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Chemical and Biological Conjugation Strategies for the Development of Multivalent Protein Vaccine Nanoparticles

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Abstract

The development of subunit vaccine platforms has been of considerable interest due to their good safety profile and ability to be adapted to new antigens, compared to other vaccine typess. Nevertheless, subunit vaccines often lack sufficient immunogenicity to fully protect against infectious diseases. A wide variety of subunit vaccines have been developed to enhance antigen immunogenicity by increasing antigen multivalency, as well as stability and delivery properties, via presentation of antigens on protein nanoparticles. Increasing multivalency can be an effective approach to provide a potent humoral immune response by more strongly engaging and clustering B cell receptors (BCRs) to induce activation, as well as increased uptake by antigen presenting cells and their subsequent T cell activation. Proper orientation of antigen on protein nanoparticles is also considered a crucial factor for enhanced BCR engagement and subsequent immune responses. Therefore, various strategies have been reported to decorate highly repetitive surfaces of protein nanoparticle scaffolds with multiple copies of antigens, arrange antigens in proper orientation, or combinations thereof. In this review, we describe different chemical bioconjugation methods, approaches for genetic fusion of recombinant antigens, biological affinity tags, and enzymatic conjugation methods to effectively present antigens on the surface of protein nanoparticle vaccine scaffolds.

Graphical Abstract

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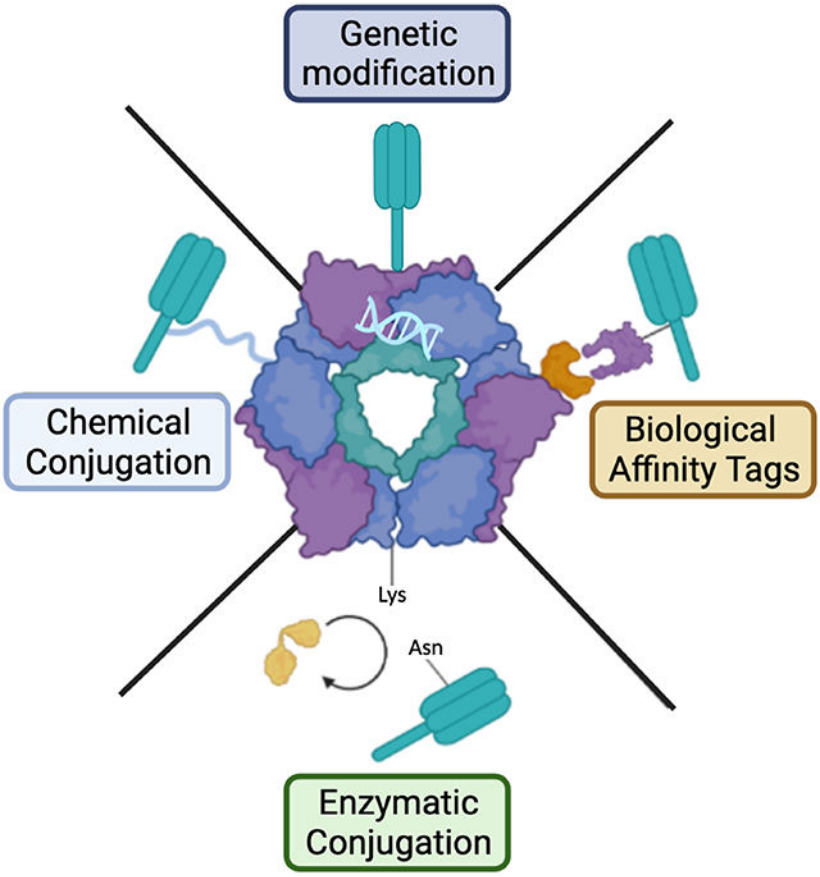
Conflict of Interest Statement
The authors declare no competing financial interest.



Keywords

Antigen conjugation; subunit vaccine; nanoparticle; immune response; multivalency

Introduction

Viruses have been one of the leading causes of diseases across the world. While live attenuated and inactivated virus vaccine have been highly effective in eradicating or curbing different types of viral infection, these vaccines have limitations.[1-3] Live attenuated vaccines consist of whole pathogens that have been weakened to be safe for administration to patients, allowing for signaling of pathogen-associated molecular patterns (PAMPs) to the immune system along with antigenic motifs for strong humoral and cellular immune responses.[4] However, the weakened virus may revert to virulence, thus causing disease. This is specifically dangerous for immunocompromised individuals.[4] Inactivated vaccines are viruses that have been killed by chemical or physical agents, such as formalin.[5] This renders them safer than live attenuated; however, the agents used in the inactivation process may damage or alter epitopes needed to elicit protective immune responses.[5, 6] Subunit vaccines are composed of recombinantly expressed proteins that contain specific antigenic components of a pathogen. These subtypes vaccines are known to have an excellent safety profile and induce prophylactic immunity against a very specific protein

or peptide epitope.[7] However, subunit vaccines often induce weaker immune responses than whole pathogen vaccines since they remove native PAMPs and lack physical properties of pathogens such as repetitive surface array of antigens.[8] To compensate for lower immunogenicity associated with subunit vaccines, nanoparticles have been used as scaffolds to display multiple antigens in order to mimic characteristics of pathogens and improve immunogenicity.[9] Nanoparticles mimic the dimensions of pathogens and can vary in composition from inorganic components, such as gold, and organic polymers or lipids, to carriers made completely out of proteins.[10, 11] Protein nanoparticles, in particular, offer chemical or genetic molecular handles to incorporate antigens and have desirable traits such as biodegradability and biocompatibility.[12] Inorganic and polymer particles may be less biodegradable and, in some cases, the degradation products may not be cytocompatible.[13-15] Protein nanoparticles can be quite stable, relative to lipid nanoparticles that require cold storage.[16-18] They can be made from natural protein nanoparticles, such as virus like particles or protein cages, from proteins conjugated together chemically to form dense nanoparticles, or from proteins engineered to self-assemble into unique nanostructures.[19] In addition, protein nanoparticles have demonstrated efficient delivery of antigens to the lymphatic system due to a high uptake of antigens in nanoparticles by dendritic cells.[20-25]

