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Protein and Peptide Biomaterials for Engineered Subunit Vaccines and Immunotherapeutic Applications

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Abstract

Although vaccines have been the primary defense against widespread infectious disease for decades, there is a critical need for improvement to combat complex and variable diseases. More control and specificity over the immune response can be achieved by using only subunit components in vaccines. However, these often lack sufficient immunogenicity to fully protect, and conjugation or carrier materials are required. A variety of protein and peptide biomaterials have improved effectiveness and delivery of subunit vaccines for infectious, cancer, and autoimmune diseases. They are biodegradable and have control over both material structure and immune function. Many of these materials are built from naturally occurring self-assembling proteins, which have been engineered for incorporation of vaccine components. In contrast, others are de novo designs of structures with immune function. In this review, protein biomaterial design, engineering, and immune functionality as vaccines or immunotherapies are discussed.

INTRODUCTION

Although most approved prophylactic vaccines use whole attenuated or inactivated pathogens, safety and efficacy concerns remain owing to the lack of control over response to certain portions of the pathogen and/or coadministered adjuvant, as well as the small possibility of pathogen reversion (1, 2). Allergy, off-target responses, side effects, and lack of lasting or universal protection across variable and complex pathogen strains are important unsolved issues (1–4). These issues are not limited to prophylactic vaccines but apply to any therapy requiring target-specific immune activation or tolerance, including cancer and autoimmune therapies. Cancer antigens are often similar to self-antigens or are not unique to cancer cells, so it is critical to have specific targeted control over the immune response (5, 6). Likewise, autoimmune disease may be exacerbated by immunotherapies if the required tolerogenic response to self-antigens is insufficient (7, 8).

Since use and study of vaccines began in the eighteenth century (9), the complex functions of the immune system and vaccines are much better understood (10). Upon administration, vaccines encounter antigen presenting cells (APCs), phagocytic cells that surveil and sample their environment. Dendritic cells (DCs) are APCs that play a major role in adaptive immune responses. They internalize extracellular materials like dead cells or foreign material, including vaccines, and process the protein antigens to present on their surfaces via major histocompatibility complex (MHC) type II. Vaccines can also directly encounter B cells, which are APCs with immunoglobulin surface receptors that bind foreign molecules and activate danger signals. This leads to increased antigen presentation, proliferation, and antibody secretion. Most cells, including APCs, present processed cytosolic peptides, containing pathogen proteins if they are infected, on MHC type I surface proteins. Pathogens, and ideally vaccines, contain pathogen-associated molecular patterns (PAMPs), which bind pattern recognition receptors (PRRs) and activate APC production of cytokines and costimulatory surface proteins essential for antigen presentation to T cells. T cells with T cell receptors (TCRs) that recognize MHC/antigen complexes proliferate and differentiate into effector cells. $CD4^+$ T cells differentiate into many subsets of T helper (Th) cells or T regulatory (Treg) cells. They provide feedback to increase and direct B and T cell proliferation, modulating the immune response for different types of infection or dangers. $CD8^+$ T cells differentiate into cytotoxic T cells (CTLs) upon TCR binding and activation. CTLs eliminate infected cells when their TCR binds MHC I/antigen complexes. Several potential interactions in this process are summarized in **Figure 1**. Whole-pathogen vaccines contain antigens and PAMPs necessary for an adaptive immune response. However, some components may be unnecessary or excessive. Better understanding of the adaptive immune response has enabled components from multiple pathogens or synthetic materials to be combined to produce different activation profiles, ultimately controlling the immune response type and strength. This facilitates use of only necessary portions of pathogens, called subunits, making vaccines safer and more tunable.

Subunit vaccines use parts of a pathogen or antigen recognizable as foreign and, therefore, have potential to induce an adaptive immune response. Subunits can be membranes, capsules, toxins, polysaccharides, proteins, or small peptides. The research shift toward subunit vaccines is likely due to advantages including safety, control over the strength and type of immune response, and potential to protect against multiple pathogen strains (11). Generally, the more specific the subunit, the more control. However, smaller and more specific subunits are often less immunogenic.

To address this, many approaches are employed in subunit vaccine design to improve their immunogenicity. A common solution is to coadminister an adjuvant, an immune stimulatory agent. Although effective in producing costimulatory signals necessary for a strong immune response, very few are approved because of side effects and dangers associated with uncontrolled immune stimulation (12, 13). This is likely because individuals react differently to adjuvants (9) and because stimulation does not always occur within the same cell that receives the antigen (8, 14, 15).

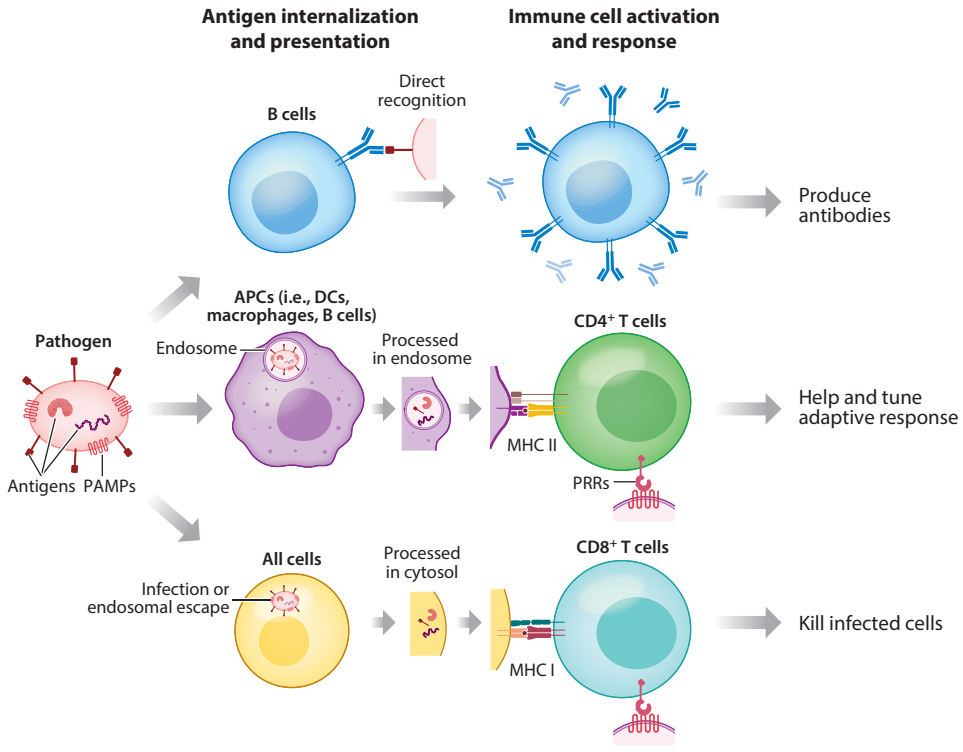

Figure 1

Summary of possible adaptive immune response pathways. Abbreviations: APC, antigen presenting cell; DC, dendritic cell; MHC, major histocompatibility complex; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor.

This can lead to immunotolerance to the target antigen, cytokine storm, or unwanted reactions to non-target or self-antigens (4, 9, 16). Conjugation of antigen to adjuvant can address some of these issues (17, 18). However, other reasons for low subunit immunogenicity, such as low internalization or rapid clearance from the body owing to subunit size or surface characteristics, remain generally unsolved by conjugation. Use of carrier materials enables tuning of size, shape, and function, which helps modulate diffusion, circulation time, biodistribution, and targeting of certain cell types. There exists a diverse array of delivery platforms customizable for different types of antigens and specific immunological challenges. They can also have a self-adjuvanting effect, which mitigates the need for strong adjuvants. Adjuvants may be incorporated into materials with antigen, however, ensuring joint stimulation. A wide range of materials used in drug delivery have also been used for vaccine delivery, including polymers, inorganics, polysaccharides, and lipids, which have been comprehensively reviewed recently (19–23).

