physiological levels. Figure created with Biorender.com

2018b). The presence of nanoclusters for longer than 2 and 5 days for i.d. and i.m. injection, respectively, indicates that immune cells in the tissue are persistently exposed to antigen and increases the likelihood of antigen uptake. Nanoclusters also exhibit different trafficking and biodistribution compared to soluble antigen. When PNC was administered i.d., the antigen was detected in draining lymph nodes for 4–24 h after injection, which can increase the likelihood of presentation to T cells and activation (Tsoras et al. 2020). Nanocluster peptide antigen was not detected in the spleen, and soluble peptide antigen was detected in the spleen only transiently. The route of administration is important for antigen trafficking, as protein nanoclusters administered i.m. exhibited antigen trafficking to both the draining lymph nodes and spleen that was detectable more than a week after injection (Deng et al. 2018a, b). Antigen accumulation in the spleen was significantly higher for protein nanoclusters than soluble protein antigen, though levels in the draining lymph nodes were similar. The diameter of most antigen nanoclusters is in the range of 200 nm, which is the upper limit seen for nanoparticles that traffic directly in the lymphatic system (Reddy et al. 2007; Manolova et al. 2008). Likely, most nanoclusters are taken up by APCs at the injection site and are actively trafficked through the lymphoid system whereas soluble antigens would diffuse directly.

3.2 Antigen Presentation

Given the importance of antigen uptake by APCs for both trafficking and presentation, enhanced uptake of nanoclusters by dendritic cells (DCs) also contributes to their overall function. Nanoclusters made from large protein antigens show significant increases in uptake by DCs in vitro, regardless of the size of the nanoclusters (Chang et al. 2016). This is consistent with other types of vaccine nanoparticles (Snapper 2018). However, nanoclusters made from small peptide antigens (minimal epitopes) did not show any advantage in uptake relative to soluble small peptides, with both exhibiting high uptake by DCs in vitro (Tsoras and Champion 2018). Once internalized, nanoclusters appeared to traffic differently than soluble antigen in cells, as the intracellular pH experienced by soluble antigen was significantly lower than that experienced by nanoclusters. Reduced acidification can decrease antigen degradation, which may lead to improved cross-presentation, and could indicate endosomal escape (Tran and Shen 2009; Accapezzato et al. 2005).

While uptake by APCs is a critical first step, ultimately, antigen processing and presentation are needed to activate antigen-specific T cells (Murphy et al. 2012). For MHC I peptide antigens for which presentation can be measured, it was observed that DCs exposed to nanoclusters demonstrated greater levels of presentation than those exposed to soluble antigen both in vitro and in draining lymph nodes following i.d. administration in vivo (Tsoras and Champion 2018; Tsoras et al. 2020). Concurrently, nanoclusters induced increased DC display of maturation

factors CD80 and CD86 compared to soluble antigen when incubated with DCs in vitro (Tsoras and Champion 2018; Chang et al. 2016). This data demonstrates that even when there is little or no enhancement in antigen uptake via nanoclusters, the subsequent steps of presentation and maturation are more productive for nanoclusters than soluble antigen.

Similar to maturation markers, in vitro DC secretion of inflammatory cytokine IL-1β was increased for nanoclusters containing either ovalbumin (OVA) or influenza antigens matrix protein 2 ectodomain (M2e) or M2e and HA (Chang et al. 2016; Deng et al. 2017). IL-1β is a product of inflammasome activation and induces rapid cleavage of pro-inflammatory cytokines into their active form, triggering a local, innate immune response (Murphy et al. 2012). Not all vaccine nanoparticles are capable of triggering inflammasome activation (Neumann et al. 2014; Gross et al. 2011). Interestingly, in vitro DC secretion of inflammatory cytokine TNF-α was lower for OVA nanoclusters than soluble OVA and higher for HA or M2e nanoclusters compared to soluble antigen (Chang et al. 2016; Deng et al. 2017, 2018a, b), though the role of TNF-α in vaccine responses is not clear (Murphy et al. 2012).

In vivo maturation of DCs in response to SIINFEKL nanoclusters mirrored that seen in vitro. SIINFEKL is an MHC I minimal peptide epitope from OVA protein. Upon i.d. vaccination, higher levels of double-positive, antigen-presenting, and CD86 positive, DCs were observed in the draining lymph nodes for SIINFEKL nanoclusters than for soluble antigen (Tsoras et al. 2020). Unexpectedly, this was only true for nanoclusters that were crosslinked with a non-reducible crosslinker that formed amide bonds between antigens, which is incidentally the same bond cleaved by proteases (Kisselev et al. 2000; Hedstrom 2002). Nanoclusters crosslinked with a reducible crosslinker, or a crosslinker that was non-reducible and also not cleavable by proteases did not induce significant increases in DC antigen presentation and CD86 expression. We speculate that proteolytic cleavage of the nanoclusters might have enhanced presentation and maturation. Large proteins are required to be proteolytically processed by APCs, whether or not they are in nanoclusters, to extract epitopes for MHC presentation. Antigens in nanoclusters must be similarly extracted by the cellular machinery for MHC presentation. By providing crosslinks between antigens that can be readily degraded by the natural protein-degradation machinery of the cells, nanoclusters may promote antigen extraction and MHC presentation. Importantly, in vivo DC maturation was antigen-specific as the increase in CD86 positive DCs was only seen for those DCs presenting antigen, and, the increases in the fraction of DCs presenting antigen were only seen for CD86 positive DCs (Tsoras et al. 2020). This was true of all nanoclusters, regardless of the crosslinker type. Administration of soluble antigen with poly (I:C), a synthetic double-stranded RNA adjuvant, upregulated CD86 in DCs that did not present antigen, demonstrating non-specific activation that is not necessarily productive to the adaptive immune response and may be responsible for side effects.

3.3 Cellular Responses

Following successful antigen presentation, CD4 and CD8 T cells are activated. This activation depends on the type of antigen and MHC used for presentation (Murphy et al. 2012). Protein and PNC have demonstrated antigen-specific T cell activation with several different antigens and in different tissues, depending on the route of administration. M2e nanoclusters induced a strong systemic and mucosal cellular response upon intranasal (i.n.) vaccination, as cells collected from both the spleen and lungs secreted IFN-γ and IL-4 in response to M2e restimulation (Wang et al. 2014). Influenza nucleoprotein (NP) nanoclusters and nanoclusters made from peptides derived from NP were coated with M2e and administered i.m. The coated nanoclusters, but not soluble mixtures of the proteins, elicited antigen-specific responses to both M2e and NP. Upon restimulation, splenocytes from mice administered nanoclusters secreted IFN-γ, IL-4, and IL-2 at much higher levels than those from animals administered soluble protein (Deng et al. 2018b). Similarly, HA-coated M2e nanoclusters also induced specific cellular responses, as demonstrated by high IFN-γ secretion following restimulation.