Immunogenicity of protein scaffolds varies by their PAMP components. Protein scaffolds derived from viruses contain multiple antigenic components and show self-adjuvanting effects, resulting in strong immunogenicity.[26-29] However, this can result in antigen specific, but off-target, immunity against the scaffold itself, which may not be desired. Although non-viral natural carriers such as ferritin, a universal iron transport protein[30], lack immunogenicity, antigen conjugation to the carriers with highly repetitive surface can induce potent immune responses[31]. By displaying multiple antigen copies on a scaffold, BCRs can better cluster to initiate signaling cascades for strong B cell activation, resulting in potent humoral immune responses.[32] It has been shown that antigen multivalency is strongly associated with activation of effector B cell responses.[33] This also enhances antigen uptake and processing, which is critical for orchestrating B and T cell communication and activation as shown in Figure 1.[34] T cell co-stimulation is an important feature for the magnitude and breadth of the immune response, and polyfunctionality of T cells is required for secretion of cytokines, chemokines, or cytotoxic granules.[35] The importance of T cell activation has been of particular interest as T cell responses have shown to have an important role in vaccine protection against severe Covid-19 disease, for example, particularly against viral variants that partially escape neutralizing antibodies.[21] Therefore, strategies to exploit the unique biophysical properties of nanoparticles are needed for both robust humoral and cellular immune responses. For effective presentation of multiple antigens, the successful development of subunit vaccine nanoparticles requires appropriate scaffolds and strategies to decorate them with antigens or to engineer scaffolds that naturally display attached antigens. Protein nanoparticles offer a wide variety of approaches to achieve this presentation.
To date, various protein scaffolds or nanoparticles have been developed and engineered to present multiple antigens with the help of diverse conjugation methods including chemical bioconjugation, genetic modification, and biotag coupling. Rapid development of novel vaccines can be achieved by a combination of different conjugation methods and nanoparticle scaffolds, resulting in “plug-and-display” modular vaccines. In this review,

we will describe the repertoire of different approaches to conjugate antigens to protein nanoparticles for improved immunity of vaccines.

Chemical Conjugation Methods

Chemical conjugation is a method in which a covalent bond is formed between two molecules, where one is a biomolecule.[36] Due to diverse amino acids presented on protein nanoparticles, various functional groups in the protein can serve as reactive sites for chemical conjugation to peptide or protein antigens. Genetic modifications to provide reactive molecular handles can enable site specific conjugation in some cases, such as with cysteine, or can increase the number of conjugation sites, such as with lysine.[37] However, chemical bioconjugation could still result in an unintended off target conjugation of different sites of antigens and protein nanoparticles, depending the amino acid sequence of each, and disrupt the antigen structure or orientation.

EDC/NHS Conjugation

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-Hydroxysulfosuccinimide (Sulfo-NHS) are commonly used bioconjugation reagents that activate a carboxylic acid to enable coupling of free primary amine-containing biomolecules as shown in Figure 2.[38, 39] EDC/Sulfo-NHS coupling has been extensively used in designing peptide nanostructures and immobilizing proteins on the surface of nanoparticles such as virus-like particles (VLPs).[40-43] VLPs are self-assembled virus capsids with sizes ranging from 20 to 200 nm that have been emptied of their genetic contents and are often rich with amino acids that enable conjugation. VLPs, such as those made from tobacco mosaic virus (TMV), are well controlled delivery systems and effective immunogens that have been used to study the function and maturation of antigen-presenting cells (APCs) in lymphoid tissues.[44, 45] Royal et al. utilized EDC/Sulfo-NHS conjugation to present receptor-binding domain (RBD) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) on tobacco mosaic virus-like particles (VLPs).[42] The SARS-CoV-2 antigen consisted of the RBD from the spike glycoprotein (S) fused to a human IgG1 Fc domain motif to improve stability and enable purification using protein A affinity chromatography. The addition of a Fc fragment has been shown to boost cellular and humoral immune responses due to its ability for antigen delivery and presentation via Fc receptors on antigen presenting cells. This fusion antigen was conjugated to TMV via EDC/Sulfo-NHS conjugation. Antibody binding assays showed that structure of RBD was preserved following conjugation. When mice were subcutaneously immunized with the blend of TMV and soluble antigens from SARS-CoV-2, a balanced Th1/Th2 response and antibody response to the S1 domain, a highly conserved epitope, were elicited.[42, 44] However, vaccination of mice with RBD functionalized VLPs resulted in a significantly higher neutralization titer against SARS-CoV-2 pseudoviruses with higher reactivity towards the S1 domain than immunization with soluble antigens. In addition, the vaccine nanoparticles were stable for 12 months at 2–8 °C or at 22–28 °C and, therefore, could address limitations associated with cold-chain storage.

Dityrosine Reaction

Dityrosine reaction uses a reactive tag comprising of histidine and tyrosine residues at the termini of the protein molecules.[46, 47] The reaction is mediated through a solvent accessible tyrosine residue (Y) near six histidine residues. The histidine residues on different proteinscoordinate with nickel (II) ions in the solution, bringing their adjacent tyrosine residues in proximity and, thus, facilitating the formation of covalent dityrosine bonds when exposed to an oxidizer such as magnesium monoperoxyphthalic acid (MMPP) or persulfate as shown in Figure 3.[46] Wilks et al. used dityrosine reaction to crosslink recombinant matrix protein 2 ectodomain (M2e) peptides of Influenza A to generate antigen peptide nanoclusters.[47] A M2e peptide is flanked by the reactive tags, containing tyrosine-rich region $(GY)_2$ and adjacent six histidine residues. When nickel and MMPP were added to the engineered M2e peptides, stable peptide nanoclusters were assembled displaying M2e on the surfaces and within the particles. After intramuscular vaccination of mice with the crosslinked M2e nanoclusters mixed CpG oligonucleotide, an adjuvant which stimulates toll-like receptor 9, significantly high IgG antibody titers against M2e were measured. Soluble M2e peptides with CpG did not induce detectable levels of anti-M2e antibody titers. Moreover, crosslinked M2e nanoclusters with CpG favored a IgG2a response, which is vital for clearance of virus.[48] Upon challenging with A/California/07/2009 (H1N1), mice vaccinated with the dityrosine crosslinked M2e nanoclusters and CpG adjuvant showed 100% survival rate while vaccination with a mixture of soluble M2e and CpG resulted in only a 40% survival rate. The M2e antigen was ~5 kDa and it remains to be seen if stable particles can be created from much larger protein antigens. Furthermore, this method requires removal of toxic nickel ions following fabrication of nanoparticles.

