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Nanoparticle-Based Peptide Vaccines

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8.1 INTRODUCTION

Over the past decade, exciting progress has been made in the field of nanotechnology. Many research groups are studying a wide variety of nanomaterials with different components, sizes, shapes, and surface characteristics for applications in the field of medicinal chemistry and pharmaceutics. The application of nanotechnology for vaccine delivery is of particular interest for the treatment of many infectious diseases, such as HIV/AIDS, because of the many limitations and adverse effects of classic vaccine strategies using live or killed microbes. One of the alternatives to this classic approach is peptide–based subunit vaccination, in which a peptide antigen is used as the main vaccine component, even though its antigenicity is weak compared with traditional vaccines using whole pathogens and the addition of an adjuvant is often required. Antigen size (molecular weight and length) has been identified to affect the efficacy of vaccines. The small peptide antigens are generally weakly immunogenic when administrated without adjuvant.

The formulation of nanoparticle-based peptide vaccines aims to increase the size of vaccine components and antigen molecules similar to that of pathogenic bacteria and viruses. The nanoparticle itself can also act as an immune adjuvant. Small peptide antigens can be enveloped in nanocontainers or can be covalently attached to other molecules through their N-terminal amino group, C-terminal carboxyl group, or various side chain functional groups. The use of nanoparticles can overcome the limitations of peptide-based vaccines because the nanoparticles can protect the encapsulated or attached peptide from enzymatic degradation and deliver the peptide antigen to the immune cells. Hence we classify nanoparticle-based peptide vaccine approaches into two strategies: (1) noncovalent attachment/entrapment of short peptide antigens into nanoparticles and (2) covalent conjugation of short peptide antigens to the surface of nanoparticle platforms. The former is a type of "antigen container" such as emulsions, liposomes, virosomes, immune-stimulating complexes (ISCOMs), and MF59. These antigen containers can also be constructed from well-characterized nanopolymers, for example, poly(lactic-*co*-glycolic acid) (PLGA), which is a synthetic copolymer composed of lactide and glycolide and a biodegradable and biocompatible material approved by the US Food and Drug Administration (FDA) and European Medicine Agency (EMA) for use in micro- and nanoparticle-based drug delivery devices in humans. The use of nanoparticles for the encapsulation of bioactive compounds has been previously described in detail.[1,2] This chapter will focus on the current status of the latter strategy, which involves the presentation of multiple copies of peptide antigens covalently attached on the surface of nanoparticles constructed from various materials. Such nanoparticle-based vaccines are prepared by preconstruction of nanosized platforms followed by attachment of antigens or chemical modification of peptide antigens prior to the formulation of nanoparticles.

8.2 VACCINE COMPONENTS
8.2.1 Traditional microbial vaccines

Traditionally, attenuated or killed pathogen-based vaccines are prepared from whole microorganisms (viruses and bacteria) containing a wide variety of proteins (receptors and enzymes), some of which act as antigens. In general, vaccines using whole microbes can elicit strong immune responses and often lifelong protection without need of boosting immunizations. However, when using live-attenuated vaccines, the risk of infection remains. One example is the development of vaccine-associated paralytic poliomyelitis and vaccine-derived polioviruses in the case of the live oral polio vaccine.[3] A strain of poliovirus in the oral polio vaccine may undergo occasional genetic changes and causes paralysis in about 1 in 2.5 million doses of the vaccine. Another limitation to the use of live or killed microbes for human vaccinations is their potential harmful effects, including toxicity and immune cross-reactivity with human tissues. When whole microorganisms are used as vaccine constituents, it must be kept in mind that they also contain lipids and nucleotides. Lipid components of Gram-negative bacteria, such as lipopolysaccharides

(LPSs) derived from *Salmonella minnesota* and *Escherichia coli*, bind to Toll–like receptor 4 (TLR4), which is expressed on the surface of a variety of cells such as dendritic cells (DCs), and are responsible for endotoxic shock. Additionally, certain DNA and RNA fragments derived from infectious microbes bind to other types of TLRs (3, 7, 8, and 9), and RNA fragments can also act as siRNAs that influence the host immune system in both a positive and negative manner.[4–11] The certain siRNAs that activate the host innate immunity such as an RNAi–mediated type I interferon induction[6–8] can be used as potential therapeutic tools; however, they may cause gene silencing and the following downregulation of the molecules associated with the host immune responses such as cytokines and clusters of differentiation.[9–11]

8.2.2 Downsizing to peptide antigens

Antigenic proteins (isolated or recombinant) derived from infectious microbes could be a potential candidate for a subunit vaccine. However, the immunogenicity of proteins alone is weaker compared with traditional attenuated or inactivated pathogen vaccines in general. Every protein has several epitopes (B cell, Th, CTL epitopes) which may induce sufficient immune responses for vaccine purposes. However, the risk of developing allergy and cross–reactivity to the host tissues still needs to be considered. For instance, the surface M proteins of group A *Streptococci* (GAS) are strongly immunogenic and a major virulent factor in GAS infection, which indicates that they are a potential targets for GAS vaccine development. However, immunization with M protein may elicit cross–reactivity against human heart muscle.[12] In addition, the N–terminal region of M proteins is hypervariable; consequently, more than 200 different serotypes have been identified so far.[13] These issues make it difficult to develop a broadly protective GAS vaccine based on M proteins.

The use of a well–defined peptide epitope excludes any other biocomponents from microbes and is an ideal approach for the development of safe vaccines in terms of minimizing the risk of side effects and eliciting the desired immune response. Contrary to these advantages, immunization with short synthetic peptide epitopes alone is unable to stimulate strong immune responses in animals. Further pharmaceutical development, including additional chemical modification or the utilization of carrier proteins or adjuvants in the vaccine formulation and antigen delivery system, is essential.