The responses described above were for nanoclusters without added adjuvant, where the nanocluster itself served as the delivery vehicle and "self-adjuvant". We have also investigated the effect of adjuvants co-delivered with nanoclusters, as coatings, incorporated within the nanoclusters, or as simple mixtures. Incorporation of CpG oligonucleotide, a toll-like receptor 9 agonists, within M2e nanoclusters did not have any effect on cellular or antibody responses (Wang et al. 2014). M2e and the H2 domain of HA have also been incorporated as fusion proteins within the variable domain of bacterial adjuvant flagellin (flic) (Deng et al. 2017). Flic is a toll-like receptor 5 agonist and has been demonstrated to boost anti-influenza immune responses (Kim et al. 2015; Oh et al. 2014). Flic-M2e nanoclusters and flic-H2HA coated flic-M2e nanoclusters were formed by crosslinking without desolvation and administered to mice i.n.. All nanoclusters induced high levels of M2e-specific cellular responses, measured by IL-2 secretion upon restimulation ex vivo. Though not typically considered as adjuvants, immunoglobulins from the host can serve this purpose. Anti-OVA IgM coated on the surface of OVA nanoclusters induced the formation of more central memory T cells relative to OVA-coated OVA nanoclusters (Chang et al. 2017).

Using PNCs made from SIINFEKL, a CD8 T cell antigen, we have more deeply investigated the T cell response following i.d. administration. Relative to soluble SIINFEKL, PNCs showed increased expression of CD69, an early marker of T cell activation, on CD8 T cells from draining lymph nodes, corresponding to the previously described evidence of DC presentation in those nodes (Tsoras et al. 2020). Adjuvanting soluble SIINFEKL with poly(I:C) resulted in increased CD69 expression in CD8 and CD4 T cells in both the draining lymph nodes and spleen, indicating non-specific activation. Only nanoclusters crosslinked with amide bonds, those which showed enhanced DC presentation and maturation, induced high secretion of IFN-γ upon restimulation of lymph node CD8 T cells, equivalent to

poly(I:C) adjuvanted peptide. This connection between nanocluster processing, presentation, and T cell activation is encouraging and will motivate future work on the role of antigen crosslinking and cleavage in nanoclusters. Altogether, across a wide combination of antigens, nanoclusters reliably induce a strong cellular immune response that is, importantly, antigen-specific.

3.4 Humoral Responses

In addition to cellular responses, PNCs are capable of stimulating robust humoral responses. Nanoclusters of a variety of designs presenting B cell epitopes from M2e and HA induced significant antibody production. The repetitive, multi-valent presentation of antigens on the surface of nanoclusters is likely beneficial for recognition by B cell receptors (Wang et al. 2012; Schellekens and Jiskoot 2013; Kim et al. 2006). M2e nanoclusters induced a strong humoral response upon i.n. vaccination, including serum antibody subtypes IgG1, IgG2a, and IgG2b indicative of balanced Th1 and Th2 responses (Wang et al. 2014). Further, mucosal antibody response was seen as lung and nasal washes revealed the production of both IgG and IgA. When M2e was coated on the surface of NP nanoclusters and administered i.m., a similar strong serum M2e-specific antibody response was seen (Deng et al. 2018a, b). No NP-specific antibodies were detected. However, when nanoclusters made from M2e were coated with HA, M2e-specific antibodies were still produced at high levels (Deng et al. 2018b). There was a small, but statistically significant, decrease in M2e antibody titers for HA-coated nanoclusters compared to uncoated nanoclusters. Whether immunological differences between M2e and NP antigens are responsible, or if the HA coating did not completely obscure M2e on the surface of the particle, is not clear. For HA specifically, a crosslinked coating of HA was required on the surface of nanoclusters to induce antibody production. This is likely due to the sensitive nature of the structure of trimeric HA, which was disrupted in the desolvation process. HA-coated HA nanoclusters exhibited high titers of IgG, including IgG1, IgG2a, and IgG2b, though the response was biased toward Th2 (Wang et al. 2017). The antibodies were shown to be neutralizing and inhibit hemagglutination. Serum antibody titers were dependent on the dose and route of administration, with 2 i.m. doses inducing far higher levels than 1 i.m., 1 i.n., or 2 i.n. doses. Despite the large difference in antibody response, 2 doses given either i.m. or i.n. both protected fully from weight loss and death upon H7N9 viral challenge. Similarly, when M2e nanoclusters were coated with trimeric HA, HA-specific antibody responses were strong (Deng et al. 2018a, b).

The humoral responses described above were all the result of nanocluster delivery of antigen with no external adjuvant provided. In the case of flic-M2e fusion protein nanoclusters and flic-H2HA fusion coated flic-M2e nanoclusters, strong antibody responses were induced that were specific to both M2e and HA (Deng et al. 2017). Interestingly, incorporation of the flic-HA coating improved Me2-specific serum IgG titers. In a different configuration, we found that

flagellin-coated OVA nanoclusters and soluble flagellin mixed with OVA nanoclusters induced similar levels of antibodies upon i.m. immunization. However, flagellin-coated nanoclusters induced significant affinity maturation of antibodies, while flagellin-admixed nanoclusters did not (Chang et al. 2017). Anti-OVA IgM coated on the surface of OVA nanoclusters increased IgG1 and IgG2a serum titers relative to OVA-coated OVA nanoclusters (Chang et al. 2017). Interestingly, the IgM coating prevented affinity maturation despite its induction of class switching. Affinity maturation was also observed for OVA-coated nanoclusters, which is consistent with our data showing splenic germinal center B cell proliferation upon immunization with HA-coated HA nanoclusters (Chang 2017). The reason for adjuvant-dependent differences in affinity maturation is not clear. Affinity maturation has not been studied for non-model antigens, such as influenza proteins.

3.5 Storage and Stability

There are many challenges for global vaccination campaigns. One significant challenge is the need for cold chain storage of vaccines, especially in developing countries with unreliable electricity and transportation infrastructure as well as hot climates. We assessed the ability of HA-coated HA nanoclusters to maintain their potency over storage time, as measured by hemagglutination activity in vitro, and antibody titers and hemagglutination inhibition titers following i.m. vaccination. Nanoclusters aged in solution (PBS) for 3 months at 25 °C retained their full immunogenicity, relative to fresh nanoclusters (Chang et al. 2018). M2e coated NP nanoclusters retained full activity after 2 months storage at 4 °C and had a small, but statistically significant, loss after 2 months storage at 25 °C, as measured by endpoint antibody titers (Deng et al. 2018b). Stability may depend on the exact antigen used and is also likely to be affected by the amount of crosslinking, protein concentrations, and any formulation stabilizers. These factors will be important to investigate thoroughly in future work.