Maleimide Conjugation

Maleimide is a chemical compound that is chemoselective for thiols, allowing conjugation to sulfhydryl groups in cysteine residues of proteins (Figure 4). Maleimide conjugation has been utilized in protein nanoparticle vaccine formulations.[49, 50] Jones et al. demonstrated a method to produce protein vaccine nanoparticles using malarial antigens via maleimide modification and thiolation.[49] Pfs25M, AMA1, CSPM3 antigens from *P. falciparum* were conjugated separately using SATA (N-succinimidyl S-acetylthioacetate), which added sulfhydryl groups on each lysine via N-hydroxysuccinimide (NHS) ester conjugation. Recombinant *Pseudomonas aeruginosa* Exoprotein A (EPA) was used for improved immunogenicity and was modified to add maleimide groups using EMCS (N-ε-malemidocaproyl-oxysuccinimide ester), which reacts with lysines or terminal amines. When mixed with cysteine hydrochloride at pH 6.5 in phosphate buffer, the dual antigen system formed Pfs25M-EPA, AMA1-EPA, and CSPM3-EPA protein nanoparticles with diameters ranging from 12 to 38 nm. Secondary structure of each antigen within the particles were maintained and preserved, as confirmed by circular dichroism. Upon intramuscular injection, antigen-specific antibody levels for Pfs25M-EPA and CSPM3-EPA were significantly higher compared to the unmodified proteins, suggesting the nanoparticles could be effective vaccines to combat malaria. However, AMA1-EPA particles generated similar levels of antigen-specific antibody as unmodified AMA1 protein.

In addition to formulating nanoparticles, maleimides have been used for conjugation of a peptide (S9), which mimics the protective epitope of group B streptococcal type III capsular polysaccharide, onto the surface of cowpea chlorotic mottle virus (CCMV) and cowpea mosaic virus (CMV). Pomwised et al. investigated immunity provoked by S9 peptides conjugated to pseudovirus carriers or soluble protein keyhole limpet hemocyanin (KLH) through maleimide assisted conjugation.[51] To decorate CCMV and CMV with S9 peptides, maleimides were covalently linked to S9 lysines or terminal amines using sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC). Then the peptides were conjugated to cysteines on CCMV or CMV at pH of 6.5-7.5, as shown in Figure 4. After subcutaneous vaccination of soluble KLH-S9 peptides in mice followed by intraperitoneal boost, a Th2 response was induced favoring IgG1 antibody isotype while peptide alone generated no significant immune responses. Conversely, S9 peptides conjugated to CCMV or CMV elicited a Th1 biased immune response with high IgG2a titers. It was speculated that pseudovirus carriers induce a Th1 response since they contain RNA from the assembly process and thus stimulate innate immunity through pattern recognition receptors such as endosomal toll-like receptors.[52] This suggests that conjugation of antigen, as enabled by maleimide conjugation, can tune the nature of the immune response based on the choice of carrier system.

Click Chemistry

While maleimide-sulfhydryl reaction is specific, it is not orthogonal as cysteines are present in many proteins and peptides. Click chemistry is a set of reactions for bioconjugation that joins specific biomolecules together, similar to mechanical snap fasteners that allow to “click” biomolecules and can be implemented orthogonally to all natural bioconjugation reactions. It was originally introduced to join small units together through the means of heteroatom linkages (C-x-C). This technique led to the 2022 Nobel Prize and has gained traction in medicine through the ability of conjugating biomolecules such as DNA, carbohydrates, peptides, and proteins together quickly and efficiently.[53-57] Click chemistry has been extensively used to immobilize antigens on nanoparticles as vaccine platforms.[58, 59] Copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction is one of the first click reactions for conjugation of protein and peptides antigens onto scaffolds.[58] CuAAC exploits copper as a catalyst to reduce the activation energy for reaction between azides and alkynes in the presence of tris(triazolyl)amine ligands.[59] Yin et al. demonstrated the ability to conjugate MUC1 (glyco)peptides onto Bacteriophage Qβ VLPs as an antitumor vaccine using CuAAC.[60] Mucin-1 (MUC1) is a glycoprotein that have been shown to be overexpressed in tumorous cells and can serve as an antigen to generate MUC1 antibodies and MUC1 specific cytotoxic T cells to combat against cancer.[61] The MUC1 conjugated Qβ VLPs (Qβ-MUC1) were generated through alkyne addition to Qβ followed by CuAAC using an azide group that linked to the N-terminus of MUC1 (glyco)peptide.[60] C57BL6 mice were given prime and boost immunization with Qβ-MUC1 variants which had different density of MUC1 and glycosylation on MUC1 peptide. Upon vaccination, Qβ-MUC1 nanoparticles with high local density of MUC1 on Qβ elicited the highest IgG titer 21 days after the prime vaccination and maintained similar IgG titers for longer than 6 months. In addition to humoral response, cytotoxic T cell responses