8.2.3 Size of vaccine components

The size of an immunogen is one of the most important factors for vaccine efficacy. A size comparison of different vaccine components is illustrated in Fig. 8.1. In general, human cells, including immune cells, have different sizes ranging from a few to several hundred micrometers. For example, among the antigen–presenting cells (APCs) in the human immune system, macrophages have an average size of 20–50 µm,[14] while mature DCs have an average diameter of only 10–15 µm. Immature DCs are even smaller (<10 µm) than mature DCs.[15] Infectious pathogens
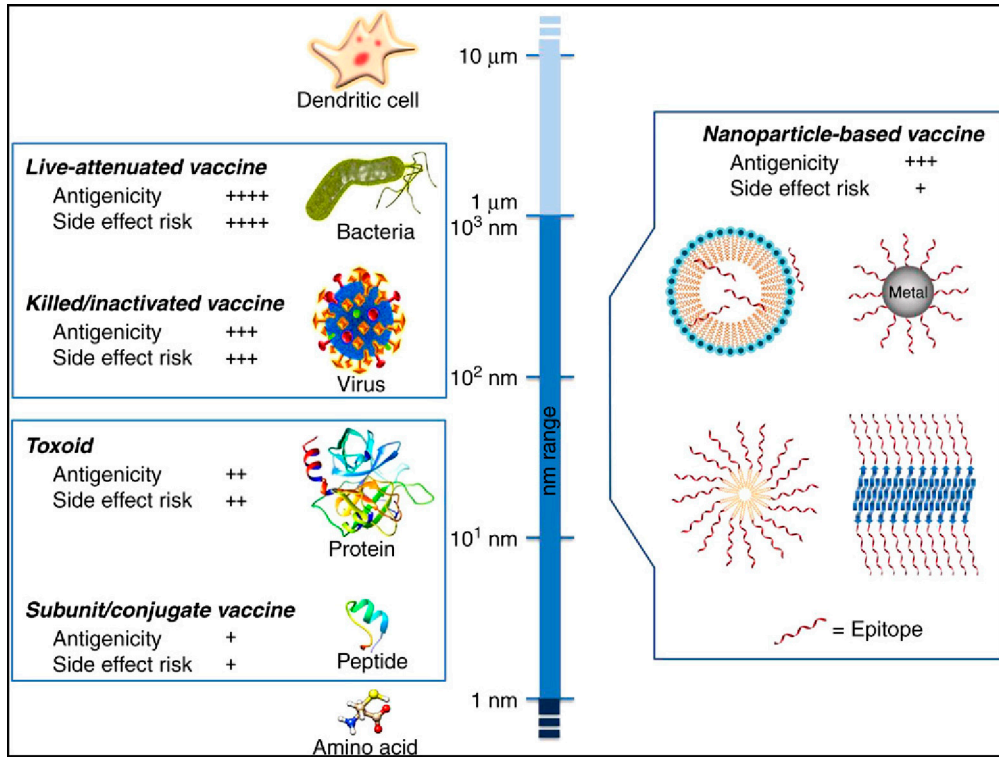
Figure 8.1 *Size comparison of different vaccine components and DCs.*

are microscopic. Bacteria show a wide diversity of shapes and sizes and are usually anywhere between 0.5 and 5 μm in length. For example, the rod-shaped *E. coli* has a width of approximately 0.5 μm and length of 2 μm. In contrast, viruses range in size from about 20–400 nm in diameter. Influenza virus, for example, is approximately 100 nm in size. Proteins are much smaller than viruses. An antibody has an average molecular weight of 150 kDa and a length of 10–15 nm. Ovalbumin (OVA), one of the most studied antigens, is composed of 385 amino acids and has a molecular weight of 45 kDa and a molecular diameter of 5.4 nm.[16] Peptide antigens used in vaccine studies are composed of a single B cell epitope, CTL epitope, or Th cell epitope, or they can be relatively longer peptides containing different epitopes like OVA$_{323-339}$, which is a 17-mer peptide with both a B cell and Th cell epitope in its sequence. Although their actual size depends on their folding, linear peptides composed of a few tens of amino acids have an approximate size of one nanometer. As mentioned previously, decreasing the size of immunogens and trimming the antigenic protein into its minimal epitope reduce the risk of side effects but also affect vaccine efficacy. Nanoparticles can be applied in peptide subunit vaccine development to recover the lost immunogenicity of "safe" peptide epitopes.

8.2.4 Size issues of particle-based vaccines

Several studies have investigated differences in particle size for peptide vaccines.[1,17–19] Particles in vaccine formulations need to have a size similar to that of pathogens in order to be taken up by APCs: nanoparticles are of the size of viruses (ranging from tens to hundreds of nanometers), and microparticles have a similar or bigger size to those of bacteria. Based on their surface-volume ratios, smaller nanoparticles have a wider surface area for attachment or absorption of peptide antigens than larger microparticles do, whereas for encapsulation, bigger particles are suitable for loading a larger amount of antigens. Peptides are more easily entrapped or encapsulated into nanovehicles or attached onto the nanoparticle surfaces in comparison with proteins due to their smaller size. Moreover, smaller particles can reach into blood and lymph nodes, while larger particles remain at the injection site. Smaller-sized particles are taken up by receptor-mediated endocytosis; in contrast, larger-sized particles are taken up by phagocytosis or micropinocytosis.[20,21] DCs and macrophages prefer smaller-sized particles and larger-sized particles, respectively. The Plebanski group studied the size-dependent immunogenicity of nanobeads–antigen conjugates using commercially available carboxylated polystyrene with a range of different sizes (0.02, 0.04, 0.1, 0.5, 1, or 2 μm size), and the conjugate using 0.04 μm nanobeads showed the strongest immune response.[17,22,23] Thus the particle size–dependent immunostimulatory effects are significant in the design of particle-based vaccines for eliciting the desired immune response. However, composition of the particle itself is also an important factor. Kirby et al. studied the vaccine efficacy of two types of PLGA-based microparticles that were prepared from the same material and had similar sizes. The first formulation was an oil–in–water single emulsion (4.7 μm) absorbing antigens on its surface, and the second was a water–in–oil–in–water double emulsion (3.0 μm) for entrapping antigens within the particle.[24] The amount of loaded protein antigen in the oil–in–water single emulsion system was approximately three times larger than that of the water–in–oil–in–water double emulsion system. The release of antigen from the oil–in–water single emulsion showed a notable burst release over the first 24 h. Mice immunized with these two types of particles showed significant differences in immune response. The oil–in–water single emulsion (absorbed antigen) system promoted humoral response, while the water–in–oil–in–water double emulsion (entrapped antigen) promoted cellular response. These findings indicate that the antigen dosage and immune response depend on the particle type as well.