4 Application of Protein Nanoclusters as Protective Vaccines

4.1 Broadly Protecting Influenza Vaccines

Seasonal influenza vaccines provide limited protection, which is better is some years and worse in others, based, in part, on how well the strains included in the vaccine match the circulating strains in the community (CDC CDC Seasonal Flu Vaccine Effectiveness Studies 2020). Additionally, zoonotic influenza strains, such as the recent avian strains, do infect humans occasionally and are serious health

Table 1 M2e sequences from different human influenza A strains and the consensus sequence (Wang et al. 2014)

Viral strains	Subtype	M2e amino acid sequence
M2e in nanoclusters	N/A[a]	MSLLTEVETP IRNEWGCRCN D
A/Philippines/2/82	H3N2	MSLLTEVETP IRNEWGCRCN D
A/Puerto Rico/8/34	H1N1	MSLLTEVETP IRNEWGCRCN **G**
A/California/04/09	H1N1	MSLLTEVETP**T**R**S**EW**E**CRC**S**D
A/Vietnam/1230/04	H5N1	MSLLTEVETP**T**RNEW**E**CRC**S**D

Reproduced from Wang et al. (2014)
[a]M2e consensus of human influenza A viruses

threats (Lai et al. 2016; Gao et al. 2013; Wu et al. 2016). A universal influenza vaccine is needed that can protect populations against seasonal and pandemic influenza strains, including future strains that have not yet evolved. One challenge of universal influenza vaccines is the high mutation rate of influenza, specifically in the immunodominant antigens, hemagglutinin, specifically the head domain, and neuraminidase (Altman et al. 2018). The highly conserved ectodomain of matrix protein 2 is a promising antigen for a universal influenza vaccine (Lamb et al. 1985), however, its immunogenicity is low. Though M2e specific antibodies can reduce viral load, they are rarely detected after natural infection or seasonal vaccination (Hughey et al. 1995; Treanor et al. 1990; Black et al. 1993; Feng et al. 2006). The first application of PNCs sought to improve the immunogenicity of M2e and evaluate the breadth of protection. We selected the M2e consensus sequence from human influenza A viruses as the antigen, as shown in Table 1.

To recapitulate the native tetrameric state of M2e, the GCN4 tetramerization motif, modified from a leucine zipper region of a yeast transcription factor (De Filette et al. 2008), was fused to M2e and expressed in insect cells (Wang et al. 2014). Nanoclusters were fabricated by desolvating the tetrameric M2e complex and crosslinking with non-reducible glutaraldehyde. Mice were vaccinated i.n. with 10 μg of M2e each in a prime and two boost doses. The M2e specific humoral and cellular responses generated by vaccination with nanoclusters protected mice from lethal challenge. Antibodies generated recognized not only the M2e consensus peptide but mutated M2e sequences from different viral strains. While no mice immunized with soluble tetrameric M2e survived, all mice vaccinated with tetrameric M2e nanoclusters did survive. This protective effect was broad, as mice survived both challenged by an influenza strain with the same M2e consensus sequence (A/Philippines/82 H3N2: Phi/82 H3N2) as the nanoclusters, and challenged by a divergent strain with four mutated residues out of 21 (A/California/04/09 H1N1: CA/09 H1N1). While survival was promising, the mice did lose weight, up to 10 and 20% for similar and divergent strains, respectively, indicating the need to further enhance the vaccine to improve the quality and breadth of protection. Passive transfer of immune sera from nanocluster-immunized mice to naïve mice protected most animals, but not all, demonstrating the importance of the cellular response in protection against influenza. There is evidence that M2e antibodies are

not neutralizing but rather mediate effector cell functions (Jegerlehner et al. 2004; El Bakkouri et al. 2011; Mozdzanowska et al. 2005; Tompkins et al. 2007).

In an effort to improve the efficacy and breadth of protection, the antigen content of nanoclusters was expanded. Instead of a single M2e consensus sequence from human influenza A, a chimeric antigen was created that linked M2e consensus sequences from human, swine, avian, and fowl influenza A strain into a single chain fused to GCN4 to induce tetramerization (Fig. 4a). The goal of this modification was to improve protection against zoonotic strains. The stalk domain of HA was also used, as it is also relatively conserved, compared to the HA head domain (Ekiert et al. 2011; Mallajosyula et al. 2014) and provides additional immune epitopes. Engineered trimeric HA stalk was created from both H1 and H3 virus (Fig. 4b). Desolvation was used to create chimeric M2e nanoclusters, which were coated with H1, H3, or a mixture of H1 and H3 trimeric stalk domains and crosslinked (Deng et al. 2018a) (Fig. 4c). Upon i.m. vaccination, mice maintained early antibody titers until at least 4 months post-vaccination. The immune responses extended beyond the nanocluster-specific humoral and cellular responses seen for the antigens in the nanoclusters. Vaccination induced antibodies capable of binding not only the antigens present in the nanoclusters, but also H2 and H5 subtypes if H1 was present in the coating, and H7 and H10 subtypes if H3 was present in the coating. Broad protection was ultimately demonstrated by viral challenge with a panel of six influenza viruses: A/Puerto Rico/8/34 H1N1 (PR8 H1N1) and A/Aichi/2/1968 H3N2 (Aic H3N2), from which the H1 and H3 stalks were derived, A/California/7/2009 H1N1pdm (p09 H1N1), reassorted A/Vietnam/1203/2004 (Vtn H5N1) virus, A/Philippines/2/1982 (Phi H3N2), and reassorted A/Shanghai/2/2013 (SH H7N9) virus. The respective reassorted viruses contained HA and NA from the parent strain (Vtn or SH) but the internal genes and M2e from PR8 H1N1. In all cases, nanoclusters containing both the chimeric M2e core and the coating of either both H1 and H3 stalk or coating of the HA stalk variant most closely related to the challenge virus (H1 for H5N1 virus and H3 for H7N9 virus) fully protected mice from lethal challenge. Vaccination with only the M2e nanocluster core protected most mice, but not all. These results demonstrate high potential for a broadly protecting influenza vaccine and the benefit of combining multiple conserved antigenic domains.

To further explore the value of other conserved influenza antigens, we fabricated nanoclusters from NP and known NP peptide antigens (Deng et al. 2018b). NP is an internal influenza protein that is highly conserved and has been shown to induce cross-protection against a challenge from diverse influenza A viruses (Zheng et al. 2014). Nanoclusters were desolvated both from full-length NP and from an engineered peptide containing three linear repeats each of the NP_{147} and NP_{55} short peptide epitopes. Following desolvation, nanoclusters were coated with the chimeric M2e tetramer used previously (Fig. 4a) and crosslinked. Mice vaccinated i.m. with both types of M2e coated NP nanoclusters were protected from lethal challenge with the reassortant H5N1 virus. Animals did lose $\sim$10% body weight and the coated nanoclusters containing NP peptide recovered faster than those