from spleens and lymph nodes were assessed. It was demonstrated that cytotoxic T cells from both lymph nodes and spleens of vaccinated mice were able to kill RMA-MUC1 cells by 20% more than those induced by empty Qβ VLPs. Overall, Qβ-MUC1 vaccine nanoparticles were shown to be highly effective against cancer by eliciting long-lasting and robust humoral responses accompanied with potent MUC1-specific cytotoxic T cell responses. However, due to the toxicity of copper, vaccine nanoparticles developed by using CuAAC reaction must be carefully purified prior to use in living organisms. Alternatively, dibenzocyclooctyne (DBCO) is a copper-free click chemistry reactant, shown in Figure 5, that is compatible with living cells and organisms and has been used for nanovaccine formulation.[62, 63] Mohsen et al. designed a cancer vaccine using engineered cucumber mosaic virus (CMV) decorated with universal tetanus toxoid (TT) epitope $TT_{830–843}$ as an universal T cell epitope to enhance the immune responses of aging populations.[64] To study the efficacy of CMV-$_{TT}$-VLPs as a cytotoxic T cell based nanoparticle vaccine, H-2D^{b} restricted p33 peptide derived from lymphocytic choriomeningitis virus was used as a model antigen. For the DBCO-mediated conjugation, solid state synthesized p33 with an azide group on the lysine at the C-termini was used. Cyclooctyne moieties with NHS esters were added to lysine residues on CMV_{TT}, which then reacted with p33 azide forming a stable triazole linkage without copper catalyst as shown in Figure 5. The vaccine was administrated with micron-sized microcrystalline (MCT) tyrosine as an adjuvant in murine B16F10p33 melanoma models, which present p33 antigen. This CMV platform demonstrated that the incorporation of tetanus toxin epitope with MCT enhanced p33 immunogenicity with increased p33 specific CD8+ IFN-γ and CD8+ TNF- α levels and 75% reduction in tumor growth compared to CMV_{TT} control. In addition, biodistribution results indicated prolonged release of CMV_{TT}MCT in the popliteal draining lymph nodes over 9 days, compared to CMV_{TT} which had rapid accumulation and release in the popliteal draining lymph nodes over 4 days. An increased population of tumor infiltrating CD8+ T cells and enhanced p33 specific CD8+ T cell infiltration was also observed in the CMV_{TT}MCT group. The formulation of CMV with p33 and MCT enables a depot effects in the lymph nodes and inhibit the growth of solid tumors. This study highlighted a method to generate vaccines using azide motifs on peptides without the use of copper catalyst or non-natural amino acid incorporation.

DBCO bioconjugation is a highly modular system that allows for rapid nanovaccine fabrication. The method has also been applied to development of an anti-human epidermal growth factor receptor-2 (HER2) breast cancer vaccine. Hu et al. conjugated the HER2-derived CH401 epitope (amino acid residues 163–182) with an azide modification at the C-terminus onto Physalis mottle virus (PhMV) using DBCO-PEG4-NHS.[65] PhMV is an icosahedron comprising 180 identical coat protein units, of which each contains of four lysine residues. So, a high number of conjugation sites for NHS-ester group are available on the external surface to attach DBCO. Densitometric analysis from SDS-PAGE indicated a ~20% conjugation efficiency or ~36 units of CH401 epitope per particle. CpG-ODN(oligodeoxynucleotides) was also added as a TLR-9 agonist to stimulate and mature dendritic cells for improved IFN-α secretion. PhMV-CH401 was subcutaneously injected into BALB/c mice with prime and boost immunization, inducing anti-CH401 IgG titers. For tumor challenge, HER2 cells were cultured and injected subcutaneously on day 45. It was

observed that PhMV-CH401 reduced tumor growth by ~30% compared to CH401 peptide control and improved survival rate up to day 38 compared to PBS, CH401, and empty PhMV controls. Overall, copper-free click chemistry is an efficient method to link antigens onto nanocarriers as a promising vaccine system.

Chemical crosslinkers

Chemical crosslinkers are molecules with terminal functional groups that undergo conjugation with proteins, often with spacing molecules in between. Crosslinkers can be used to immobilize antigen on the surface of a nanoparticle or create protein nanoparticles by linking amino acids such as lysine or cysteine. While EDC/NHS and maleimide chemistry described above are sometimes considered as “zero length” crosslinkers, this section describes crosslinkers with spacers providing length. Chemical crosslinkers can be homo- or hetero-functional and bi-, tri- and tetrafunctional. The length of the crosslinker can be modified using poly(ethylene glycol) (PEG) spacers, which also provides flexibility. Additionally, crosslinkers have been developed to have stimuli responsiveness such as activation through UV exposure or cleavable in the presence of disulfides in the spacer domain.[66] 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) is one of the commonly utilized crosslinkers for direct conjugation of antigens to fabricate protein nanoparticles, especially for the development of influenza vaccines.[67-73] DTSSP contains two NHS-ester reactive groups, which react with terminal and lysine amines, with a disulfide bond between an 8-carbon spacer arm. For the synthesis of influenza vaccine nanoparticles, DTSSP crosslinkers were used to stabilize nanoparticles formed by adding desolvating agents to proteins such as M2e or nucleoprotein as shown in Figure 6. In addition, nanoparticles were coated with an adsorbed layer of hemagglutinin (HA), which was covalently bound using DTSSP crosslinkers. Since DTSSP has a cleavable disulfide bond, DTSSP crosslinked nanoparticles can be reduced in intracellular reducing environment. As demonstrated by Deng et al., chimeric M2e nanoparticles coated with HA stalk domains and crosslinked with DTSSP elicited potent immune responses.[70] Upon intramuscular immunization of mice, anti-HA and M2e IgG titers showed strong cross-reactivity, M2e-specific T cell activation was observed, and upon challenge mice were protected against divergent influenza strains from multiple groups. Song et al. also used DTSSP crosslinkers to synthesize an influenza vaccine nanoparticle from influenza B nucleoprotein and HA stalk.[71] The nanoparticles were formed via desolvation of soluble nucleoprotein and coated with HA stalk using DTSSP. Upon intranasal vaccination, a significant increase in the population of IL-4 and IFN-γ secreting T cells was seen in mice administered DTSSP crosslinked nanoparticles compared to soluble antigens. Furthermore, nanoparticle immunization induced robust cross-reactive T cell responses. Sustained antibody responses were also observed from the nanoparticle group six months post-boost immunization with over 3-fold higher levels of IgA towards both Victoria and Yamagata lineage of influenza compared to soluble antigen control. This platform has been used with various conserved antigenic protein domains related to influenza and SARS-CoV-2, which have led to broad protection against several different stains of the corresponding viruses. [67-74] This method allows for the combination of antigens to be easily added to both nanoparticle cores and surfaces through the use of crosslinkers. However, addition of desolvant to antigens to form nanoparticles can