Synthetic carriers primarily serve a structural purpose, for example, to form a structure to increase APC internalization. They do not typically interact functionally with antigen or have specific binding locations for antigens or cell receptors. In contrast, protein biomaterials can serve a structural and functional purpose, such as carrying antigen and engaging PRRs. Knowledge of protein–protein interactions and protein self-assembly, combined with protein engineering and chemical biology tools, enables protein vaccine design to control interactions between carrier proteins, antigens, and adjuvants, and between the assembled protein material and relevant immune cells. The materials discussed in this review, outlined in **Table 1**, present a range of recent

Table 1 Summary of engineered protein biomaterial assembly, immune applications, and responses evaluated

Assembly type	Material subset	Type of antigen incorporation	Application	Antigen(s)/adjuvant	Number/type immuniza-tions	Immune response observed
Biomimetic	T7 phage VLP	Fusion protein	Influenza (27)	HA, M2e	1, i.p. + 2, s.c.	N-Ab, Chal.
	Hepatitis B VLP		Melanoma (28)	8 melanoma antigens	1, s.c.	Ab
	P22 phage		Respiratory syncytial virus (30)	M, M2	2, i.n.	CD8, CD4, Chal.
	Qβ phage	Click chemistry	Pneumonia (31)	Tetrasaccharide/NKT cell adjuvant	2, i.m.	N-Ab, Chal.
			Leishmania (32, 33)	Trisaccharide	2, s.c.	N-Ab, Chal., CD4
			Cancer (34)	MUC-1	3, s.c.	N-Ab, CTL kill
	Filamentous phage fd	Fusion protein, insertion	Melanoma (35)	OVA, α-galactosylceramide	1, i.t.	iNKT, Chal.
	Cowpea mosaic virus/potato virus X	Crosslinking	Breast/ovarian cancer (36)	Her2 peptide	1, s.c.	LN, APC, Ab
	E2 cage		Melanoma (37, 38)	gp100/CpG	1, s.c.	CTL, Chal.
	Human ferritin cage	Fusion protein	Influenza (40)	M2e	3, i.n.	T cell, Chal.
	Thermotoga maritima encapsulin		Melanoma (42)	SIINFEKL/Poly(I:C)	1–2, s.c.	CD8, Chal.
			Influenza (43)	M2e, GFP/CFA	3, s.c.	Ab
	Vault	Fusion protein	*Chlamydia* (47)	PmpG	3, i.n.	CD4, Chal.
Structurally ordered assemblies	β-sheet fibers	Fusion peptide	*Mycobacterium tuberculosis* (55)	ESAT6, TB10.4, Ag85B/Pam2Cys	1, s.c., + 1, i.n.	Ab, CD8, Chal.
			Staphylococcus aureus (52)	$OVA_{323–339}$, E214/PADRE	3, s.c.	Ab, T cell
			Malaria (53)	$(NANP)_3$	2, s.c.	Ab
			Influenza (51)	$PA_{224–23}$	2, i.n. or s.c.	Ab, CD8, T cell, Chal.
			OVA model (48)	$OVA_{323–339}$	2, s.c.	s.c.: CD8, i.n.: CD8, CTL kill
	α-helix fibers		Cancer (EGFRvIII), OVA model (59)	PEPvIII, SIINFEKL/ PADRE	3, s.c.	EGFRvIII: Ab, CD4 OVA: APC, CD8
	α-helix self-assembling peptide nanoparticles		Malaria (66)	PbCSP	3, i.p.	Ab, CD4, Chal.
			Toxoplasmosis (63)	5 $CD8^+$ T cell epitopes/ PADRE, flagellin, GLA-SE	3, i.m.	CD8, Chal.
			Toxoplasmosis (62)	GRA7/PADRE	3, s.c.	CD4, CD8, Chal.
			HIV-1 (65)	2F5, 4E10/IFA (i.p. only)	1, i.p. or i.d.	Ab
			SARS (61)	HRC1	4, i.p.	Ab
			Influenza (64)	M2e/CFA+IFA	3, i.m.	Ab, N-Ab, Chal.
			Influenza (67)	Helix C, M2e/flagellin, PADRE	3, i.m.	N-Ab

(*Continued*)

Table 1 (*Continued*)

Assembly type	Material subset	Type of antigen incorporation	Application	Antigen(s)/adjuvant	Number/type immuniza-tions	Immune response observed
Hydrophobic assemblies	Peptide-lipid amphiphile	Chemical conjugation	Group A streptococcus (73)	J14	3, s.c.	Ab
			Cervical cancer (72)	E6, E7	1, s.c.	CD8, Chal.
			OVA model (74)	$OVA_{323–339}$	2, s.c.	Ab, LN
			OVA model (76)	$OVA_{323–339}$ /Pam2Cys	2, s.c.	APC, Ab, T cell
			Group A streptococcus (75)	J8/MPLA, Pam2Cys	4, s.c.	Ab
	Peptide-lipid nanodisc	Cysteine addition/ disulfide binding	OVA model, melanoma (78)	SIINFEKL, Adpgk, Trp2, M27, M30/CpG	3, s.c.	APC, LN, CD4, CD8, Chal.
			Melanoma (79)	Adpgk, Trp2, M27, M30/CpG	3, s.c. or i.m.	LN, CD8, T cell, Chal.
	Peptide–polymer amphiphile	Chemical conjugation	Cervical cancer (80, 81)	E6, E7	1–3, s.c.	Chal.
	Nanoclusters	Crosslinking	Influenza (85)	M2e/CpG	3, i.n.	Cross-reactive Ab, T cell, Chal.
		Cysteine addition/ crosslinking	Hematologic cancer (88)	Oncofetal antigen	1, i.d.	APC, LN
		Crosslinking	OVA model (89, 90)	OVA/IgM, flagellin	2, i.m.	APC, Ab, affinity mat., T cell
			Influenza (86)	HA	1–2, i.m. or i.n.	APC, N-Ab, Chal.
			Influenza (87)	Nucleoprotein, tM2e/monovalent inactivated vaccine	2, i.m. or 1, i.m. + 1, i.d.	APC, N-Ab, T cell, Chal.
Electrostatic assemblies	Immune poly-electrolyte monolayers	Electrostatic interactions	Diphtheria (91)	Diphtheria toxoid/trimethyl chitosan	3, i.d. or s.c.	Ab, N-Ab
		Arginine addition/ electrostatic interactions	OVA model (93)	SIINFEKL + poly(I:C)	3, i.d.	APC, CD8
			Melanoma (92)	Trp2/CpG	2, i.d.	APC, CD8, Chal.
			Multiple sclerosis (8)	MOGR/GpG	2, s.c.	APC, CD4, Treg proliferation, Chal.
	Polyplex		Multiple sclerosis (94)	MOGR/GpG	1–3, s.c.	APC, CD4, Chal.