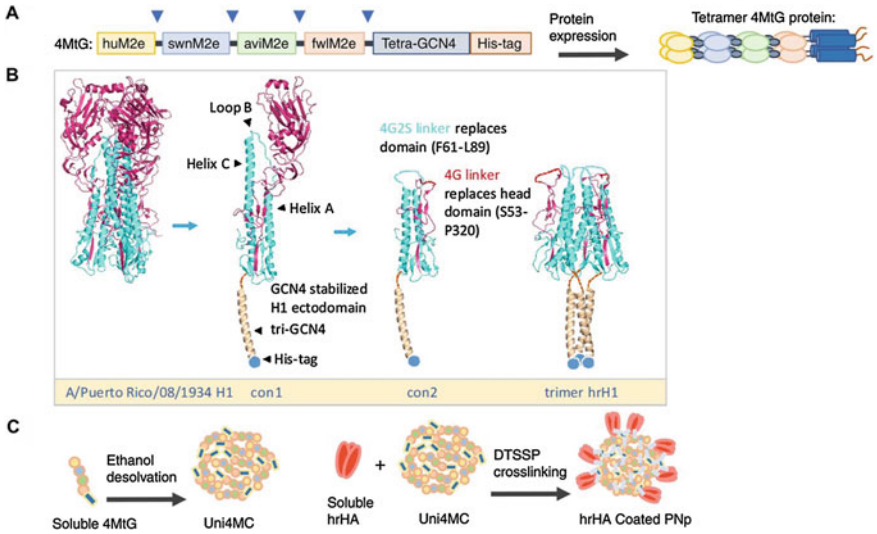

Fig. 4 Antigen design of (**a**) chimeric M2e tetramers and (**b**) stabilized HA1 stalk antigen. Coated PNCs containing both antigens were made by the method described in Sect. 2 (**c**) (Deng et al. 2018a). Figure adapted from Deng et al. (2018a) under the Creative Commons license

containing NP protein. We also investigated whether nanoclusters could benefit from codelivery with inactivated (PR8 H1N1) influenza vaccine, as previous work had shown that codelivery of soluble flic-M2e fusion proteins and inactivated vaccines broadened the protection (Zhu et al. 2017). Using both i.m. and microneedle delivery routes, mice co-vaccinated with M2e coated NP PNC and inactivated PR8 were protected against both PR8 challenge and Phi H3N2 challenge. Mice vaccinated only with inactivated PR8 were only protected from PR8 challenge. Again, the value of antigen combination in nanoclusters is evident and there is potential for further combination with existing vaccines to boost immunogenicity.

A universal influenza vaccine would ideally protect against all potential influenza strains. However, strain-specific vaccines could be useful for enhancing responses against specific antigenic sequences in the case of an emerging outbreak. Additionally, in the case of a recombinant influenza virus engineered to evade a broadly protective vaccine, an antigen-matched formulation may be the only means of generating protective immunity. We have demonstrated that PNCs made with hemagglutinin H1 and H3 stalk domains inserted into the flic protein can trigger subtype-specific protective immunity (Deng et al. 2017). Overall, PNCs made from a variety of influenza A antigens can elicit strong, protective immune responses against homo- and heterosubtypic challenges.

4.2 Progress Towards Cancer Vaccines

Protein nanoclusters have also been recently explored as therapeutic cancer vaccines. In particular, the E.G7-OVA mouse T cell lymphoma cell line generates tumors when injected subcutaneously and can be used as a measure of OVA nanocluster efficacy. OVA nanocluster-immunized mice had drastically lower tumor volumes and growth rates compared to mice immunized with soluble OVA, which had almost no effect on tumor volume (Dong et al. 2019). CpG-coated nanoclusters also slightly, but significantly, reduced tumor volumes as compared to unadjuvanted OVA nanoclusters. Another study demonstrated the protective effects of OVA nanoclusters crosslinked with indocyanine green. This crosslinker has the bonus of generating reactive oxygen species (ROS) upon near-infrared irradiation, which was shown to be essential for endosomal escape and therapeutic effect (Cao et al. 2018).

Given the high specificity needed for cancer vaccines, many antigens are minimal peptide epitopes. We have fabricated PNCs from oncofetal antigen (OFA) peptide epitopes (Tsoras and Champion 2018). OFA is a highly conserved protein antigen expressed in many types of cancer cells including breast, head/neck, and hematologic malignancies, but it is not detectably expressed in healthy cells (Siegel et al. 2003; Barsoum and Schwarzenberger 2014). The lack of reactive groups for sufficient crosslinking required the addition of a terminal cysteine to enable stabilization of the nanoclusters using a tri-thiol crosslinker. Evaluation of human cancer antigens in humanized mice or clinical trials is needed to determine efficacy, likely in combination with checkpoint inhibitors, given the highly immunosuppressive environment of many tumors (Sharma et al. 2017).

5 Conclusion and Future Outlook

PNCs are designed to present specific antigens and epitopes in the context of enhanced immunogenicity conferred by particulate matter. Our work has demonstrated successful immunization with PNCs, yet opportunities still remain for enhancing their immunogenicity. The immunomodulatory effects of nanoparticles have been shown to be a function of many factors, including nanoparticle size (Xiang et al. 2006; Stano et al. 2012; Mottram et al. 2007), shape (Kumar et al. 2015; Vaine et al. 2013), charge (Neumann et al. 2014), surface chemistry (Huang et al. 2013), and administration route (Zolnik et al. 2010). HSA protein nanocluster density has recently been shown to affect delivery to cells of the immune system in vitro and in vivo (Roh et al. 2019). Future vaccine nanocluster work should examine whether nanocluster density is an intrinsic property of the antigen, or if synthesis conditions can tune nanocluster density and the resulting immune response. Non-desolvated protein nanoclusters may better preserve the native

structure of certain antigens and are also being explored for cancer (Dong et al. 2019; Cao et al. 2018) and influenza (Deng et al. 2017) vaccines.

We have also demonstrated the importance of the outer coat layer on protein nanoclusters for effective immune responses. The outer layer of protein nanoclusters is especially important for cell- and tissue-specific targeting. Recent studies of non-vaccine HSA nanocluster drug carriers have used small molecules (Akbarian et al. 2020), nucleic acid aptamers, and antibodies (Keuth et al. 2020) to target nanocluster delivery. The protein corona on the respiratory syncytial virus (RSV) and herpes simplex virus type 1 (HSV-I), has been shown to affect immune cell activation and viral pathogenesis (Ezzat et al. 2019). The corona of protein nanoparticle vaccines, including PNCs, has not been studied yet, though is very likely to impact biodistribution, cellular uptake, and/or immunogenicity.

The use of antibodies and other proteins of the immune system for targeting is especially relevant for vaccine nanoclusters, given that the Fc receptor is a key mediator of antigen presentation to B cells (Bergtold et al. 2005). We have tested flagellin and immunoglobulin as adjuvants to nanoclusters (Chang et al. 2017), yet other protein types should be explored. In particular, the complement protein C3d can bridge the innate and adaptive immune response by flagging particulate antigens as targets for humoral immune responses (Del Nagro et al. 2005).