8.3 UTILITY OF SYNTHETIC PEPTIDES FOR SUBUNIT VACCINES

The chemical synthesis of peptides and proteins, with regard to the development of protecting groups and coupling methods, has significantly progressed since the studies of Curtius and Fischer in the early 19th century. The development of solid phase peptide synthesis (SPPS) by Merrifield[25] and the native chemical ligation technique by Kent

and coworkers[26] as well as the recent application of microwave irradiation to SPPS have improved the coupling efficacy, reaction time, and reduction of by–products, especially for the synthesis of longer peptides.[27] As such, chemical peptide synthesis can currently be carried out on a large scale, allowing for the production of grams or even kilograms of peptide. Peptides can be stored or shipped as lyophilized powder, while proteins are in general more labile and cold chain is often required to maintain their immunological properties. Another advantage over proteins is that the structure and sequence of synthetic peptides can be arbitrarily modified on demand in the following manner:

1. Natural proteins and peptides are typically linear. The backbone of synthetic peptides can be designed as not only a linear form but also a branching or cyclic form. A Lys residue, for example, has two amino groups, which can be modified to create a branched peptide backbone. A Lys-based dendritic scaffold was used in the multiple antigen peptide (MAP) system.[28,29] Cyclization of a peptide affects its secondary structure, which is relevant to its biological activity in some cases (eg, B cell epitopes need to maintain their native conformation to trigger desired humoral immunity). Head–to–tail cyclization avoids peptide hydrolysis by exoacting enzymes due to the absence of both N– and C–termini.

2. Nonproteinogenic amino acids including D-amino acids and β-amino acids, as well as artificial amino acids, such as lipoamino acids, can be readily introduced into a peptide epitope during SPPS. Generally, the addition and/or substitution of non-coded amino acids renders peptides reduce the susceptibility to enzymatic degradation. Pam3Cys and Pam2Cys are amino acid derivatives with lipophilic modification of bacterial lipid components and the ligands for the TLR2/TLR1 heterodimer and TLR2/TLR6 heterodimer, respectively. Immunization with both bacterial and synthetic peptide antigens containing Pam3Cys or Pam2Cys elicits strong immune responses because of the maturation of DCs via TLR2 activation. Such TLR ligand–antigen conjugate vaccine approaches to enhance immune responses have been reviewed elsewhere.[30]

3. Peptides and proteins have several functional groups on their side chains, such as the hydroxyl group of Ser, Thr, and Tyr; the amino group of Lys; and the thiol group of Cys. Some of these groups are physiologically targeted by posttranslational modification (eg, lipidation, glycosylation, phosphorylation, or disulfide bond formation). These modifications affect the solubility, folding, and biological activity, including the immune response, of peptides and proteins. In some cases, it is difficult to express recombinant proteins with the desired posttranslational modification. However, SPPS can achieve the faithful reproduction or site-specific mimicking of peptide fragments derived from naturally modified proteins, such as bacterial lipoproteins/peptides or glycoproteins/peptides. Lipophilic modification, similar to the addition of Pam3Cys or Pam2Cys mentioned earlier, can be carried out by attachment of long alkyl chains to peptide epitopes, and the resulting lipopeptides show amphiphilic properties and

enhanced immunogenicity compared with the peptide antigen without lipid chains. With regard to glycosylation, tumor-associated carbohydrates are linked to proteins such as MUC1, which is highly overexpressed on tumor cells as compared to the expression for almost all epithelial tissues, and the glycosylation of MUC1 on tumor cells is considerably different from that on normal cells.[31,32] Tumor-associated carbohydrate antigens (TACAs), including Tn antigen (α-GalNAc-Thr/Ser) and sialylated Tn (sTn) antigen, are attractive targets for the development of anticancer vaccines. TACAs were empirically shown to elicit a weak immune response without covalent attachment to a carrier protein or Th cell epitope.[33] Via peptide chemistry, peptide-based vaccines can be readily synthesized with TACAs in a single construct.[34–36] DCs have mannose receptors (eg, CD206 as a human mannose receptor), and the uptake of mannosylated protein antigens derived from bacteria and fungi is one of the important features of DCs. Mannose receptor-mediated uptake of antigens enhances HLA class II-restricted antigen presentation.[37] Therefore, the mannose receptor on the surface of DCs is an attractive target for the development of new antigen delivery systems. Immunization of a mannosylated ovalbumin peptide (Man–OVA$_{323-339}$) showed enhanced antigen presentation and induced proliferation of T cells.[38] In a study on human papillomavirus (HPV)-associated cervical cancer attachment of a mannose moiety on the N-terminus of the peptide epitope 8Q, derived from HPV type-16 E7 protein, efficiently reduced tumor sizes in mice as compared with nonmannosylated peptides.[39]