Abbreviations: Ab, antigen-specific antibodies; APC, antigen presenting cell maturation and/or antigen presentation; CD4/CD8/iNKT, $CD4^+$/$CD8^+$/ invariant natural killer T cell activation and/or proliferation; CFA, complete Freund's adjuvant; Chal., challenge protection; CTL kill, antigen-specific CTL killing ability; HA, hemagglutinin; i.d., intradermal; IFA, incomplete Freund's adjuvant; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratumoral; LN, lymph node trafficking; M2e, extracellular domain of matrix 2 protein; N-Ab, pathogen-neutralizing antibodies; NKT, natural killer T cell; OVA, ovalbumin; SARS, severe acute respiratory syndrome; s.c., subcutaneous; T cell, general T cell activation from splenocytes or lymph nodes; VLP, virus-like particle.

approaches combining proteins or protein conjugates to create desired structures and immune functions. Broadly, there are two categories: biomimetic and rationally designed. Biomimetic protein nanomaterials originate from natural self-assembling protein nanostructures, such as viruses or protein cages, and have been engineered to incorporate antigen for use as vaccines. Rationally designed protein materials are not based on any naturally occurring protein scaffolds and exhibit a wide range of properties. They include peptides designed with secondary structures specific for self-assembly and peptides designed to assemble less specifically via hydrophobic or electrostatic interactions. This review describes the functions, benefits, and limitations of recently developed protein biomaterial assemblies from an immunoengineering standpoint and discusses current challenges.

BIOMIMETIC ENGINEERED PROTEIN MATERIALS

This section covers protein nanomaterial vaccines that most closely resemble traditional whole-pathogen vaccines. Virus-like particles (VLPs) are close mimics of their infectious counterparts, chemically and physically. From a materials perspective, VLPs are protein containers, and vaccine engineering has also been applied to protein cages and vaults, naturally occurring containers with no pathogenic origin. These materials mirror viruses in their structural symmetry and exhibit a range of sizes and geometries, which can be selected and repurposed for a particular vaccine application.

Virus-Like Particles

VLPs are noninfectious, highly homogeneous nanoparticles that self-assemble from viral structural proteins and often do not contain nucleic acid cargo (24). VLPs present antigen and interact with immune cells similar to viruses, making them beneficial for use as vaccines. Gardasil® and Gardasil®9 are Food and Drug Administration (FDA)-approved adjuvanted human papillomavirus (HPV) VLP vaccines produced by Merck & Co., Inc., for protection against infection and cancers caused by infection (25, 26). New VLP designs seek to eliminate the need for adjuvants and give more control over the immune response induced. For VLP vaccines made from the capsid of the target pathogen, surface antigens on the VLP are typically modified to increase or bias the immune response. Alternatively, a VLP made from an irrelevant virus can be engineered to present antigens from the target pathogen. This strategy has been applied to many bacterial (bacteriophage) and plant viruses. Because VLPs have been reviewed extensively and there are many minor variations of particular VLPs (24), this review covers very recent progress in modifying VLPs with various antigen types using different fabrication strategies.

Protein antigens. Antigens are most frequently incorporated on the surface of VLPs to increase their recognition. They can be genetically fused or chemically conjugated to the capsid to produce dense arrays of surface antigen. The hepatitis B core protein (Hbc), which assembles to form the viral capsid, is challenging to modify recombinantly, as VLP assembly is easily disrupted. Hbc was fused into a dimer connected by a short linker. Influenza antigens hemagglutinin (HA) and extracellular domain of matrix 2 protein (M2e) from three flu strains were inserted within the Hbc domains to present on the VLP exterior (27). With this design, VLPs properly assembled, and three immunizations in mice induced antibodies against M2e and five different group 1 HA types and protected completely from H1N1 challenge. However, M2e-only VLPs also protected all animals.

VLPs are not limited to human viruses. Mutated neoantigens from the B16-F10 melanoma model were incorporated on the T7 bacteriophage surface through genetic fusion, 415 antigen

copies per VLP (28). Although most cancer vaccines focus on T cell immunity, humoral responses are also valuable. Small, 11-residue antigens induced greater antibody titers than larger, 34-residue antigens. Likewise, there was no benefit in presenting linear trimers of the 11-residue antigens. This contrasts with 23-residue M2e influenza antigen, which has greater immunogenicity when three copies are linearly connected and presented on influenza VLPs, suggesting that both the antigen and carrier may affect optimal presentation (29). When 8 different antigens were tested in a single dose of mixed T7, 5 elicited antibody responses and 3 did not, indicating T7 phages are not entirely capable of overcoming the natural immunogenic variability of the peptide antigens. In T7 vaccinated mice, the draining lymph nodes had a high percentage of B cells with class-switched antibodies, expansion of clonal B cell populations, and reduced antibody diversity, evidence of a robust antibody response to the 5 immunogenic antigens.

One advantage of VLPs is the repetitive, dense presentation of antigen recognizable by B cells, and much infectious disease VLP research assesses only the humoral response. However, VLPs can also induce infection-protective T cell responses via APC interactions. *Salmonella typhimurium* bacteriophage P22 VLPs were engineered to co-encapsulate two respiratory syncytial virus (RSV) protein antigens, matrix (M) and matrix 2 (M2), which generate $CD8^+$ and $CD4^+$ T cell responses, respectively (30). In an atypical VLP approach, RSV antigens were not presented on the surface. Instead, they were genetically fused to P22 scaffold protein, which is naturally encapsulated by P22 coat protein, to produce ~60-nm P22 VLPs containing ~142 copies of fusion protein with an exact 1:1 M:M2 ratio. When mice were vaccinated and boosted intranasally, antigen-specific $CD8^+$ and $CD4^+$ T cell responses were observed. Upon RSV challenge, vaccinated mice exhibited 1,000-fold lower viral load and greater frequency and number of antigen-specific $CD8^+$ T cells in their lungs compared with unvaccinated or empty P22–vaccinated animals.

Glycan antigens. VLPs have also been used recently to induce protective antiglycan antibodies, which are difficult to achieve because polysaccharide antibody responses are T cell independent, eliminating B cell activation signals needed from Th cells for affinity maturation and memory. However, T cells are capable of recognizing some glycopeptides and glycolipids containing small saccharides. Two azide-functionalized tetrasaccharides from different *Streptococcus pneumoniae* serotypes were conjugated by copper-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry to modified Qβ bacteriophage VLPs functionalized with alkyne groups on surface-accessible amines (31). By controlling reaction stoichiometry, homogeneous protein-conjugated glycan nanoparticles were produced containing 20–200 presented glycans. Mice immunized and boosted with VLPs containing 80 glycans or more and combined with a Natural Killer (NK) T cell adjuvant produced protective antiglycan antibodies. When adoptively transferred, these antibodies provided significantly improved protection from *S. pneumoniae* challenge, whereas the FDA-approved vaccine, Prevnar 13, did not. Critically, these nanomaterials induced T cell help, which is essential for a robust, high-affinity humoral response. This approach has been extended to other polysaccharides, including the trisaccharide Galα(1,3)Galβ(1,4)GlcNAcα (α-gal) found on *Leishmania* parasites (32). In this case, Qβ was functionalized with azide, and alkyne-functionalized α-gal was conjugated by CuAAC for 540+/−40 copies per VLP (33). Subcutaneous vaccination with two boosts induced high titers of anti-α-gal antibodies, and mice had undetectable or nearly undetectable levels of two *Leishmania* strains following challenge. Control glucose-functionalized Qβ did not induce α-gal antibodies or protect.

Qβ was also recently used to present glycopeptide antigen MUC-1, which is overexpressed on many cancer cell types (34). Glycosylated and non-glycosylated azide-functionalized MUC-1 peptides were conjugated at high density to alkyne-functionalized Qβ VLPs. When mice were

immunized and boosted twice, high MUC-1 glycopeptide antibody titers were generated that induced selective killing via complement-mediated cytotoxicity in vitro. Additionally, CTLs were induced in vivo and exhibited antigen-specific killing of tumor cells. Qβ without antigen induced Qβ-specific antibody production but did not induce CTLs.