In addition to humoral immune responses, viral vaccines need to induce robust cell-mediated immunity, which is a much more difficult challenge. Protein nanoclusters deliver large quantities of antigen per particle to antigen-presenting cells, increasing the chances of successful T cell epitope antigen presentation. In mice, PNCs show enhanced antigen retention in lymph nodes and the spleen (Deng et al. 2018b; Tsoras et al. 2020), important organs for antigen presentation. However, the presentation of antigen alone is not sufficient for stimulating cell-mediated immunity. T cell differentiation and polarization are mediated by cytokine transfer from the APC to the cognate T cell (Murphy et al. 2012; Munoz-Wolf and Lavelle 2018). Exogenous cytokine administration can be dangerous if not appropriately targeted (Tisoncik et al. 2012), and protein nanoclusters could provide a means of such targeting. Pathogenic pattern recognition receptor activation also leads to specific inflammatory cytokine production, and engagement of these receptors by adjuvants has been shown to be critical to successful T cell responses (Kumar et al. 2019). Whether engaging cytokine receptors directly or inducing pro-inflammatory cytokine production pathways in APCs, adjuvant-coated protein nanoclusters should be explored as a means of inducing specific cell-mediated immune phenotypes.

Though a significant benefit of PNCs is the lack of external adjuvants, PNC vaccines of the future may contain both molecular adjuvants and particulate antigens, especially as we learn more about how different host- and pathogen-derived components trigger specific aspects of the immune system. Future research should address how different adjuvant combinations, as well as antigen nanoparticle properties, can optimize immune responses to combat viral, bacterial, and/or oncological threats.

References

Accapezzato D, Visco V, Francavilla V, Molette C, Donato T, Paroli M, Mondelli MU, Doria M, Torrisi MR, Barnaba V (2005) Chloroquine enhances human CD8(+) T cell responses against soluble antigens in vivo. J Exp Med 202(6):817–828. https://doi.org/10.1084/jem.20051106

Akbarian A, Ebtekar M, Pakravan N, Hassan ZM (2020) Folate receptor alpha targeted delivery of artemether to breast cancer cells with folate-decorated human serum albumin nanoparticles. Int J Biol Macromol 152:90–101. https://doi.org/10.1016/j.ijbiomac.2020.02.106

Al-Husseini JK, Stanton NJ, Selassie CRD, Johal MS (2019) The binding of drug molecules to serum albumin: the effect of drug hydrophobicity on binding strength and protein desolvation. Langmuir 35(52):17054–17060. https://doi.org/10.1021/acs.langmuir.9b02318

Altman MO, Angeletti D, Yewdell JW (2018) Antibody immunodominance: the key to understanding influenza virus antigenic drift. Viral Immunol 31(2):142–149. https://doi.org/10.1089/vim.2017.0129

Amighi F, Emam-Djomeh Z, Labbafi-Mazraeh-Shahi M (2020) Effect of different cross-linking agents on the preparation of bovine serum albumin nanoparticles. J Iran Chem Soc 17(5):1223–1235. https://doi.org/10.1007/s13738-019-01850-9

Barsoum AL, Schwarzenberger PO (2014) Oncofetal antigen/immature laminin receptor protein in pregnancy and cancer. Cell Mol Biol Lett 19(3):393–406. https://doi.org/10.2478/s11658-014-0203-7

Baseer A, Koenneke A, Zapp J, Khan SA, Schneider M (2019) Design and characterization of surface-crosslinked gelatin nanoparticles for the delivery of hydrophilic macromolecular drugs. Macromol Chem Phys 220(18):1900260. https://doi.org/10.1002/macp.201900260

Bergtold A, Desai DD, Gavhane A, Clynes R (2005) Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. Immunity 23(5):503–514. https://doi.org/10.1016/j.immuni.2005.09.013

Black RA, Rota PA, Gorodkova N, Klenk HD, Kendal AP (1993) Antibody response to the M2 protein of influenza A virus expressed in insect cells. J Gen Virol 74(Pt 1):143–146. https://doi.org/10.1099/0022-1317-74-1-143

Cao FQ, Yan MM, Liu YJ, Liu LX, Lu L, Wang H, Zhang C, Sun HF, Kong DL, Ma GL (2018) Photosensitizer-induced self-assembly of antigens as nanovaccines for cancer immunotherapy. Biomater Sci 6(3):473–477. https://doi.org/10.1039/C7BM01082F

CDC CDC Seasonal Flu Vaccine Effectiveness Studies. https://www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm (accessed 10 July 2020)

Chang TZ (2017) Protein nanoparticle vaccines. Georgia Tech

Chang TZ, Stadmiller SS, Staskevicius E, Champion JA (2016) Effects of ovalbumin protein nanoparticle vaccine size and coating on dendritic cell processing. Biomater Sci

Chang TZ, Diambou I, Kim JR, Wang B, Champion JA (2017) Host- and pathogen-derived adjuvant coatings on protein nanoparticle vaccines. Bioeng Transl Med 2(1):120–130. https://doi.org/10.1002/btm2.10052

Chang TZ, Deng L, Wang B-Z, Champion JA (2018) H7 Hemagglutinin nanoparticles retain immunogenicity after > 3 months of 25 °C storage. PLoS ONE 13(8):e0202300–e0202300. https://doi.org/10.1371/journal.pone.0202300

De Filette M, Martens W, Roose K, Deroo T, Vervalle F, Bentahir M, Vandekerckhove J, Fiers W, Saelens X (2008) An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. J Biol Chem 283(17):11382–11387. https://doi.org/10.1074/jbc.M800650200

Del Nagro CJ, Kolla RV, Rickert RC (2005) A critical role for complement C3d and the B cell coreceptor (CD19/CD21) complex in the initiation of inflammatory arthritis. J Immunol 175(8):5379. https://doi.org/10.4049/jimmunol.175.8.5379

Deng L, Kim JR, Chang TZ, Zhang H, Mohan T, Champion JA, Wang BZ (2017) Protein nanoparticle vaccine based on flagellin carrier fused to influenza conserved epitopes confers full protection against influenza A virus challenge. Virology 509:82–89. https://doi.org/10.1016/j.virol.2017.06.001

Deng L, Mohan T, Chang TZ, Gonzalez GX, Wang Y, Kwon Y-M, Kang S-M, Compans RW, Champion JA, Wang B-Z (2018a) Double-layered protein nanoparticles induce broad protection against divergent influenza A viruses. Nat Commun 9(1):359. https://doi.org/10.1038/s41467-017-02725-4

Deng L, Chang TZ, Wang Y, Li S, Wang S, Matsuyama S, Yu G, Compans RW, Li J-D, Prausnitz MR, Champion JA, Wang B-Z (2018b) Heterosubtypic influenza protection elicited by double-layered polypeptide nanoparticles in mice. Proc Nat Acad Sci 115(33):E7758. https://doi.org/10.1073/pnas.1805713115

Doan CD, Ghosh S (2019) Formation and stability of pea proteins nanoparticles using ethanol-induced desolvation. Nanomaterials 9(7). https://doi.org/10.3390/nano9070949

Dong X, Liang J, Yang A, Qian Z, Kong D, Lv F (2019) A visible codelivery nanovaccine of antigen and adjuvant with self-carrier for cancer immunotherapy. ACS Appl Mater Interfaces 11(5):4876–4888. https://doi.org/10.1021/acsami.8b20364