damage antigen structure, as reported by Park et al. using molecular dynamics and circular dichroism.[75] Salt precipitation was instead used to form ovalbumin (OVA) nanoparticles, which were crosslinked by DTSSP. Interestingly, the desolvated OVA nanoparticles elicited lower OVA-specific IgG titers than salt-precipitated nanoparticles, which preserved the native conformation of OVA. Furthermore, the desolvated nanoparticles shifted the dominant isotypes of antibody from IgG1 to IgG2a against ovalbumin although it normally favors allergen-associated antibody responses including IgG1 and IgE. This work demonstrated that it was desolvation, not crosslinking that changed the structure of antigen and affected immunity.

A natural alternative to synthetic crosslinkers is genipin, a crosslinker derived from the gardenia fruit. Genipin is reactive to free amine groups of lysine or hydroxylysine residues. Dong et al. demonstrated genipin-mediated crosslinking of OVA proteins to form nanoparticles.[76] The strategy allows antigens to be displayed both within and on the surface of nanoparticles. The OVA incorporated in the nanoparticle exhibited no structural change from soluble ovalbumin based on circular dichroism spectroscopy, and the diameter of nanoparticles was 86.34 ± 3.21 nm. Antibody production of anti-OVA IgG, IgG1 and IgG2a was detected from serum of immunized mice 7 days after injection of the OVA nanoparticles. Vaccination also triggered significant OVA-specific cellular immune responses, resulting in ~10% and ~25% of activated CD4+ and CD8+ T cells, respectively. While a variety of crosslinked nanoparticles have been made from different antigens, using both synthetic and natural crosslinkers, fabrication procedures must be optimized and not all antigens have sufficient surface accessible reactive residues to form crosslinked nanoparticles.

Biological Conjugation Methods

Biological conjugation is the alternative covalent method to chemical conjugation and is also frequently used for conjugation of antigens to protein nanoparticle scaffolds. The process involves genetic modification of an antigen for site-specific conjugation via a biological interaction, which leads to proper orientation of the antigen. Antigen orientation on particles has been shown to be an important factor for humoral immune responses,[77-79] and site-specific biological conjugation can enhance vaccine effectiveness of protein nanoparticles. Biotag coupling requires the addition of a tag, usually at a terminus of an antigen. While some coupling systems, such as Spycatcher/Spytag, can be directly used for antigen attachment, some methods, including sortase transpeptidation, need an enzyme to activate the biotag for conjugation. In contrast to the biotag coupling systems, one terminus of an antigen can also be genetically fused directly to a protein scaffold. In all cases, genetic modification of the antigen must not compromise its expression or folded structure nor interfere with formation of the protein nanoparticle.

Genetic Fusion of Recombinant Antigens to Natural Self-Assembling Scaffolds

As the name implies, genetic fusion connects two proteins at the DNA level to be expressed as a single protein. In the case of vaccine design, this can be utilized by fusing an antigen to

a protein that naturally self assembles into nanoparticles, of which there are many, including previously described VLPs and many protein cages. A robust and well-characterized self-assembling scaffold, such as ferritin, is desirable for displaying the fused recombinant antigen. Ferritin is an intracellular iron-carrying protein that naturally assembles into a hollow, spherical nanocage. The nanocage consists of 24 subunits of 4 alpha-helix bundles, which form interfaces along 4-fold and 3-fold symmetric axes by joining four and three subunits, respectively. The threefold symmetry of the ferritin nanocage makes it amenable to display of trimeric antigens, such as HA, on its surface as demonstrated in Figure 7a-b.[80] After vaccination of female BALB/c mice with the self-assembling influenza ferritin nanoparticles, hemagglutination inhibition antibody titers from the sera were higher than those from mice administered with licensed trivalent inactivated influenza vaccine (TIV) by more than 10-fold. The enhanced immune responses were also demonstrated by challenging vaccinated ferrets with 2007 Bris virus. A significant reduction in viral loads were only detected in ferrets immunized with the self-assembling influenza vaccine nanoparticles a day after challenge. On the same day, TIV-vaccinated ferrets did not show a significant reduction in the viral loads compared to sham control. Various viruses such as respiratory syncytial virus (RSV)[81, 82], hepatitis C virus[83], cytomegalovirus[84-86], and SARS-CoV-2[87] carry trimeric glycoprotein antigens, and ferritin nanocages have served as scaffolds to present multiple trimeric antigens against SARS-CoV-2, influenza, Middle East respiratory syndrome (MERS-CoV), and RSV.[24, 88-90] The use of ferritin nanocages as a SARS-CoV-2 vaccine platform was demonstrated by genetically fusing the S protein to N-terminus of ferritin protein. The S-trimer-ferritin nanoparticles conferred humanized k18-hACE2 mice and macaques protection against SARS-CoV-2 challenge by effectively neutralizing the virus compared to unvaccinated controls.[91, 92]