In an alternative approach to activate T cells with glycan antigens, α-galactosylceramide (αGC), a glycolipid antigen recognized by antitumor invariant NK T (iNKT) cells, was immobilized by lipid insertion in filamentous phage fd alongside model ovalbumin (OVA) peptide antigen fused to the phage coat proteins (35). This vaccine resulted in presentation to and repeated stimulation of iNKT cells by DCs, whereas soluble αGC induced iNKT cell anergy. In a B16-OVA melanoma model, both αGC-only phage and αGC-OVA phage injected intratumorally significantly reduced tumor volume relative to soluble antigen controls.

Antigen delivery effects. Aside from the antigens presented, VLP physical properties can significantly affect the immune response. Her2 cancer antigen peptides were conjugated via terminal cysteines to surface lysines on differently shaped plant VLPs. Filamentous potato virus X (PVX), which has dimensions of 515 nm by 13 nm, had ~70% greater antigen surface loading than cowpea mosaic virus (CPMV), which is composed of 30-nm spheres (36). The spherical VLPs exhibited increased lymph node trafficking and retention, APC uptake and activation, and Her2-specific antibodies. This is likely because PVX naturally avoids endo- and phagocytosis and traffics to B cell–rich regions. This demonstrates that viruses, and VLPs derived from them, may also have evolved biases toward targeting certain cell types that should be carefully evaluated before selection for particular applications.

Cages and Vaults

Protein cages and vaults are similar to VLPs in their symmetric, nanoscale, container-like geometries. However, they are derived from prokaryotes and eukaryotes that use these structures for functions such as biocatalysis or molecular transport. Like VLPs, cages and vaults can be modified recombinantly or chemically with antigens.

E2, a 25-nm cage derived from the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*, has been investigated for presentation of both peptide antigens and CpG nucleic acid adjuvant using chemical crosslinking. A cysteine mutation introduced on the internal cavity surface enabled covalent encapsulation of aldehyde-modified CpG, while gp100, a melanoma peptide antigen with a terminal cysteine, was conjugated to native external surface lysines (37). This combination led to an enhanced gp100-specific CTL response compared with soluble components and delayed tumor growth when mice were immunized and boosted prior to tumor inoculation. This system was also applied to two human antigens frequently expressed in glioblastomas and melanomas (38). Transgenic mice with human MHC I that were immunized and boosted with E2 vaccines containing one type of antigen and CpG exhibited increased T cell activation and antigen-specific target cell lysis. Importantly, when a mixture of two different antigen-loaded E2 vaccines was given, there was no decrease in T cell response, and an improvement in lysis ability was seen. When CpG was conjugated to surface E2 lysines via a polyethylene glycol (PEG) linker, greater E2 uptake by APCs and increased lymph node accumulation were observed (39).

Human heavy chain ferritin is an iron storage protein that assembles into 13-nm cages and was recombinantly fused to three linear repeats of the conserved influenza peptide M2e for cage surface presentation, as illustrated in **Figure 2*a*** (40). Mice were vaccinated and boosted twice, which induced M2e serum immunoglobulin G (IgG) and specific T lymphocytes in the spleen.

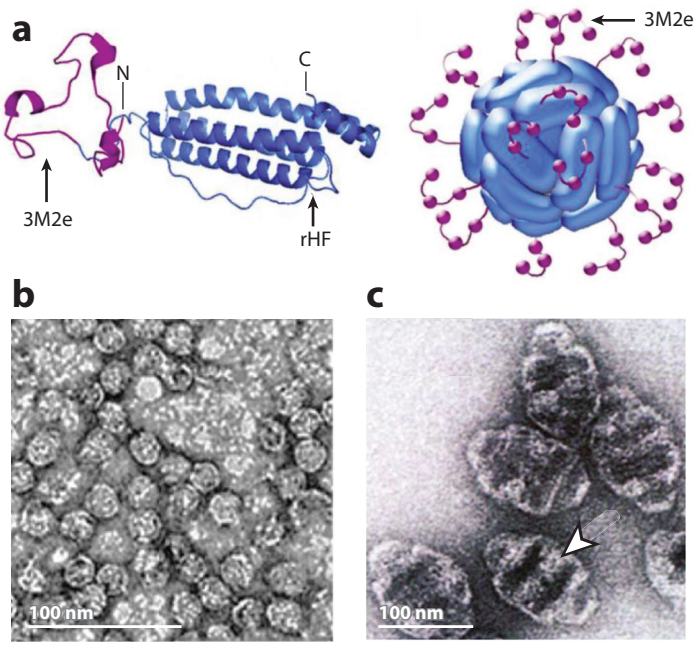

Figure 2

Example fusion protein designs and assembled structures of protein cages. (*a*) Fusion protein schematic of human heavy chain transferrin cage with three linear M2e antigens (3M2e, *purple*) fused to the N terminus of each recombinant human heavy chain transferrin protein (rHF, *blue*) to position the antigens on the cage exterior (reprinted with permission from Reference 40). (*b*) Encapsulin (reprinted with permission from Reference 42, copyright 2018 American Chemical Society) and (*c*) vault (45; **https://creativecommons.org/licenses/by/4.0/**) structures visualized by transmission electron microscopy.

Only when mice were vaccinated intranasally, and not intramuscularly, were IgG and IgA detected in nasal washes and animals protected against challenge from different virus subtypes.

Encapsulins, shown in **Figure 2*b***, are a class of icosahedral cage structures from bacteria and archaea, with sizes and functions dependent on the species (41). SIINFEKL, the model $CD8^+$ T cell antigen peptide from OVA, was recombinantly fused in three different positions to the *Thermotoga maritima* encapsulin protein, resulting in different presentation locations of 60 peptide copies on each ~24-nm cage (42). The exterior-facing C-terminal fusion performed better in ex vivo DC stimulation of $CD8^+$ T cell proliferation than the interior-facing N-terminal and loop 42 fusions. When vaccinated subcutaneously with poly(I:C) adjuvant, C-terminal SIINFEKL-fused encapsulins induced activation of antigen-specific $CD8^+$ T cells. In both a prime and boost prophylactic and single-administration tumor therapeutic regimen, SIINFEKL encapsulin reduced B16-OVA melanoma tumor growth similarly to soluble whole OVA protein. M2e, which has much lower immunogenicity than OVA, was also recombinantly fused to the surface of *T. maritima* encapsulins (43). Green fluorescent protein (GFP) was simultaneously encapsulated in the cages by fusion to a protein tag that binds the internal cavity. In a three-dose subcutaneous vaccination with Freund's adjuvant, the M2e antibody responses of encapsulin were comparable to those of similarly sized MS2 bacteriophage VLPs displaying the same M2e antigen. However, the encapsulin induced lower levels of anti-GFP antibodies compared with soluble GFP vaccination. These studies demonstrate the importance of external placement of antigens on encapsulins whether for a

cellular (SIINFEKL) or humoral (M2e, GFP) response, in contrast to the M22 VLP that induced T cell responses with encapsulated antigens (30).

Protein vaults are eukaryotic cage-like structures assembled from ribonucleoproteins. As shown in **Figure 2*c***, they are barrel-shaped structures, approximately 70 nm × 40 nm × 40 nm, made from major vault protein (MVP) (44). Antigen fusion with mINT, the MVP interaction protein, enables noncovalent association of antigen with the vault interior and encapsulation during vault assembly. In earlier work, vaults were used to simultaneously encapsulate *Chlamydia* major outer membrane protein (MOMP) and present IgG Fc binding peptides on the surface (45). In this example, as well as with OVA encapsulation, primarily T cell responses were induced by vaults (45, 46). A peptide from polymorphic membrane protein G-1 (PmpG) of *Chlamydia muridarum* has also been recently encapsulated in vaults (47). With three intranasal vaccinations, PmpG vaults reduced genital bacterial load and inflammation following challenge and increased antigen-specific $CD4^+$ T cell responses. Interestingly, MOMP vaults induced anti-vault antibodies, but PmpG vaults did not.