Ekiert DC, Friesen RHE, Bhabha G, Kwaks T, Jongeneelen M, Yu WL, Ophorst C, Cox F, Korse H, Brandenburg B, Vogels R, Brakenhoff JPJ, Kompier R, Koldijk MH, Cornelissen L, Poon LLM, Peiris M, Koudstaal W, Wilson IA, Goudsmit J (2011) A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333(6044):843–850. https://doi.org/10.1126/science.1204839

El Bakkouri K, Descamps F, De Filette M, Smet A, Festjens E, Birkett A, Van Rooijen N, Verbeek S, Fiers W, Saelens X (2011) Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J Immunol 186(2):1022. https://doi.org/10.4049/jimmunol.0902147

Estrada LH, Chu S, Champion JA (2014) Protein nanoparticles for intracellular delivery of therapeutic enzymes. J Pharm Sci-Us 103(6):1863–1871. https://doi.org/10.1002/jps.23974

Ezzat K, Pernemalm M, Pålsson S, Roberts TC, Järver P, Dondalska A, Bestas B, Sobkowiak MJ, Levänen B, Sköld M, Thompson EA, Saher O, Kari OK, Lajunen T, Sverremark Ekström E, Nilsson C, Ishchenko Y, Malm T, Wood MJA, Power UF, Masich S, Lindén A, Sandberg JK, Lehtiö J, Spetz A-L, El Andaloussi S (2019) The viral protein corona directs viral pathogenesis and amyloid aggregation. Nat Commun 10(1):2331. https://doi.org/10.1038/s41467-019-10192-2

Feng J, Zhang M, Mozdzanowska K, Zharikova D, Hoff H, Wunner W, Couch RB, Gerhard W (2006) Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. Virol J 3:102. https://doi.org/10.1186/1743-422x-3-102

Fleischer CC, Payne CK (2014) Secondary structure of corona proteins determines the cell surface receptors used by nanoparticles. J Phys Chem B 118(49):14017–14026. https://doi.org/10.1021/jp502624n

Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu Y (2013) Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med 368(20):1888–1897. https://doi.org/10.1056/NEJMoa1304459

Gregory AE, Titball R, Williamson D (2013) Vaccine delivery using nanoparticles. Front Cell Infect Mi 3. Unsp 13. https://doi.org/10.3389/fcimb.2013.00013

Gross O, Thomas CJ, Guarda G, Tschopp J (2011) The inflammasome: an integrated view. Immunol Rev 243(1):136–151. https://doi.org/10.1111/j.1600-065X.2011.01046.x

Hedstrom L (2002) Serine protease mechanism and specificity. Chem Rev 102(12):4501–4524. https://doi.org/10.1021/cr000033x

Herrera Estrada L, Wu H, Ling K, Zhang G, Sumagin R, Parkos CA, Jones RM, Champion JA, Neish AS (2017) Bioengineering bacterially derived immunomodulants: a therapeutic approach to inflammatory bowel disease. ACS Nano 11(10):9650–9662. https://doi.org/10.1021/acsnano.7b03239

Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol 9(8):847–856. https://doi.org/10.1038/ni.1631

Huang RX, Carney RP, Stellacci F, Lau BLT (2013) Protein-nanoparticle interactions: the effects of surface compositional and structural heterogeneity are scale dependent. Nanoscale 5 (15):6928–6935. https://doi.org/10.1039/C3nr02117c

Hughey PG, Roberts PC, Holsinger LJ, Zebedee SL, Lamb RA, Compans RW (1995) Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. Virology 212(2):411–421. https://doi.org/10.1006/viro.1995.1498

Jahanshahi MB (2008) Z, Protein nanoparticle: a unique system as drug delivery vehicles. Afr J Biotechnol 7(25):4926–4934

Jegerlehner A, Storni T, Lipowsky G, Schmid M, Pumpens P, Bachmann MF (2002) Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation. Eur J Immunol 32 (11):3305–3314. https://doi.org/10.1002/1521-4141(200211)32:11%3c3305:Aid-immu3305%3e3.0.Co;2-j

Jegerlehner A, Schmitz N, Storni T, Bachmann MF (2004) Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. J Immunol 172(9):5598–5605. https://doi.org/10.4049/jimmunol.172.9.5598

Kallewaard NL, Corti D, Collins PJ, Neu U, McAuliffe JM, Benjamin E, Wachter-Rosati L, Palmer-Hill FJ, Yuan AQ, Walker PA, Vorlaender MK, Bianchi S, Guarino B, De Marco A, Vanzetta F, Agatic G, Foglierini M, Pinna D, Fernandez-Rodriguez B, Fruehwirth A, Silacci C, Ogrodowicz RW, Martin SR, Sallusto F, Suzich JA, Lanzavecchia A, Zhu Q, Gamblin SJ, Skehel JJ (2016) Structure and function analysis of an antibody recognizing all influenza A subtypes. Cell 166(3):596–608. https://doi.org/10.1016/j.cell.2016.05.073

Kanekiyo M, Wei CJ, Yassine HM, McTamney PM, Boyington JC, Whittle JRR, Rao SS, Kong WP, Wang LS, Nabel GJ (2013) Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. Nature 499(7456):102–106. https://doi.org/10.1038/nature12202

Karch CP, Matyas GR, Burkhard P, Beck Z (2018) Self-assembling protein nanoparticles: implications for HIV-1 vaccine development. Nanomedicine 13(17):2121–2125. https://doi.org/10.2217/nnm-2018-0222

Keuth J, Nitschke Y, Mulac D, Riehemann K, Rutsch F, Langer K (2020) Reversion of arterial calcification by elastin-targeted DTPA-HSA nanoparticles. Eur J Pharm Biopharm 150:108–119. https://doi.org/10.1016/j.ejpb.2020.03.007

Kim Y-M, Pan JY-J, Korbel GA, Peperzak V, Boes M, Ploegh HL (2006) Monovalent ligation of the B cell receptor induces receptor activation but fails to promote antigen presentation. Proc Nat Acad Sci USA 103(9):3327. https://doi.org/10.1073/pnas.0511315103

Kim JR, Holbrook BC, Hayward SL, Blevins LK, Jorgensen MJ, Kock ND, De Paris K, D'Agostino RB, Aycock ST, Mizel SB, Parks GD, Alexander-Miller MA (2015) Inclusion of flagellin during vaccination against influenza enhances recall responses in nonhuman primate neonates. J Virol 89(14):7291–7303

Kirchdoerfer RN, Wang N, Pallesen J, Wrapp D, Turner HL, Cottrell CA, Corbett KS, Graham BS, McLellan JS, Ward AB (2018) Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis. Sci Rep 8(1):15701. https://doi.org/10.1038/s41598-018-34171-7

Kisselev AF, Songyang Z, Goldberg AL (2000) Why does threonine, and not serine, function as the active site nucleophile in proteasomes? J Biol Chem 275(20):14831–14837. https://doi.org/10.1074/jbc.275.20.14831

Krammer F, Palese P (2013) Influenza virus hemagglutinin stalk-based antibodies and vaccines. Curr Opin Virol 3(5):521–530. https://doi.org/10.1016/j.coviro.2013.07.007