Similarly, other self-assembling proteins can be utilized to construct scaffolds for the development of multivalent vaccines. Motifs such as coiled coils, α-helix, and β-sheets alone can be linked to antigens to generate scaffolds with distinct shapes.[24, 94-96] In some cases these fusions are small enough to be produced via solid state synthesis instead of recombinant methods. A potent HIV vaccine was developed by fusing trimeric coiled coils to pentameric coiled coils, which assemble into a spherical protein nanoparticle and present multiple HIV epitopes, resulting in a significantly more potent antibody immune response from vaccinated rats than unvaccinated rats.[97, 98] Wu et al. demonstrated the use of α-helical nanofiber (Coil29) and β-sheet fibril (Q11) to incorporate ovalbumin (OVA) peptides and induce OVA-specific immune responses.[99] Interestingly, OVA functionalized Coil29 (OVA-Coil29) induced stronger antibody and cellular immune responses from mice than OVA functionalized Q11 (OVA-Q11). The trend was associated with better geminal center B cell formation, stronger activation of dendritic cells, and better follicular helper T cell engagement seen in mice vaccinated with OVA-Coil29, indicating conjugation of antigen to different vaccine platforms may be crucial for identifying enhanced vaccine effectiveness. However, Q11 was found to be stable without any undiminished efficacy at 45 °C even for six months, rendering it an attractive platform for long term storage.[100] The effectiveness of Q11 nanofibers as a vaccine platform was also demonstrated by fusion with human papillomavirus 16 (HPV-16) oncoprotein E7 (E7-Q11). Compared to empty Q11 and unassembled E7-Q11, immunization of mice with β-sheet assembled E7-Q11 was shown to

be more effective in the reduction of HPV associated tumor size and induction of CD8+ T cells.[101] Aside from oncoantigens, Q11 has been used for a wide variety of antigens including viral antigens such as HIV gp120 envelope glycoprotein[102, 103] and self-antigens such as anti-TNF B cell epitopes[104, 105]. When antigens were used in conjunction with T cell epitopes and adjuvants, such as STING and TLR9 agonists[106] or complement protein C3dg[104], enhanced immunogenicity was seen in immunized mice. Q11 has been shown to be a versatile platform to display various antigens for different vaccine applications.[107, 108]

To improve potency of vaccine nanoparticles, antigen can also be fused to immunogenic proteins that can self-assemble into a nanoparticle and serve as an alternative to conventional adjuvants. Kang et al. used BP26, a highly immunogenic Brucella outer membrane protein, to form a hollow nanobarrel and decorated the scaffold with influenza M2e.[93] As shown in Figure 7c, monomeric BP26 was genetically fused to M2e and assembled into a hexadecameric nanobarrel, resulting in antigen display at each end of the nanobarrel. Vaccination of BALB/c mice with BP26-M2e elicited much stronger humoral immune responses than a mixture of soluble M2e and BP26, even with alum adjuvant. In addition, after challenge, immunization with BP26-M2e resulted in 90% and 75% survival rates against a lethal dose of Puerto Rico/8/34 (H1N1) influenza A and California/04/2009 influenza A, respectively. Conversely, the PBS control and a mixture of soluble M2e and BP26 with alum adjuvant didn't confer mice any protective immune response against the virus challenges.

Just as VLPs have been frequently used with chemical conjugation of antigens, antigens can also be easily linked to VLPs recombinantly, making them an attractive approach for the rapid development of new vaccine nanoparticles against various pathogens. Venereo-Sanchez et al. used group specific antigen (gag), a viral structural protein of HIV-1, to generate VLPs presenting HA and neuraminidase (NA) from mammalian cells as gag HIV-1 was more efficient for the production of influenza VLPs from HEK293 cells than influenza matrix protein 1 (M1).[109] Compared to naïve and empty VLP groups, higher nasal and serum IgA, serum IgG1, and serum IgG2a titers were seen in mice intranasally immunized with influenza VLPs. Although the antibody titers from influenza VLP-vaccinated mice were comparable to those from soluble recombinant H1N1 HA (A/Puerto Rico/8/34), serum IgG2a titers were slightly higher than those from the soluble H1N1 HA control. After mice were vaccinated with the influenza VLPs or soluble H1N1 HA, all survived from a lethal influenza challenge. Naïve and empty VLP groups did not provide protection against influenza virus or any detectable antibody immune response. Antigen can also be fused to VLPs manufactured in plants. Plant-derived influenza VLPs, such as recombinant quadrivalent VLP (QVLP) influenza vaccine, can be generated by delivering an expression cassette encoding influenza HA carried by agrobacterium into plants via physical or vacuum infiltration, a process called agroinfiltration. Plant-derived VLPs with HA anchored in the plasma membrane bud from the plants. The plant-derived influenza VLPs have shown to be immunogenic in both human and animals.[110, 111] Mice administered with QLPVs showed cross-reactive antibody responses against A/Brisbane H1N1, A/Uruguay H3N2, B/Malaysia, and B/Massachusetts and strong CD4 T cell responses against A/California H1N1, A/Victoria H3N2, A/Hong-king H3N2, B/Brisbane, B/Wisconsin, and B/Massachusetts. Although QVLP vaccines resulted in modest antibody immune responses

in human recipients, robust CD4 T cell immune responses were seen, resulting in 33-57% vaccine efficacy across adults aged 18-64 years. In addition, plant-derived VLPs are marked by their rapid production and scalability as demonstrated by agroinfiltration of *Nicotiana benthamiana* plants for transient expression of HA bearing VLPs.[112-114] Plant-derived VLPs were also developed against Covid-19 by transfecting *Nicotiana benthamiana* with full-length S glycoprotein of SARS-CoV-2 and the vaccine nanoparticles were evaluated through different phases of clinical trials.[115-117] During phase 3, a total of 24,141 participants were intramuscularly vaccinated with a combination of the plant-derived coronavirus-like particles (CoVLP) and Adjuvant System 03 (AS03) or placebo, resulting in vaccine efficacy ranging from 69.5% in preventing any symptomatic Covid-19 infection to 78.8% in preventing moderate-to-severe disease. In general, these studies suggest plant-derived VLPs with anchored antigens on the surface are a promising vaccine candidate against various pathogens.