RATIONALLY ENGINEERED PROTEIN MATERIALS

Given the successes and challenges of manipulating biomimetic protein assemblies, many de novo protein assemblies have been engineered with different properties for immune applications. In this class of materials, protein or peptide design must achieve self-assembly with both desired material physicochemical properties and desired immune interactions. Though the molecular interactions to induce assembly are similar to those found in naturally self-assembling materials, the design to achieve these interactions arguably requires more engineering effort, as a bottom-up design approach. However, there is complete control over the components that are used, minimizing unnecessary or uncharacterized molecules. In this section, we discuss the rational design and evaluation of protein carriers that have been engineered specifically for vaccine improvement through antigen administration in an assembled material form.

Structurally Ordered Assemblies

Proteins assemble first through the formation of secondary structure, where the primary sequence of amino acids interacts nonlinearly to create β-sheets, α-helices, or random coils. These secondary structures further interact to form a macromolecular tertiary structure. Quaternary structure is formed when the macromolecular interactions involve multiple proteins and assemble into multimeric structures, such as in VLPs and cages. Using β-sheet or α-helical secondary structure motifs, peptides have been rationally engineered to achieve modular, tunable self-assembly into predictable quaternary structures that present immune epitopes.

β-Sheet. The engineered β-sheet-forming peptide Q11 (QQKFQFQFEQQ), which assembles into nanofibers, has been evaluated extensively as a vaccine platform. Solid-state synthesis is used to append Q11 to a peptide antigen C terminus, connected by a short linker sequence. Q11 forms β-sheet stacks that assemble into fibrils with the epitope sticking outward from the fibril surface. This method of antigen incorporation is robust, as fibrils have been assembled with a variety of peptides, including model OVA epitopes (48–51), universal Th cell epitope PADRE (52), a malarial *Plasmodium falciparum* circumsporozoite epitope (53), *Mycobacterium tuberculosis* epitopes (54, 55), *Staphylococcus aureus* epitope E214 (52), and H1N1 influenza acid polymerase epitope $PA_{224–233}$ (51). β-Sheet-forming peptide subunits with different epitopes can also be mixed at desired ratios to form multi-epitope fibrils. For example, Q11 was synthesized with PADRE, to increase T cell

activity, and E214, an *S. aureus* B cell epitope, coassembling into fibers that elicited E214 antibody responses and T cell activation cytokines when restimulated with PADRE (52). Furthermore, adjuvanting properties of fibrils can be tuned by modifying the Q11 sequence to switch the neutrally charged sheet to negatively charged, or by glutamine dimethylation to disrupt β-sheet formation. When fibers were coassembled with $OVA_{323–339}$-Q11 and charge- or structure-modified Q11, there was a significant decrease in $OVA_{323–339}$-specific antibody titers (56). This was likely due to decreased internalization of negatively charged or poorly formed fibers. Another β-sheet-forming peptide, KFE8 (FKFEFKFE), was shown to have similar adjuvanting effects to Q11, indicating the specific Q11 sequence is less important than its self-assembled structure. Mice immunized subcutaneously with Q11 or KFE8 alone did not produce significant antibodies (48). Mice immunized intranasally with $PA_{224–233}$-Q11 fibers showed antigen-specific $CD8^+$ T cell activation and PA-specific killing ability compared with saline control (51). $OVA_{323–339}$-KFE8 nanofibers administered orally were able to induce mucosal anti-OVA antibodies. When these fibers were encapsulated in 2-μm $CaCO_3$ microparticles, this response increased significantly, likely due to gastric protection and mucosal barrier penetration by the particles (57).

α-Helix. Although α-helix coiled coils are normally smaller structures, they can also be engineered to form fibrils that induce immune activation, as is the case for α-helical peptide Coil29 (58, 59). Similar to Q11, solid-state synthesis was used to fuse Coil29 with peptide epitopes via a short linker sequence. Following ordered assembly of Coil29 into fibers, the epitopes were displayed perpendicular to the fiber length. The epitopes [tumor-associated antigen B cell epitope derived from epidermal growth factor receptor class III variant (EGFRvIII), SIINFEKL, and PADRE] were chosen to assess differences in immune response based on different combinations. PADRE-conjugated fibers were shorter owing to PADRE's hydrophobicity, which likely interfered with fiber assembly. However, PADRE-Coil29 monomers mixed with 20:1 EGFRvIII-Coil29 monomers looked similar to original Coil29 fibers. EGFRvIII peptide–specific titers were significant after vaccination in mice but were sustained only when it was coassembled with PADRE, implying that humoral responses require a T cell epitope. SIINFEKL-Coil29 fibers alone, however, induced comparable $CD8^+$ T cell activation to CFA-adjuvanted SIINFEKL.

Self-assembling protein nanoparticles (SAPNs) also use α-helical coiled coils. A monomer fusion peptide was designed with a pentamer-forming and a trimer-forming segment connected by a $(glycine)_2$ linker and expressed recombinantly. The monomers' segments formed pentameric and trimeric coiled coils, ultimately assembling 60 monomers into an icosahedral nanoparticle 20–30 nm in diameter (60). **Figure 3** shows an example of monomer design and self-assembly. Antigens and adjuvants were inserted before the pentameric helix (61, 62), after the trimeric coil sequence (63, 64), or on both ends (65, 66), depending on the expected structures based on in silico modeling. Full proteins and random coil peptides were added at the termini, whereas α-helical structures, including PADRE, were incorporated into the coiled sequence.

Subunit vaccine SAPNs demonstrated improved immune responses targeting several diseases. When C-terminal malaria epitope PbCSP was added, protection was conferred against two malarial sporozoite challenges after a prime and two boost immunizations (66). SAPNs induced mostly IgG1 antibodies with some IgG2a, indicating a response profile biased toward eliminating extracellular parasites. Immunized mice also showed a $CD4^+$ T cell response, suggesting the particles contain a T cell–reactive epitope, though none was explicitly added. In a more complex SAPN, C-terminal flagellin was added, and a series of 5 toxoplasmosis $CD8^+$ T cell epitopes and PADRE were inserted as a linear sequence within the flagellin sequence (**Figure 3**) (63). These SAPNs showed increased T cell response to the epitopes, but much more significantly when stimulated

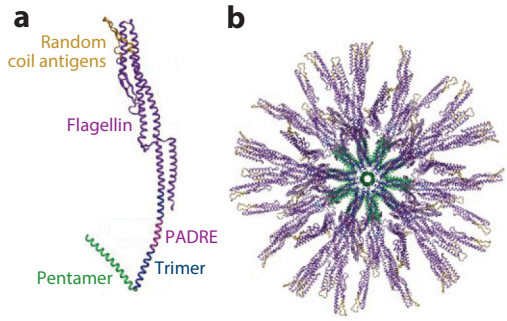

Figure 3

Schematic of α-helical self-assembling peptide nanoparticle (SAPN). (*a*) Peptide monomer containing a pentameric assembling region, a trimeric assembling region attached to the pentameric region via $(glycine)_2$ linker, the universal $CD4^+$ epitope PADRE in the trimeric assembling region, and a C-terminally incorporated flagellin–antigen fusion construct. (*b*) A model of 60 monomers coassembled into an icosahedral SAPN. Adapted from Reference 63, **https://creativecommons.org/licenses/by/4.0/**.

simultaneously with PADRE. Prevention of parasitic brain cysts was seen upon immunization with SAPNs supplemented with TLR4 ligand emulsion adjuvant GLA-SE. Two antigenic epitopes from the HIV-1 gp41 surface glycoprotein, 2F5 and 4E10, were incorporated into SAPNs at the N terminus (65). Rats that were immunized both intraperitoneally with adjuvant and intradermally without adjuvant raised antigen-loaded SAPN-specific antibodies. Only moderate titers against the full gp41 protein were produced, indicating that the structure antigens assume in SAPNs affects antibody specificity when introduced in another conformation. The antigen structure challenge was also studied with a severe acute respiratory syndrome (SARS) epitope, HRC, a natural trimeric coiled coil (61). When incorporated on the N terminus adjacent to the trimeric coiled coil sequence, native HRC trimerization was preserved and anti-HRC titers in immunized mice were substantial, though variable. Titers from full SARS CoV S protein, from which HRC is derived, were higher than monomeric HRC titers yet lower than HRC SAPN titers.