Kreuter J (1991) Nanoparticle-based dmg delivery systems. J Controlled Release 16(1):169–176. https://doi.org/10.1016/0168-3659(91)90040-K

Kumar S, Anselmo AC, Banerjee A, Zakrewsky M, Mitragotri S (2015) Shape and size-dependent immune response to antigen-carrying nanoparticles. J Control Release 220(Pt A):141–148. https://doi.org/10.1016/j.jconrel.2015.09.069

Kumar S, Sunagar R, Gosselin E (2019) Bacterial protein toll-like-receptor agonists: a novel perspective on vaccine adjuvants. Front Immunol 10:8. https://doi.org/10.3389/fimmu.2019.01144

Lai S, Qin Y, Cowling BJ, Ren X, Wardrop NA, Gilbert M, Tsang TK, Wu P, Feng L, Jiang H, Peng Z, Zheng J, Liao Q, Li S, Horby PW, Farrar JJ, Gao GF, Tatem AJ, Yu H (2016) Global epidemiology of avian influenza A H5N1 virus infection in humans, 1997-2015: a systematic review of individual case data. The Lancet. Infect Dis 16(7):e108–e118. https://doi.org/10.1016/S1473-3099(16)00153-5

Lamb RA, Zebedee SL, Richardson CD (1985) Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell 40(3):627–633. https://doi.org/10.1016/0092-8674(85)90211-9

Langer K, Balthasar S, Vogel V, Dinauer N, von Briesen H, Schubert D (2003) Optimization of the preparation process for human serum albumin (HSA) nanoparticles. Int J Pharm 257(1–2):169–180. https://doi.org/10.1016/S0378-5173(03)00134-0

Li HF, Willingham SB, Ting JPY, Re F (2008) Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. J Immunol 181(1):17–21

Li W, Joshi MD, Singhania S, Ramsey KH, Murthy AK (2014) Peptide vaccine: progress and challenges. Vaccines 2(3):515–536. https://doi.org/10.3390/vaccines2030515

Lindman S, Lynch I, Thulin E, Nilsson H, Dawson KA, Linse S (2007) Systematic investigation of the thermodynamics of HSA adsorption to N-iso-propylacrylamide/N-tert-butylacrylamide copolymer nanoparticles. Effects of particle size and hydrophobicity. Nano Lett 7(4):914–920. https://doi.org/10.1021/nl062743

Liu L, Ma P, Wang H, Zhang C, Sun H, Wang C, Song C, Leng X, Kong D, Ma G (2016) Immune responses to vaccines delivered by encapsulation into and/or adsorption onto cationic lipid-PLGA hybrid nanoparticles. J Control Release 225:230–239. https://doi.org/10.1016/j.jconrel.2016.01.050

Luebbert CC, Clarke TM, Pointet R, Frahm GE, Tam S, Lorbetskie B, Sauve S, Johnston MJW (2017) Nanoparticle size and production efficiency are affected by the presence of fatty acids during albumin nanoparticle fabrication. Plos One 12(12). https://doi.org/10.1371/journal.pone.0189814

Mallajosyula VVA, Citron M, Ferrara F, Lu X, Callahan C, Heidecker GJ, Sarma SP, Flynn JA, Temperton NJ, Liang X, Varadarajan R (2014) Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. Proc Nat Acad Sci 111(25):E2514. https://doi.org/10.1073/pnas.1402766111

Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF (2008) Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol 38(5):1404–1413. https://doi.org/10.1002/eji.200737984

Markovsky E, Koroukhov N, Golomb G (2007) Additive-free albumin nanoparticles of alendronate for attenuating inflammation through monocyte inhibition. Nanomedicine 2(4):545–553. https://doi.org/10.2217/17435889.2.4.545

Marty JJ, Oppenheim RC, Speiser P (1978) Nanoparticles—a new colloidal drug delivery system. Pharm Acta Helv 53(1):17–23

Mohammad-Beigi H, Shojaosadati SA, Morshedi D, Mirzazadeh N, Arpanaei A (2016) The effects of organic solvents on the physicochemical properties of human serum albumin nanoparticles. Iran J Biotechnol 14(1):45–50. https://doi.org/10.15171/ijb.1168

Mottram PL, Leong D, Crimeen-Irwin B, Gloster S, Xiang SD, Meanger J, Ghildyal R, Vardaxis N, Plebanski M (2007) Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus. Mol Pharmaceut 4(1):73–84. https://doi.org/10.1021/Mp060096p

Mozdzanowska K, Furchner M, Zharikova D, Feng J, Gerhard W (2005) Roles of CD4 + T-cell-independent and -dependent antibody responses in the control of influenza virus infection: evidence for noncognate CD4 + T-cell activities that enhance the therapeutic activity of antiviral antibodies. J Virol 79(10):5943–5951. https://doi.org/10.1128/jvi.79.10.5943-5951.2005

Munoz-Wolf N, Lavelle EC (2018) A guide to IL-1 family cytokines in adjuvanticity. FEBS J 285 (13):2377–2401. https://doi.org/10.1111/febs.14467

Murphy K, Travers P, Walport M, Janeway C (2012) Janeway's immunobiology. Garland Science, New York

Neumann S, Burkert K, Kemp R, Rades T, Dunbar PR, Hook S (2014) Activation of the NLRP3 inflammasome is not a feature of all particulate vaccine adjuvants. Immunol Cell Biol 92 (6):535–542. https://doi.org/10.1038/icb.2014.21

Oh JZ, Ravindran R, Chassaing B, Carvalho FA, Maddur MS, Bower M, Hakimpour P, Gill KP, Nakaya HI, Yarovinsky F, Sartor RB, Gewirtz AT, Pulendran B (2014) TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. Immunity 41(3):478–492

Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neill CP, Lee LK, Swartz MA, Hubbell JA (2007) Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotechnol 25(10):1159–1164. https://doi.org/10.1038/Nbt1332

Roh YG, Shin SW, Kim SY, Kim S, Lim YT, Oh BK, Um SH (2019) Protein nanoparticle fabrication for optimized reticuloendothelial system evasion and tumor accumulation. Langmuir 35(11):3992–3998. https://doi.org/10.1021/acs.langmuir.8b03776

Roldão A, Mellado MC, Castilho LR, Carrondo MJ, Alves PM (2010) Virus-like particles in vaccine development. Expert Rev Vaccines 9(10):1149–1176. https://doi.org/10.1586/erv.10.115

Russell CJ, Hu M, Okda FA (2018) Influenza hemagglutinin protein stability, activation, and pandemic risk. Trends Microbiol 26(10):841–853. https://doi.org/10.1016/j.tim.2018.03.005

Schellekens H, Jiskoot W (2013) Pharm Biotech (Springer)

Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A (2017) Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell 168(4):707–723. https://doi.org/10.1016/j.cell.2017.01.017

Sharp FA, Ruane D, Claass B, Creagh E, Harris J, Malyala P, Singh M, O'Hagan DT, Petrilli V, Tschopp J, O'Neill LAJ, Lavelle EC (2009) Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci USA 106(3):870–875. https://doi.org/10.1073/pnas.0804897106

Siegel S, Wagner A, Kabelitz D, Marget M, Coggin J Jr, Barsoum A, Rohrer J, Schmitz N, Zeis M (2003) Induction of cytotoxic T-cell responses against the oncofetal antigen-immature laminin receptor for the treatment of hematologic malignancies. Blood 102(13):4416–4423. https://doi.org/10.1182/blood-2003-01-0198

Singh S (2007) Vaccine adjuvants and delivery systems. Wiley-Interscience, Hoboken, N.J.