4. Furthermore, during SPPS, a variety of functional groups can be added to either *N*-terminal amino group or side chain-functional groups (Fig. 8.2) in order to conjugate peptide epitopes to other molecules, such as other epitopes, carrier proteins, and micro/nanoparticles, or even to cyclize peptides by chemoselective ligation methods, for example, thiol addition between maleimide and the thiol group, thioether ligation between the haloacetyl group and thiol group,[40–42] copper(I) catalyzed [3 + 2] azide–alkyne cycloaddition (CuAAC, Huisgen reaction),[43,44] oxime ligation between the aldehyde (-CHO) group and hydroxylamine (-NH$_2$O) group,[45] and hydrazone ligation between the aldehyde group and hydrazine (-NH$_2$NH) group (Fig. 8.2).[46]

8.4 UPSIZING PEPTIDE ANTIGENS

It has been experimentally proven that the immunogenicity of antigens, that is, both short peptides and polysaccharides, can be enhanced by coadministration or covalent conjugation with carrier proteins such as tetanus toxoid (TT), diphtheria toxoid (DT), keyhole limpet hemocyanin (KLH), OVA, and bovine serum albumin (BSA). Carrier proteins act as a source of Th cell epitopes. They also increase the molecular size of antigens and thereby facilitate their uptake by immune cells such as macrophages and DCs. Antigen size plays an important role in eliciting the immune response; however, carrier protein
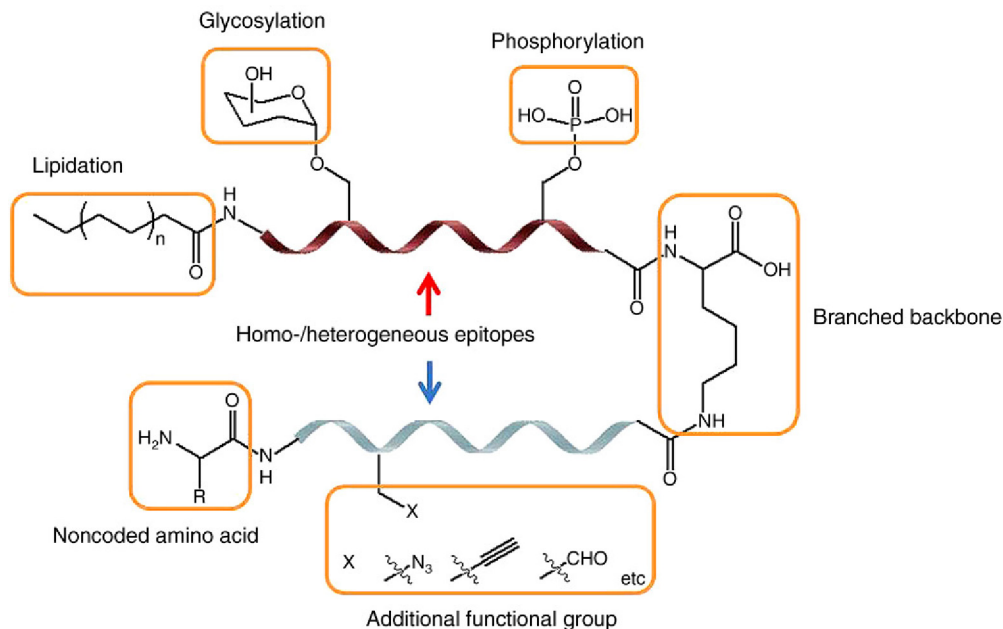
**Figure 8.2** *Design of synthetic peptide-based vaccine molecules with chemical modifications.*

causes undesirable effects, such as the suppression of antipeptide antibody production, the production of antibodies against carrier proteins, and local reactions (eg, extensive redness and swelling) in the case of excessive doses of toxoid vaccines (DT and TT).[47]

8.4.1 Multiple antigen peptide system

In 1988, Tam demonstrated that synthetic peptides can be used as multiple-antigen–presenting platforms. This alternative approach is called the multiple antigen peptide (MAP) system.[28] As shown in Fig. 8.3, 8 copies of short peptide epitopes (9–16 amino acid residues) are simultaneously attached on both the α- and ε-amino groups of a Lys-based dendrimer, resulting in a protein-sized single molecule presenting multiple peptide epitope copies. More importantly, this Lys-based dendritic scaffold is nonimmunogenic. Immunization of MAP vaccine candidates in mice or rabbits successfully elicited stronger antibody production when compared with the corresponding peptide monomers or peptide epitope–carrier protein conjugates. Tam's Lys-based dendrimer is the first artificial platform used for the development of multiple antigen–presenting vaccines and opened up new ways of vaccine design to overcome the weak immunogenicity of short peptides.