A similar method employing tetramer- and trimer-forming coiled coils was designed to accommodate tetrameric influenza M2e (tM2e) (64) and trimeric Helix C (67). This design formed an octahedral nanoparticle structure ~40 nm in diameter. Helix C and M2e from two different strains of avian influenza (H5N2, H1N1) were incorporated into SAPNs with flagellin (67). H5N2 SAPN vaccines induced neutralizing antibodies, and all mice immunized with H1N1 SAPNs survived a lethal H1N1 challenge, matching the activity of inactivated whole pathogen. Furthermore, H5N2 SAPNs produced neutralizing antibodies to more strains of influenza than inactivated H5 vaccine.

In β-sheet and α-helical fibers, several epitopes can be incorporated in one fiber simply by mixing monomers with different epitopes. SAPNs are more labor intensive per construct, including strategic mapping for optimal location of antigen or adjuvant, vector design, and protein expression and purification. However, precise control over the size and structure of SAPNs is possible (68) and may be important in establishing a reliable platform for vaccines with safety and control over immune response.

Unstructured Hydrophobic Assemblies

Proteins use hydrophobic interactions, among others, to create secondary structure and self-assemble. However, hydrophobic interactions can also form less-uniform materials, like

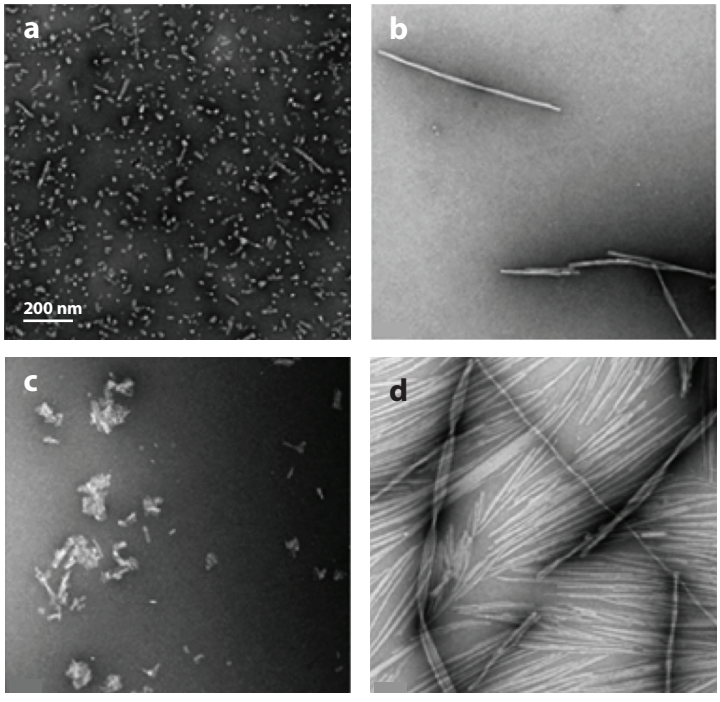

Figure 4

Example of modified peptide amphiphilic micelle monomers that result in different structures. (*a*) $Palm_2K$-$OVA_{323–339}$-$(KE)_4$ and (*b*) PalmK-$OVA_{323–339}$-$(KE)_4$ monomers have a zwitterionic peptide sequence inserted C terminally and result in spherical/cylindrical micelles or twine-like fibers, respectively. (*c*) $Palm_2K$-$(EK)_4$-$OVA_{323–339}$ and (*d*) PalmK-$(EK)_4$-$OVA_{323–339}$ monomers have a zwitterionic peptide sequence inserted between lipid and antigen, resulting in clustered micelles or braided fibers, respectively. Adapted with permission from Reference 74, Copyright 2018 American Chemical Society.

amphiphilic assemblies or aggregates. These types of interactions have been manipulated to form structures that incorporate antigens and adjuvants for use as vaccines.

Peptide-lipid micelles. Similar to liposomes (69), hydrophilic peptide antigens were synthesized and chemically conjugated to lipid tails for assembly into peptide amphiphile micelles (PAMs) (70–73). The size and shape of PAMs are affected by different lipids and peptide modifications, as shown in **Figure 4**. PAMs can form mixtures of spheres and short cylindrical micelles ~70 nm in diameter or long cylindrical fibers ~15 nm in diameter and 0.1–2 μm long (74, 75). They have increased immunogenicity of T cell (70, 74, 76) and B cell (71, 75) epitopes. In one study, several lipid variants were conjugated to two HPV epitopes, E6 and E7, using CuAAC addition and produced PAMs ranging from 350 to 750 nm or large polydisperse aggregates (72). The nano-sized PAMs conferred varying levels of increased protection against tumoral challenge. A fluorinated lipid-peptide conjugate formed using CuAAC addition assembled into much smaller, 10–15-nm particles and demonstrated a robust humoral response against group A streptococcus (73). Change in size and shape affects intracellular fate, strength of immunogenic response, and in vivo trafficking. In one study, all PAMs were internalized at higher rates than soluble peptides, but smaller PAMs with a PEG linker between the peptide and a di-stearyl lipid were exocytosed at higher rates than larger PAMs with a di-palmitic tail (77). Di-palmitic lipids attached to $OVA_{323–339}$ formed

spherical and cylindrical micelles or clusters (**Figure 4*a*,*c***) that were internalized more by macrophages and induced more anti-$OVA_{323–339}$ antibodies than their mono-palmitic lipid PAM counterparts, which formed fibrous structures (**Figure 4*b*,*d***) (74). Addition of a C-terminal anionic peptide $(E)_8$ to the OVA-lipid conjugate maintained lymph node trafficking but reduced internalization and Ab titers, whereas a cationic peptide $(K)_8$ reduced lymph node trafficking but maintained Ab titers. Addition of a zwitterionic peptide $(KE)_4$ maintained both traits, which are essential for a robust immune response.

Lipid TLR agonists including monophosphoryl lipid A and Pam2Cys were also used as the hydrophobic tail (75). Interestingly, the way adjuvants were incorporated affected the immune response. Pam2Cys-SK, an adjuvant lipid-nonantigen peptide conjugate, was incorporated into the same PAM as a nonadjuvant lipid-$OVA_{323–339}$ conjugate, or Pam2Cys was directly conjugated to $OVA_{323–339}$ and incorporated into PAMs with nonadjuvant lipid-$OVA_{323–339}$ at different ratios (76). Higher Ab titers and lymph node resident cell activation resulted from a prime and boost immunization of the Pam2Cys-SK-incorporated PAMs than any Pam2Cys-$OVA_{323–339}$-incorporated PAMs. This may also indicate that Pam2Cys activity differs when conjugated to different peptides.