Snapper CM (2018) Distinct immunologic properties of soluble versus particulate antigens. Front Immunol 9:598–598. https://doi.org/10.3389/fimmu.2018.00598

Stano A, Nembrini C, Swartz MA, Hubbell JA, Simeoni E (2012) Nanoparticle size influences the magnitude and quality of mucosal immune responses after intranasal immunization. Vaccine 30(52):7541–7546. https://doi.org/10.1016/j.vaccine.2012.10.050

Storp B, Engel A, Boeker A, Ploeger M, Langer K (2012) Albumin nanoparticles with predictable size by desolvation procedure. J Microencapsul 29(2):138–146. https://doi.org/10.3109/02652048.2011.635218

Subara D, Jaswir I, Alkhatib MFR, Noorbatcha IAN (2017) Process optimization for the production of fish gelatin nanoparticles. Int Food Res J 24:S501–S507

Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG (2012) Into the eye of the cytokine storm. Microbiol Mol Biol Rev MMBR 76(1):16–32. https://doi.org/10.1128/MMBR.05015-11

Tompkins SM, Zhao Z-S, Lo C-Y, Misplon JA, Liu T, Ye Z, Hogan RJ, Wu Z, Benton KA, Tumpey TM, Epstein SL (2007) Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg Infect Dis 13(3):426–435. https://doi.org/10.3201/eid1303.061125

Tran KK, Shen H (2009) The role of phagosomal pH on the size-dependent efficiency of cross-presentation by dendritic cells. Biomaterials 30(7):1356–1362. https://doi.org/10.1016/j.biomaterials.2008.11.034

Treanor JJ, Tierney EL, Zebedee SL, Lamb RA, Murphy BR (1990) Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J Virol 64(3):1375–1377

Tsoras AN, Champion JA (2018) Cross-linked peptide nanoclusters for delivery of oncofetal antigen as a cancer vaccine. Bioconjug Chem 29(3):776–785. https://doi.org/10.1021/acs.bioconjchem.8b00079

Tsoras AN, Wong KM, Paravastu AK, Champion JA (2020) Rational design of antigen incorporation into subunit vaccine biomaterials can enhance antigen-specific immune responses. Front Immunol. https://doi.org/10.3389/fimmu.2020.01547

Vaine CA, Patel MK, Zhu JT, Lee E, Finberg RW, Hayward RC, Kurt-Jones EA (2013) Tuning innate immune activation by surface texturing of polymer microparticles: the role of shape in inflammasome activation. J Immunol 190(7):3525–3532. https://doi.org/10.4049/jimmunol.1200492

Wang W, Singh SK, Li N, Toler MR, King KR, Nema S (2012) Immunogenicity of protein aggregates–concerns and realities. Int J Pharm 431(1–2):1–11. https://doi.org/10.1016/j.ijpharm.2012.04.040

Wang L, Hess A, Chang TZ, Wang YC, Champion JA, Compans RW, Wang BZ (2014) Nanoclusters self-assembled from conformation-stabilized influenza M2e as broadly cross-protective influenza vaccines. Nanomed-Nanotechnol 10(2):473–482. https://doi.org/10.1016/j.nano.2013.08.005

Wang L, Chang TZ, He Y, Kim JR, Wang S, Mohan T, Berman Z, Tompkins SM, Tripp RA, Compans RW, Champion JA, Wang B-Z (2017) Coated protein nanoclusters from influenza H7N9 HA are highly immunogenic and induce robust protective immunity. Nanomed Nanotechnol Biol Med 13(1):253–262. https://doi.org/10.1016/j.nano.2016.09.001

Weber C, Coester C, Kreuter J, Langer K (2000) Desolvation process and surface characterisation of protein nanoparticles. Int J Pharm 194(1):91–102. https://doi.org/10.1016/S0378-5173(99)00370-1

Willett TA, Meyer AL, Brown EL, Huber BT (2004) An effective second-generation outer surface protein A-derived Lyme vaccine that eliminates a potentially autoreactive T cell epitope. Proc Natl Acad Sci USA 101(5):1303–1308. https://doi.org/10.1073/pnas.0305680101

Wu P, Peng Z, Fang VJ, Feng L, Tsang TK, Jiang H, Lau EHY, Yang J, Zheng J, Qin Y, Li Z, Leung GM, Yu H, Cowling BJ (2016) Human infection with influenza A(H7N9) virus during 3 major epidemic waves, China, 2013–2015. Emerg Infect Dis 22(6):964–972. https://doi.org/10.3201/eid2206.151752

Xiang SD, Scholzen A, Minigo G, David C, Apostolopoulos V, Mottram PL, Plebanski M (2006) Pathogen recognition and development of particulate vaccines: does size matter? Methods 40(1):1–9. https://doi.org/10.1016/j.ymeth.2006.05.016

Xiang SD, Wilson K, Day S, Fuchsberger M, Plebanski M (2013) Methods of effective conjugation of antigens to nanoparticles as non-inflammatory vaccine carriers. Methods 60(3):232–241. https://doi.org/10.1016/j.ymeth.2013.03.036

Zhang Y, Xu C, Zhang H, Liu GD, Xue C, Cao Y (2019) Targeting hemagglutinin: approaches for broad protection against the influenza A virus. Viruses 11(5):405. https://doi.org/10.3390/v11050405

Zhao L, Seth A, Wibowo N, Zhao C-X, Mitter N, Yu C, Middelberg APJ (2014) Nanoparticle vaccines. Vaccine 32(3):327–337. https://doi.org/10.1016/j.vaccine.2013.11.069

Zheng M, Luo J, Chen Z (2014) Development of universal influenza vaccines based on influenza virus M and NP genes. Infection 42(2):251–262. https://doi.org/10.1007/s15010-013-0546-4

Zhu W, Pewin W, Wang C, Luo Y, Gonzalez GX, Mohan T, Prausnitz MR, Wang BZ (2017) A boosting skin vaccination with dissolving microneedle patch encapsulating M2e vaccine broadens the protective efficacy of conventional influenza vaccines. J Control Release 261:1–9. https://doi.org/10.1016/j.jconrel.2017.06.017

Zolnik BS, Gonzalez-Fernandez A, Sadrieh N, Dobrovolskaia MA (2010) Minireview: nanoparticles and the immune system. Endocrinology 151(2):458–465. https://doi.org/10.1210/En.2009-1082