RNA bacteriophage derived VLPs, such as Qβ [118], are also widely engineered to present multivalent antigens. AP205 (Figure 8) is a bacteriophage derived VLP that is highly ordered and can display multiple antigens on the surface. Its versatile application as a vaccine scaffold was shown by Tissot et al.[119] According to the report, epitopes including angiotensin II (Ang II), *S.typhi* outer membrane protein (D2), HIV1 Nef, influenza matrix protein 2 (M2), and CXCR4, an HIV entry coreceptor, were fused to AP205 VLPs to demonstrate that the VLPs can display peptides with different lengths on the surface. Short and long protein spacers were introduced between AP205 and epitopes to separate proteins domains to improve protein folding and effectively present large epitopes. In the study, accessibility of epitopes with different lengths of linkers on the VLPs were assessed by examining immunogenicity. Although D2 epitope with a long spacer at the N-terminus of AP205 induced lower IgG titer than that with a short linker at the N-terminus, D2 peptides fused at the C-terminus using short and long spacers did not affect the immunogenicity, implying that the effect of spacers didn’t play critical role in enhancing accessibility of the epitopes. Upon vaccination, influenza matrix protein 2 (M2) fused to AP205 VLPs (AP205-M2) induced a strong humoral immune response against both AP205 and M2 antigen. AP205-M2 conferred mice protection against a lethal influenza challenge, whereas AP205-vaccinated mice didn’t survive the challenge. Furthermore, the VLPs were also conjugated with gonadotropin releasing hormone (GnRH) to treat prostate cancer, and endogenous GnRH was inhibited in mice administered with AP205-GnRH VLPs. The immunized mice also exhibited a rapid onset of antibody production against GnRH after a single shot was given. Altogether, genetic fusion of antigens to self-assembling scaffolds has been an effective strategy to induce potent immune responses. However, the approach can be limited by disrupted folding of the antigen or scaffold protein or decreased expression of the new fusion proteins. To address the issue, self-assembling scaffolds can be decorated with multiple antigens via biotag coupling as this approach involves post-translational modification.

Nonenzymatic Biotag Coupling of Recombinant Antigens

Plug-and-display has been a strategy to quickly formulate multivalent vaccines thanks to its simple and modular display of multiple antigens. With the help of nonenzymatic biotag

coupling, vaccine platforms such as self-assembling protein nanoparticles or VLPs can be easily decorated with multiple antigens without direct genetic fusion to nanoparticles, facilitating the development of new subunit vaccines against different pathogens. Among different biotags, SpyCatcher has been extensively utilized to present antigens on the surface of subunit vaccine nanoparticles.[24, 120-122] Immunoglobulin-like collagen adhesin domain (CnaB2) from *Streptococcus pyogenes* (Spy) was split into a large N-terminal protein (SpyCatcher) and a small C-terminal peptide (SpyTag). The N-terminal lysine of SpyCatcher can spontaneously form an intramolecular isopeptide bond with the C-terminal aspartic acid of SpyTag (Figure 9a).[123] The SpyCatcher system was recently applied to construct various SARS-CoV-2 vaccines. Kang et al. reported the use of 24-mer ferritin, 60-mer mi3, and 120-mer I53-50 to increase the valency of RBD antigen presented on the surface, as shown in Figure 9b.[121] The mi3 nanoparticle is a mutated dodecahedral i301 nanocage, whose design is based on 2-keto-3-deoxy-phosphogluconate aldolase from the Entner-Doudoroff pathway to improve its stability,[124]. Spycatcher mediated conjugation of mi3 scaffold (SpyCatcher-mi3) is a well-established platform.[124, 125] The I53-50 nanoparticle is a rationally designed icosahedral nanocage that can be formed from a combination of 20 copies of trimeric I53-50A1.1PT1 and 12 copies of pentameric I-53-50B.4PT1.[126] Each cage scaffold was fused to SpyCatcher so that RBD tagged with SpyTag could rapidly react and be conjugated to the scaffolds. This approach ensured the antigens were properly oriented and presented on the surface with multiple copies. Owing to the high valency of RBD, the nanoparticles resulted in higher neutralization titers of antibodies from mice than vaccination with RBD monomer. The SpyCatcher-mi3 platform was also employed to generate vaccine nanoparticles with 60 copies of RBDs from SARS-CoV-2 beta and seven different animal sarbecoviruses (mosaic-8b) with C-terminal SpyTags to favor interactions between BCRs and conserved RBD domains to elicit broad protection against SARS-CoV-2 variants.[120] The same RBD epitopes with SpyTag coupled to mosaic-8b with SpyCatcher did not interact with bivalent BCRs effectively due to a mixture of eight different RBDs fused onto the surface of mi3 nanoparticles. This, in turn, favored strong interactions of bivalent BCRs with high valency of conserved RBD epitopes, although they are less accessible than variable sites of RBD, and mounted dominant humoral immune responses in mice against the conserved RBD epitopes. Mosaic-8b induced significantly higher antibody and neutralization titers in mice than homotypic mi3 nanoparticles decorated with SARS-Cov-2 beta RBDs against various strains including SARS-2 WA1, beta, and delta, SARS-1, WIV1-CoV, SHC014 CoV, SHC014-CoV, BM-4831-CoV, and Yun11-CoV. Furthermore, mosaic-8b conferred broadly protective immune responses in humanized K18-hACE2 mice and rhesus macaques against SARS-CoV-2 and SARS-CoV challenges, whereas mice immunized with mi3 control showed protective immune responses only against SARS-CoV-2 challenge.