A similar design was employed to create nanodisc vaccines. Synthetic apolipoprotein A-1 mimetic peptides were chemically conjugated to lipids to form ~10-nm high-density lipoprotein nanodiscs (78, 79). These discs partially incorporated dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[3-(2-pyridyldithio) propionate], which is a lipid attached to a pyridine group by a disulfide bond. Peptide antigens modified with C-terminal cysteines partially replaced the pyridines via disulfide replacement, loading ~6 antigens per nanodisc. Lipid-conjugated adjuvants like CpG have also been incorporated via hydrophobic insertion into the disc with ~98% efficiency. Several different cysteine-modified peptide epitopes have been incorporated, including SIINFEKL; murine carcinoma neoantigen Adpgk; and a mixture of three B16F10 melanoma epitopes, M27, M30, and TRP2 (78). Two to three subcutaneous inoculations with any nanodisc formulation induced $CD8^+$ T cell responses to all antigens, and tumor suppression was induced relative to Montanide-adjuvanted soluble combinations (78, 79). Furthermore, subcutaneous vaccination with Adpgk nanodiscs improved APC uptake, $CD8^+$ T cell responses, and lymph node localization and increased survival after B16-F10 melanoma challenge compared with intramuscular vaccination (79).

Peptide-polymer micelles. Hydrophobic star or dendrimer polymers conjugated to hydrophilic peptide antigens are another variation of peptide amphiphiles (80–84). HPV antigens E6 and E7 were conjugated to variants of alkyne-functionalized poly(*tert*-butyl acrylate) via CuAAC. The resulting particles were 10–150 μm in diameter, depending on the polymer. Mice immunized subcutaneously with peptide-polymer micelles one week after tumor challenge showed reduced tumor volume and increased survival to varying degrees. Most variations were significantly improved over soluble peptide with ISA51 adjuvant (80). The same reduction in tumor volume was seen in similar particles made from the minimal E7 epitope called 8Q conjugated via CuAAC to a 4-arm poly(*tert*-butyl acrylate) star polymer, which formed polydisperse peptide–polymer microparticles (81).

Nanoclusters. A less organized hydrophobic assembly vaccine has been made from desolvated protein and peptide nanoclusters in a process similar to the use of anti-solvents for crystallization and precipitation. Protein or peptide antigens were solubilized in a favorable solvent, and under mixing, a solvent-miscible poor solvent or desolvent was added to force nucleation and growth of protein precipitates via hydrophobic interactions. The protein clusters were then covalently

crosslinked for stabilization. Nanoclusters contain only antigen and crosslinker, with the goal of increasing loading and eliminating off-target sequences or molecules.

Several proteins have been desolvated, including influenza antigens nucleoprotein, HA, and tM2e (85–87); three small peptide tumor epitopes from oncofetal antigen (OFA) (88); and OVA (89, 90). Incorporation or adsorption of adjuvants such as CpG, flagellin, and IgM has also been successful via entrapment during desolvation, adsorption or protein fusion, and affinity immobilization, respectively (85, 90). When soluble tM2e or tM2e nanoclusters were administered intranasally in a prime and two-boost regimen, nanoclusters induced more M2e-specific antibodies, increased lung and spleen T cell activation, and improved survival upon viral challenge with four different influenza strains (85). Interestingly, CpG incorporation actually weakened challenge protection. Trimeric HA nanoclusters induced significant and long-lasting neutralizing antibodies (up to six months) and conferred protection against viral challenge after two intramuscular immunizations (86). The same regimen administered intranasally provided survival after challenge but lower antibody levels. OFA peptide nanoclusters increased DC maturation and antigen presentation compared with soluble formulations in vitro (88). Nanoclusters also demonstrated increased injection site retention compared with soluble intradermal inoculation.

Different combinations of peptides and lipids lead to different carrier shape and size, which affects immunogenicity (72–74). Further modifications of the peptide or lipid may be needed to compensate, which could be a potential limitation for some peptide vaccines. Polymer-amphiphilic materials may provide an alternative for antigens that benefit from larger carriers. However, they also introduce potential unknown effects, including toxicity and non-biodegradability. Peptide amphiphilic assemblies may also need antigen modification to achieve sufficient hydrophilicity, which may affect immunogenicity or specificity of the response. On the contrary, peptide nanoclusters can use different desolvation synthesis conditions (i.e., different solvents) to adapt to different antigen properties. Although this may be an advantage during assembly, the final properties of nanoclusters may still be antigen dependent. Moreover, use of organic solvents and crosslinkers in nanocluster synthesis may alter secondary or quaternary structure, especially for full protein antigens (86, 89).

Electrostatic Assemblies

Layer-by-layer assembly uses electrostatic interactions to build structures by alternating deposition of positively and negatively charged materials, including polymers, nucleic acids, and/or proteins, onto a mold or template. It has recently been used to layer negatively charged antigen diphtheria toxoid (DT) and positively charged adjuvant *N*-trimethyl chitosan onto microneedles (91). Similar levels of neutralizing DT antibodies were produced when mice were given a prime and two boosts of intradermal microneedle vaccines or subcutaneous injection of eightfold more of the soluble mixture. High IgG1:IgG2a ratios implied that the response was largely antibody mediated, but an increase from 2 to 10 coating bilayers reduced the ratio approximately tenfold, indicating higher doses may increase T cell activity.

Layer-by-layer coatings with charge-modified antigen, named immune polyelectrolyte multilayers (iPEMs), have been evaluated as vaccines (92, 93) and autoimmune therapies (8). To achieve electrostatic interactions with neutral peptides, antigens are synthesized with 1 to 9 additional C-terminal arginines to create a cationic antigen. Antigens were layered with anionic adjuvants TLR3 agonist poly(I:C) or TLR9 agonist CpG. Trp2 melanoma antigen and CpG were layered onto a polymer microneedle array, which was released during dermal administration (92). $CD8^+$ T cell proliferation was seen after both prime and boost immunizations. Alternatively, a $CaCO_3$ nanoparticle was used as a sacrificial template and dissolved after coating, leaving only the

antigen/adjuvant shell (93). SIINFEKL fused with 9 C-terminal arginines was layered with poly(I:C) adjuvant on $CaCO_3$, resulting in 5–15-μm microparticles, depending on fabrication conditions. These particles induced significantly more SIINFEKL-specific $CD8^+$ T cells after a prime/boost immunization compared with soluble components.

MOG, a derivative of myelin, a self-antigen attacked in multiple sclerosis (MS), was also used on sacrificial $CaCO_3$ templates for tolerogenic immunotherapy (8). GpG, a negatively charged TLR9 antagonist, was layered with arginine-modified MOG peptide, MOGR. Myelin-reactive transgenic mice were challenged with CpG, the TLR9 agonist that induces MS-like symptoms, and MOGR/GpG iPEMs were subsequently administered in a prime/boost regimen. T cell proliferation occurred in all treatment groups; however, soluble GpG and MOGR/GpG iPEM groups displayed reduced inflammatory cytokines and increased frequency of Treg markers, indicating a polarization of MOG-specific non-inflammatory T cell proliferation. This result was confirmed ex vivo with MS patient T cells. MOGR/GpG iPEM prime and boost administration showed significant reduction in symptoms in mice with experimental autoimmune encephalomyelitis (EAE) compared with untreated mice. A similar experiment was also performed with a polyplex material in which GpG and myelin peptides with one or two arginines were mixed in solution at several ratios to create a range of charged 100–200-nm particles (94). The loading and zeta potential of polyplexes were controlled by the MOGR:GpG ratio. Upon application of TLR9 agonist CpG and polyplex treatment, inflammatory cytokines, DC activation markers, and myelin-specific $CD4^+$ Th1 cell proliferation decreased significantly. In EAE, three treatments of MOGR/GpG polyplexes induced a significant decrease in MS symptoms. The immune-stimulating or autoimmune-suppressive activity of electrostatically assembled materials demonstrates the versatility of these materials for diverse applications based on the antigen and costimulatory components.