8.4.2 Multiple antigen-presenting nanoparticles

The assembly of multiple copies of peptide epitopes on Lys-based dendrimers using the conventional stepwise SPPS is often affected by problems associated with the purification
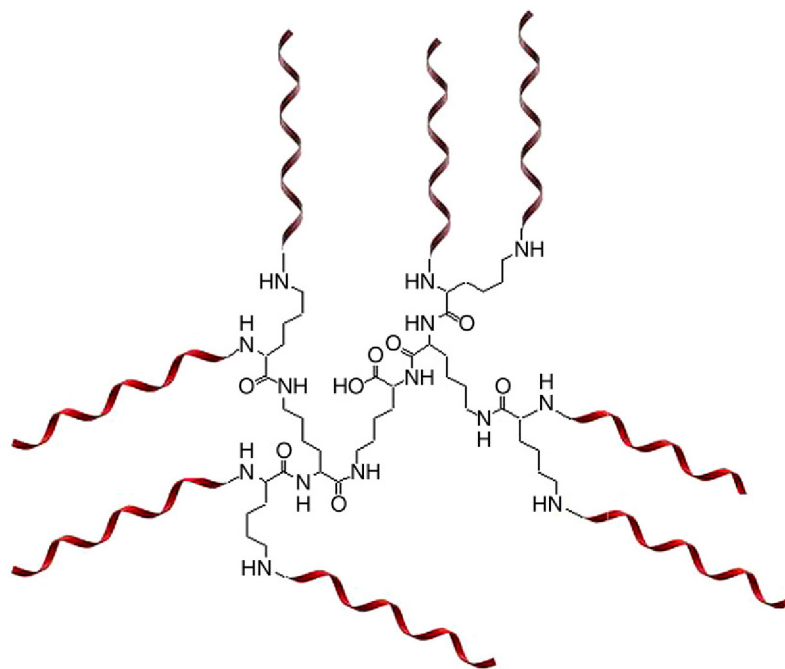
Figure 8.3 *Lys-based multiple antigen peptide (MAP) system.*

of the final products. The use of purified and well-characterized short peptide epitopes for fragment condensation is one of the fundamental solutions to obtain highly pure homogeneous product bearing multiple copies of peptide antigens. Linear and cyclic peptide scaffolds with multivalent functional groups have been developed as multiple-antigen–presenting platforms not only for peptide vaccines but also for carbohydrate vaccines.[48–52] Carbohydrates have multiple hydroxyl groups, and those functional groups can be modified to covalently attach multiple antigen copies.[53,54] Free radical–induced polymerization of acryloyl ($-CH_2 = CHCO$) groups yields linear alkane polymers. Acryloyl peptide epitopes (homologous or heterologous epitopes) can be converted to large immunogens bearing hundreds of epitopes attached to the alkane backbone. Jackson et al. have evaluated the vaccine efficacy of polymeric immunogens containing eight different epitopes derived from the GAS M protein.[55,56] The polymeric GAS vaccine molecules showed outstanding broad immunogenicity and protection from GAS infection in mice. Antibodies against each of the individual epitopes presented in the polymers were successfully elicited.[55,56] Recently, multiple copies of purified peptide epitope were covalently attached to synthetic dendrimers via click chemistry (CuAAC). The conjugate self-assembled into nanoparticles (∼20 nm) that were capable of inducing a strong antibody production,[44] and also size-dependent responses were reported for this conjugate, showing that smaller nanoparticles induced stronger humoral immunity.[57] The Plebanski group studied the conjugation of commercially available carboxylated polystyrene

nanobeads (~50 nm) to peptide epitopes derived from OVA or from the viral protein (VP)-1 capsid protein in foot-and-mouth disease virus (FMDV).[58,59] Vaccination with nanobeads conjugated with either both CD4 and CD8 T cell epitopes derived from OVA or the B cell epitope from FMDV showed induction of T cell and antibody responses[58,59] and inhibition of tumor growth.[58]

Inorganic materials are also known to form nanoparticles and can be attractive scaffolds for nanoparticle-based vaccines. Colloidal gold nanoparticles (GNPs) are ideal for this purpose primarily because of their biocompatibility and lack of immunogenicity.[60,61] They can be easily prepared from gold salt [H(AuCl$_4$)] in water, and their particle sizes are controllable within the nano range. The peptides containing Cys can be readily conjugated to the surface of GNPs via their thiol groups, resulting in the presentation of multiple peptides on GNPs surface. Chen et al. studied the immunological efficacy of nanosized (2, 5, 8, 12, 17, 37, and 50 nm in diameter) GNP-epitope conjugates containing epitopes derived from the VP1 protein of FMDV with an extra Cys residue, which provided thiol functionality for the conjugation. The conjugates using midsized nanoparticles (8 and 12 nm) showed a stronger antibody production compared with other sizes of particles or the KLH carrier protein conjugate. The midsized particles (8 and 12 nm) also showed the highest level of biodistribution in the spleen.[62] Tao et al. studied the stability of peptide–gold nanoparticle conjugates as lyophilized dry powders.[63] Lyophilized bare gold nanoparticles exhibited aggregation and could not be resuspended. By contrast, the lyophilized peptide–gold conjugates were uniformly resuspended in water and did not aggregate. The conjugation of peptides onto gold nanoparticles leads to increased stability and reproducibility of the conjugates and has the potential advantage of storage in a solid form following lyophilization. Furthermore, addition of CpG (TLR9 ligand) as an adjuvant to the conjugate did not disturb the stability of the colloidal suspension.

8.5 LIPOPEPTIDE-BASED NANOVACCINES

Lipopeptides produced by bacteria, fungi, and viruses have a wide variety of biological activities, including antimicrobial activity.[64–67] Due to the lipid tail attached to hydrophilic peptides, lipopeptides have amphiphilic physicochemical properties. The lipid chains of these peptides cause them to self-assemble into nanoparticles such as micelles and fibrils with a core composed of lipidic moieties, while a high density of peptide epitopes is oriented outward.[68] As shown in Fig. 8.4, a lipopeptide is a building block for aggregation into nanoparticles that present multiple peptide antigen copies.