DISCUSSION AND FUTURE OUTLOOK

When comparing the wide variety of biomimetic and rationally designed protein biomaterials that have been engineered for a range of vaccine applications, there are many aspects to consider, including the inherent immunogenicity; the type of antigen that can be incorporated and whether modifications are necessary; size, shape, and monodispersity of the structures; and the platform potential, or ability to be used for a wide variety of antigens and applications. Although side-by-side comparisons are rarely published, the following discussion highlights the key benefits and limitations.

Biomimetic materials, VLPs in particular, are expected to have greater immunogenicity by evolution. One aspect often not emphasized is the effect that native VLP or cage cargo removal has on the immunogenicity of the carriers. Although nucleic acids are removed from VLPs derived from infectious viruses, they are not always removed for bacteriophage and plant VLPs. Encapsulated nucleic acids or other native cargoes could serve as adjuvants, and their presence and function should be assessed. Furthermore, although increased immunogenicity is generally a benefit of VLPs, the coat proteins may behave more like antigens than adjuvants and can become the target of the immune response. Though many studies do not measure production of anti-VLP antibodies, it is reported for some, including Qβ, CPMV, and PVX, and can depend on whether a target antigen is present, as well as its identity (31, 36). Even vaults, which are abundant in eukaryotes, can trigger anti-vault antibodies, and, like in VLPs, this depends on the antigen presented (46). In theory, rationally designed protein materials would avoid anti-carrier responses. This is true for Q11 and KFE8 β-sheet fibers administered alone (48), though Coil29 fibers and SAPNs with antigens do elicit anti-carrier antibodies (59, 65). The antibodies induced by the carriers' assembly proteins could limit the number of times a material can be used in a patient, causing neutralization

or reactive toxicities upon repeated dosing (95). Carrier antigens could compete with the target antigen and alter the antibody repertoire or memory function against the desired antigen. In VLPs, they have been reported to not significantly affect T cell responses (96). Some approaches avoid this issue altogether, as iPEMs and nanoclusters contain only antigen, and adjuvant in the case of iPEMs. PAMs and peptide-polymer micelles do not have other protein components, but it is possible for polymers and lipids to induce antibodies (97, 98). This highlights the importance of full immune response evaluations of carrier materials alone and with antigen to ensure off-target responses will not interfere with the desired immune response, repeated administration, or health of the patient. If carrier-specific responses do exist, it may be possible to use protein engineering to mutate residues contributing to immunogenicity (99). Although this could be difficult, it is more feasible in protein biomaterials if directed evolution or barcode high-throughput screening can be used (100).

Perhaps the most studied aspect of protein materials is antigen incorporation. Many antigens have been tested, and some combinations are more beneficial than others. Highly hydrophobic antigens of any size may most easily be encapsulated in VLPs, cages, or nanoclusters, whereas hydrophilic peptides are ideal for PAMs or amphiphilic fibers. Protein carriers that attempt to maintain native antigen structure, like SAPNs, which use rational and complex design of epitope incorporation (67), and repetitive, dense VLPs and cages, may be better suited for vaccines against extracellular pathogens, as B cell receptors can interact directly with these antigens (65). Alternatively, hydrophobic or electrostatic assemblies may be best for viral or cancer antigens because they can be incorporated even if they are unfolded or minimal epitopes, and they can still be processed and presented on MHC I and II for both cellular and humoral responses.

Regardless of antigen identity, all antigens generally must be modified to be incorporated, whether through fusion, sequence modification, or covalent chemistry. This can be considered a drawback of this class of vaccine materials. Fusion and sequence modification minimize additional chemistry or processing steps to achieve assembly but are more affected by antigenic variability in their ability to self-assemble and preserve antigen recognition. Recombinant fusion is feasible for biomimetic materials, as is recombinant or solid-state fusion for α-helix/β-sheet assemblies. It may be necessary to test different fusion locations or linker modifications to identify the best option for protein expression or peptide synthesis, correct antigen secondary structure, antigen location on or in the carrier, and proper carrier self-assembly (27, 42, 64, 74). Recombinant fusion VLPs and cages produce highly monodisperse assemblies with exact antigen number and placement. However, fusion is restricted to protein and peptide antigens, and some fusion proteins simply may not be feasible owing to steric or folding constraints.

Chemical conjugation to other proteins, lipids, or polymers requires more processing steps but may be applicable for more types of antigens. Commonly, crosslinking between cysteine or lysine residues on the antigen and carrier protein is used. This may require sequence modification of the antigen or carrier but is less intrusive than fusion. Antigens already containing lysines or cysteines may not be ideal because the covalent bonds may prevent processing and MHC presentation. Click chemistry itself is bio-orthogonal, but the carrier and antigen must still be modified chemically or recombinantly with nonnative alkyne and azide side chains. This approach has been applied to glycan, lipid, polymer, and protein components (31, 34, 80). Linkers may also be incorporated to extend the antigen farther from the assembling portion of the particle, and linker length and chemistry may be modified for best performance in terms of both immunogenicity and self-assembly (34, 39, 77). Amino acid linkers are used in fusions, but the design space is limited. Although assemblies using chemical conjugation or solid-state fusions like PAMs and self-assembling fibers can be less homogeneous than recombinant fusions like SAPNs and biomimetic materials, the average number of antigens per assembly can still be varied through reaction or mixing stoichiometry.

Although it is efficient and economically attractive to engineer a platform that could be used for many different kinds of vaccines, there is clearly no silver bullet vaccine design. However, for many diseases, it is beneficial to use materials that can incorporate a wide range of antigens and adjuvants in different combinations to design a cocktail for a specific immune response profile. For example, for cancer vaccines, a robust and precise cocktail of antigens and stimulants, including checkpoint inhibitors, is needed to overcome severely immunosuppressive signals produced by the tumor microenvironment, which often render cancer vaccines ineffective (101). Furthermore, several critical infectious diseases, including Zika, HIV, malaria, and pandemic influenza, still lack protective vaccines and require more sophisticated antigen design, presentation, and delivery methods to overcome their specific challenges. The biomaterials discussed have made progress in addressing some of these challenging diseases, with the ability to incorporate antigens from multiple strains or stages of pathogen development or improve immunogenicity of broadly conserved antigens.

A few practices can be improved to mitigate some of the challenges and issues observed with protein-based vaccines. Biomaterials should always be evaluated for both humoral and cell-mediated responses regardless of the ultimate immune profile goals. This is to evaluate whether a balanced response is achieved and to ensure that unexpected and off-target responses do not occur. Although multiple immunizations may demonstrate significantly improved results, single immunizations using routes that have high patient compliance should be prioritized, as these are extremely important factors in vaccine success practically speaking, especially for developing countries. Also, at least two administration routes should be evaluated in vivo to observe the importance of route in the material's ability to induce a desired response, as there are many examples of administration route significantly affecting the immune response (40, 51, 79, 86). Taken together, biomaterial vaccine carriers have shown success in mitigating issues current vaccines still face, including improving lasting protection and memory cell development (30, 31, 45, 47), increasing cross-protection (40, 85–87), reducing inflammatory responses congruent with lowering risk of allergy (47, 50, 51), and reducing off-target materials administered (85, 86, 88, 92, 93). Many questions and challenges remain in finding the best balance between maintaining antigens' native properties while manipulating them for incorporation into carriers and inducing specific interactions with the immune system for each vaccine application. Isolating several factors in a complex system through careful design allows comparison that will enable progress toward clinically valuable vaccines.

DISCLOSURE STATEMENT

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