Lipophilic modification of peptide antigens has been studied in the field of vaccine development for more than 30 years. To date, several lipophilic moieties have been successfully used (Fig. 8.5). Pam3Cys is the N-terminal part of lipopeptides derived from the inner and outer membrane components of Gram-negative bacteria,
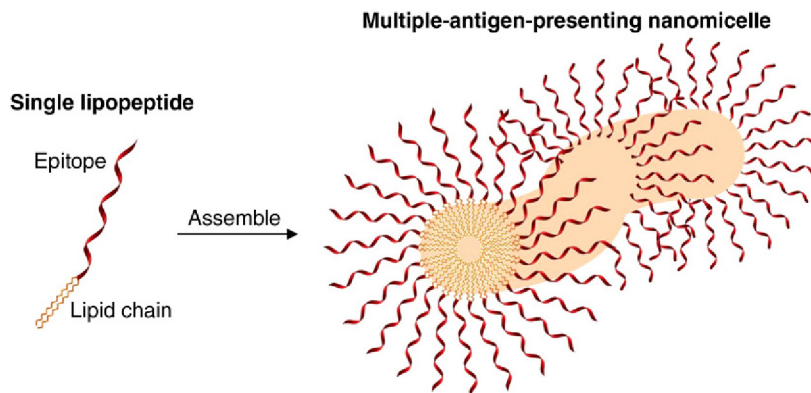
Figure 8.4 *Self-assembly of amphiphilic lipopeptides leads to the formation of micelles with multiple peptides at high density on the surface.*

while Pam2Cys is the lipid component of macrophage–activating lipopeptide 2 isolated from mycoplasma. Other amino acid derivatives, for example, O–acyl serine,[69] N–palmitoyl lysine,[70] and lipoamino acids (LAAs),[71] have been utilized for lipophilic modification (Fig. 8.5). Before the discovery of the TLR family,[72] the mechanism of lipopeptide vaccines was thought to be based on the ability of lipopeptides to attach to the membrane of APCs. After internalization into the cytoplasm, they were thought to promote MHC class I/II presentation or induce cytokine secretion. The discovery of the human TLR family revealed that Pam3Cys and Pam2Cys are the ligands of TLR2/1 and TLR2/6 heterodimers, respectively.[73,74] TLR activation triggers the innate immune system response through the activation of the NF-κB signaling pathway. Both Pam3Cys- and Pam2Cys–containing lipopeptides induce humoral and cellular immune responses against the attached peptide antigen without addition of adjuvant or carrier proteins.[75] Cocrystal structures of Pam3Cys and Pam2Cys with TLR2/1 and TLR2/6 heterodimers have been obtained recently, which have aided modern adjuvant development.[76,77] Several structure-activity relationship studies of Pam3Cys and Pam2Cys derivatives have been reported.[78–80] Metzger et al. reported
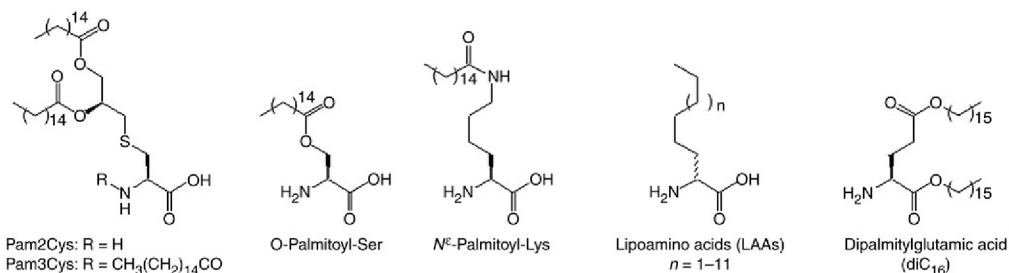
Figure 8.5 *Amino acid derivatives used in lipopeptide-based subunit vaccines.*

that the chirality of the glyceryl unit strongly affects adjuvant efficacy and that the R-configuration in the glyceryl unit is the preferred bioactive form.[78] Reichel et al. studied the physicochemical relationship between the stereochemistry of Pam3Cys and its self-assembling properties. Using transmission electron microscopy (TEM), a variety of shapes (vesicle, layer, and fibril or tubular network) with different sizes ranging from 20 nm to more than 3 μm were observed, indicating that the chirality of the glyceryl unit and the additional serine residue exert an influence on the mode of aggregation and the monolayer properties.[81] Hamley et al. investigated the self-assembly properties of amphiphilic lipopeptides [Pam3Cys-, Pam2Cys-, and PamCys-SerLysLysLysLys (PamCys: N-palmitoyl-cysteine)] using the CD spectrum, small-angle X-ray scattering, and cryogenic TEM.[82] They found that the number of acyl chains at the peptide terminus influences the secondary structure and nanostructure of lipopeptides. The Pam3Cys-peptide adopts a β-sheet structure and self-assembles into worm-like micelles (with a thickness of ∼5 nm), while the other two peptides form spherical micelles with a diameter smaller than 5 nm. Wilkinson et al. studied an anticancer lipopeptide vaccine in which three components—Pam3Cys, the Th epitope PADRE, and the tumor-associated antigens VNTR domain form MUC1 containing tumor-associated carbohydrate antigens—were covalently conjugated.[36] As expected, the tricomponent vaccine molecules were found to self-assemble into nanosized particles (average size of 20 nm), and administration of the tricomponent vaccine to mice induced strong humoral immune responses, including production of IgG1 isotype (Th2 skewed response) and IgG3 isotype (anticarbohydrate antibody).

Toth and colleagues investigated lipoamino acids (LAAs) with different lengths of alkyl chains for the purpose of synthetic lipopeptide subunit vaccines against GAS infection,[71,83–86] hookworm infection,[87] and *Schistosoma mansoni* infection[88] or as an anticancer vaccine.[39,89] Elongation of the peptide epitope by the coupling of several copies of LAAs enhances antibody production against the attached epitope without addition of adjuvant.[71,83] The structural arrangement of epitopes (the B cell epitope and Th cell epitope) and lipid moieties in a single designed molecule influenced the induction of immunological responses,[71,85] and lipid moieties composed of synthetic LAA activate TLR2.[84] Recent studies investigating the amphipathic character of lipopeptides composed of B cell epitopes, T cell epitopes, and lipid moieties showed that they form nanosized particles in PBS.[85,86] Double copies of LAA tended to lead to the formation of larger particles, meaning that the size of the nanoparticle may be controlled by the number of LAA residues as well as the length of the LAA alkyl chain.[90]

Accardo and coworkers studied an amphiphilic peptide vaccine against herpes simplex virus (HSV).[91] *N,N*-Dioctadecyl-succinamic acid, as a lipid unit, was chemically conjugated to epitopes derived from HSV envelope glycoprotein B or D via a linker, and the resulting lipopeptides aggregated into micelles (with hydrodynamic radii between 50 and 80 nm). The micelles triggered human U937 and mouse RAW264.7 macrophage

cells to release higher level of cytokines, in particular IL–23, IL–6, IL–8, MIP–2, and TNF–α, when compared to the corresponding single epitopes without lipid moiety.

The Tirrell group investigated lipopeptide vaccines using di–palmityl ester of glutamic acid (diC$_{16}$) as a lipoid moiety, shown in Fig. 8.5. The diC$_{16}$ unit was attached to the N– or C–termini of peptides: ovalbumin CTL epitope OVA$_{253-266}$[92] or B cell epitope J8 derived from GAS M protein.[93] TEM and atomic force microscope (AFM) measurements showed that amphiphilic diC$_{16}$–OVA$_{253-266}$ self-assembles into cylindrical micelles of approximately 8 nm in diameter and 50–300 nm in length. Unlike Pam2/Pam3Cys-containig lipopeptides, diC$_{16}$–OVA$_{253-266}$ did not induce TLR2 stimulation. Subcutaneous immunization of diC$_{16}$–OVA$_{253-266}$ micelles efficiently activated MHC I presentation and CTL immune response, resulting in a reduction of tumor size. Lipopeptide micelles composed of a diC$_{16}$ moiety and B cell epitope J8 were able to elicit antibody production without any adjuvant when injected in mice.

All of these findings underscore the considerable relevance of self-assembling lipopeptide systems as antigen delivery devices for the induction of desirable immune responses.
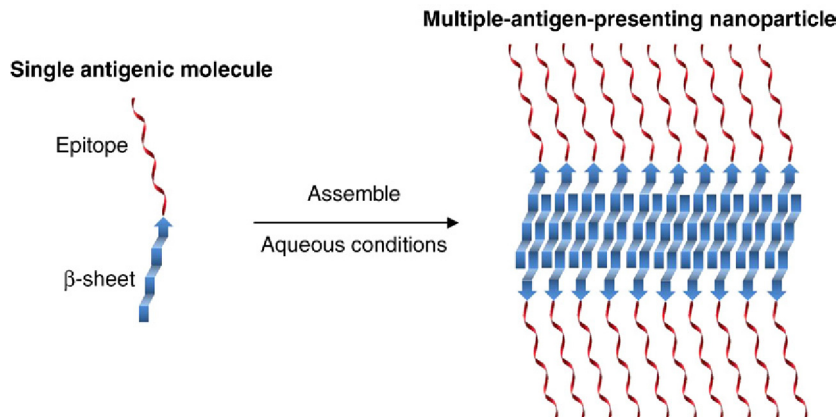
8.6 SELF-ASSEMBLING PEPTIDES

Certain peptide sequences that adopt a secondary structure (α-helix, β-strand, or β-sheet) can self-assemble in aqueous solutions and subsequently form nanoparticles via noncovalent interactions (eg, hydrogen bonds, electrostatic interactions, van der Waals bonds, or π–π stacking interactions). Such peptides are also called self-assembling peptides. Recently, self-assembling peptides have attracted a lot of attention as topologically defined building blocks. To date, a number of self-assembling peptide sequences have been developed, and their chemical, physical, and biological properties[94] as well as their application in the field of medicinal chemistry and pharmaceutics including drug delivery have been reported.[95] Their application for vaccine delivery and peptide-based nanovaccines is of particular interest. The theoretical elongation of self-assembling peptide sequences onto peptide epitopes, with or without linker, yields a single antigenic molecule, which subsequently assembles by itself into nanosized particles presenting multiple repetitive copies of a given epitope at high density, as shown in Fig. 8.6.

8.6.1 Self-assembling protein antigens

A polyglutamine (polyQ) domain is found in several types of aggregation–prone proteins associated with neurodegeneration (eg, Huntington's disease). Short polyQ domains (<35 Qs) are soluble, while longer polyQ domains (>36 Qs) tend to aggregate.[96,97] Ilyinski et al. investigated a model fusion protein, in which a polyQ domain (longer than 100 glutamine residues) was attached to the weak immunogenic green fluorescent protein (GFP).[98] When used as a plasmid DNA vaccine, polyQ–associated

Figure 8.6 *Systematic self-assembly of a peptide-based nanovaccine.* A peptide, composed of a self-assembling sequence and an epitope, assembles by itself into nanosized fibers presenting multiple epitope copies.

aggregation of expressed antigen strongly enhanced both antibody and cytotoxic responses against GFP in mice immunization, suggesting that the linkage of an elongated polyQ domain to antigens has the potency of an adjuvant. Peptides that are able to adopt a coiled–coil conformation tend to aggregate into fibers. Burkhard et al. studied self-assembling peptide-based nanoparticles, which consisted of a pentameric coiled–coil oligomerization domain derived from cartilage oligomeric matrix protein (COMP)[99] and a de novo designed trimeric coiled–coil sequence.[100] Subsequently, they applied them in the design of multiple antigen–presenting vaccines.[101,102] The self-assembling peptide, conjugated with the C-terminal heptad repeat region (HRC1) epitope derived from the severe acute respiratory syndrome coronavirus (SARS-CoV) S protein as a B cell epitope, formed nanoparticles that were about 25 nm in size and had multiple copies of HRC1 epitopes on their surface. Immunological evaluation of the conjugate containing SARS epitope showed the production of specific antibodies and their neutralization activity against SARS-CoV infection.[101] The same building block has been used for a self-assembling peptide-based vaccine against malaria. The conjugate with a B cell epitope (DPPPPNPN)$_2$D derived from the circumsporozoite protein of the malaria parasite *Plasmodium berghei* self-assembled into nanoparticles (~25 nm in diameter). The conjugate containing the malaria epitope elicited a high level of antibody production after immunization in mice. The immunized mice showed a long-lasting protection against the parasite for up to 6 months. This self-assembling peptide-based nanosystem has also been used for the development of an HIV vaccine[103] and antitumor vaccine.[104]

8.6.2 Self-assembling peptide-based nanovaccines

The systems discussed previously use large recombinant polypeptides/proteins (longer than 100 amino acids). Collier and colleagues have utilized the short β-sheet fibrilliz-ing peptide Q11 (QQKFQFQFEQQ) for the development of biomaterials and bio-medicines. Q11 assembles in salty aqueous solutions to form networks of β-sheet–rich nanofibers with a width of 15 nm. Importantly, it appears that Q11 is nonantigenic and the coadministration of Q11 with a strong adjuvant complete Freund's adjuvant (CFA) in mice did not induce antibody production against Q11. Q11-fibrillar peptide gels can be considered as a multiple antigen–displaying platform for self-assembling peptide vaccines. A 17-mer peptide, containing B cell and T cell epitope sequences derived from chicken egg OVA, was conjugated to the N-terminus of Q11 via a short linker (Ser-Gly).[105,106] The OVA–Q11 conjugate assembled into nanosized fibers, and when these were subcutaneously administrated into mice, they successfully induced a strong anti–OVA antibody production in the absence of additional adjuvant. The antibody titers were remarkably higher when compared with the mixture of the OVA epitope and CFA without inflammation at the injection site.[107] Furthermore, the study of Q11-containing peptide vaccines is being continued, not only for prophylactic vaccine (Q11–Malaria epitope conjugate)[108] but also for cancer therapeutic vaccine (Q11–MUC1 conjugate: MUC1 is a tumor-associated antigen).[109] In addition to Q11, the Collier group has also reported the use of another self-assembling sequence, KFE8 (FKFEFKFE), which was not antigenic by itself when coadministered with CFA. The KFE8–OVA conjugate also formed nanofibers and was able to successfully induce OVA-specific antibody produc-tion,[106] showing that KFE8 is another potential antigen-display platform.

Recently, the Toth group investigated the applicability and synergistic effects of the β-sheet fibrillizing peptide Q11 and the TLR2-active lipid moiety for self-assembling peptide-based subunit nanovaccines.[86,110] They synthesized 5 peptides composed of the B cell epitope J14 derived from GAS M protein, Q11, and/or the TLR2-active lipid moieties (single or double copy of LAA C16, 2-amino-hexadecanoic acid). All pep-tides formed nanoparticles (10–300 nm); however, visible fibril network formation and β-sheet predominance were not observed by TEM and CD spectra measurement, re-spectively. Among the five tested peptides, those containing a double copy of C16, J14–Q11–C16C16 and J14–C16C16, showed better results with regard to cellular uptake and antibody production when compared with peptide J14–Q11. It can be assumed that the failure in β-sheet fibril formation leads to a loss of adjuvant activity.

The self-assembling peptide strategy is a breakthrough in the development of peptide-based subunit nanovaccines. Notably, a short peptide with a sequence of FFY and its derivatives, which form nanofibrous hydrogels for using as a delivery system, were recently applied in the study of a plasmid DNA vaccine.[111] However, these short

self-assembling peptides are not used for peptide-based vaccines yet. It is predicted that the new design of combinations between self-assembling peptide nanomaterial and peptide epitopes—either B cell epitopes, Th cell epitopes, or CTL epitopes—has great potential for creating peptide-based nanovaccines that induce suitable long-term immune responses without side effects.

8.7 CONCLUDING REMARKS

Great efforts have been undertaken to improve the antigenicity of short peptides, efficiently stimulate suitable immunity, evoke adaptive immunity, and avoid side effects by using different types of nanomaterials. Currently, the number of approved vaccine adjuvant-delivery systems for human use is limited, but the development of nanotechnology has sparked remarkable progress in nanoparticle-based vaccine development. This includes the use of immune adjuvant and antigen delivery systems aiming at a slow controlled release that allows single-injection vaccine formulations or even a needle-free mucosal vaccine as the ultimate goal for improving the quality of life of people. The application of well-defined nanoparticles to peptide-based subunit vaccines offers many advantages and has significant potential for the development of next-generation vaccines. From both a manufacturing and a preservation perspective, highly pure synthetic peptide antigens containing self-assembling sequences or lipid moieties are of exceptional interest, as they can be easily produced by SPPS and lyophilized to an amorphous form that is suitable for stable storage and medical use. Furthermore, peptides with the capability for self-assembly can form nanoparticles whenever required. Animal immunization studies have already demonstrated the utility of nanoparticle-based peptide subunit vaccines for induction of the desired immune response (cytokine productions, recruitment of Th1 and Th2 lymphocytes, and cellular and/or humoral responses). However, despite the enormous amount of studies on the correlation between the nanomaterial properties (eg, size and shape) and their biological effects (eg, immunogenicity and toxicity), the issue of safety still needs to be addressed. Increased technical inventiveness and the ability to analyze immunological phenomena will aid in obtaining a purpose-designed formulation that is optimal for both prophylactic and therapeutic vaccines.

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