Similar to SpyCatcher/SpyTag, a single pilus-associated adhesin in *Streptococcus pneumoniae* (RrgA) can split into a peptide and a protein to establish a nonenzymatic biotag coupling system .[127] The peptide/protein pair (SnoopTag/SnoopCatcher) can spontaneously form a isopeptide bond without enzyme (Figure 9a). It was demonstrated that SnoopTag reacted with its cognate partner SnoopCatcher with more than 99% yield while it did not show any trace of cross-reaction with SpyTag/SpyCatcher pair. Therefore,

SnoopTag/SnoopCatcher can be harnessed in concert with SpyTag/SpyCatcher to present two different antigens in order to elicit robust immune responses. As demonstrated by Brune et al., SnoopTag/SnoopCatcher and SpyTag/SpyCatcher were used to present dual malarial antigens, Pfs25 and Pfs28, on self-assembling coiled-coil IMX313 nanoparticles, a heptamerizing domain of complement inhibitor C4 binding protein.[128, 129] SpyCatcher and SnoopCatcher were genetically fused to the N-terminus and C-terminus of IMX313, respectively, to covalently link Pfs25 with SpyTag and Pfs28 with SnoopTag to the modular scaffold. When mice were vaccinated with the IMX313 nanoparticles presenting Pfs25 and Pfs28, endpoint titers of serum antibodies against Pfs25 and Pfs28 significantly increased in comparison to soluble Pfs25 or Pfs28 antigen. As they can spontaneously be covalently linked to one another via transamidation without cross-reaction to SpyTag/SpyCatcher, antigen conjugation by SnoopTag/SnoopCatcher and SpyTag/SpyCatcher was a very effective approach for dual antigen presentation. As with any fusion proteins, there could be negative affects on protein folding or expression when adding tag or catcher components. However, given how these systems work, fusion of catcher proteins has already been successful with a number of cages and these can be used by many groups in future work with new antigens fused to the tag domain, which is much smaller and less likely to interfere with antigen folding. Furthermore, the bacterial source of the catcher and tag proteins could induce undesired immune responses specific to those sequences.

Enzymatic Biotag Coupling of Recombinant Antigens

Antigens can also be conjugated to protein nanoparticles or other protein scaffolds by enzyme-mediated biotag coupling. Tags such as LPXTG sortase A (SrtA) have shown effective specific site conjugation of antigens to protein nanoparticles. Antigen recombinantly tagged with acyl donor can be recognized by SrtA, a bacterial transpeptidase that cleaves the acyl donor and ligates the C-terminal threonine of the tag onto an N-terminal glycine of a target (Figure 10a). Sortase transpeptidation has been successfully used in the modular development of various vaccines. Saunders et al. reported higher neutralization titers of antibodies in macaques against SARS coronavirus 2 (SARS-CoV-2) induced by ferritin nanoparticles sortase conjugated with RBD of SARS-CoV-2 than mRNA lipid nanoparticles encoding spike ectodomain, analogous to Covid-19 vaccines approved for emergency use.[130] For selective conjugation, *Helicobater pylori* ferritin was tagged with an N-terminal SrtA glycine acceptor peptide that was ligated to the C-terminal LPETGG motif of monomeric RBD upon SrtA-mediated transpeptidation. Through the enzymatic biotag coupling, RBD was fused to the ferritin nanoparticles with a proper orientation and high valency. The same approach was also utilized to decorate VLPs. Rod-shaped papaya mosaic virus (PapMV) VLPs were conjugated with influenza nucleoprotein (NP) and simian immunodeficiency virus group-specific protein antigen (GAG). PaPMV VLPs decorated with NP or GAG elicited higher antibody titers and stronger IFN-γ T cell responses in mice than soluble NP or GAG.[131] Modular application of SrtA was also demonstrated by surface modification on bamboo mosaic virus (BaMV) VLPs.[132] BaMV VLPs were genetically modified to include glycine residues that served as SrtA acceptor for ligation to the recombinant envelope protein domain III (rEDIII) of Japanese encephalitis virus (JEV) tagged with SrtA donor sequence. The highly ordered presentation of rEDIII antigens with

high valency on the surface BaMV VLPs (designated BJLPET5G) resulted in higher murine IgG titer and neutralizing antibody titer than BaMV VLPs or soluble rEDIII.

SnoopLigase conjugation is another enzyme-mediated method for modular plug-and-display assembly that was applied for malaria and cancer vaccines.[133] Malarial antigens, cysteine-rich inter-domain region (CIDR) and cysteine-rich protective antigen (CyRPA), were genetically fused to SnoopTagJr while DogTag peptide was linked to self-assembling coiled-coil nanoparticle IMX (IMX-DogTag). DogTag of IMX was then reacted with SnoopTagJr to covalently conjugate CIDR or CyPRA to IMX. The isopeptide bond between DogTag and SnoopTagJr was formed by SnoopLigase, which was obtained by splitting *Streptococcus pneumoniae* adhesin into three domains (Figure 10b). The size of DogTag, a new Catcher protein, is much smaller than SnoopCatcher or SpyCatcher, reducing the size of fusion proteins and, thus, minimizing misfolding of recombinant proteins. After three injections of the vaccine nanoparticles were given to mice, higher endpoint titers of serum antibodies against CIDR and CyPRA were observed compared to soluble CIDR and CyPRA. Due to the small size of the fusion protein, SnoopLigase-mediated assembly can be a promising approach to reduce off-target immune responses for the development of future vaccine nanoparticles.

In addition to SrtA tag and DogTag, AviTag allows site specific conjugation via BirA enzyme-mediated biotinylation (Figure 10c).[134] Due to the strong binding affinity of biotin to streptavidin,[135] the conjugation has been extensively employed for multivalent antigen presentation.[136, 137] Chiba and Frey et al. harnessed AviTag to display multiple copies of the SARS-CoV-2 S protein on biotinylated bacteriophage MS2 VLPs by fusing AviTag to both S and MS2 coat proteins. Streptavidin was used to link MS2 to S protein after the AviTag of each protein was biotinylated by the enzyme BirA.[138] A single immunization of Syrian hamsters with the multivalent VLPs resulted in higher RBD-specific endpoint titer and neutralizing antibody titer against a SARS-CoV-2 virus than immunization with MS2 VLPs without S proteins (MS2-SA VLP) and PBS. After SARS-CoV-2 infection, hamsters administered with MS2-SA VLP and PBS showed high viral shedding in the lungs while a significant reduction in the viral shedding was observed in hamsters vaccinated with the MS2-SA VLPs decorated with multiple copies of S proteins.

Naturally occurring ligation can also be a powerful tool to conjugate antigen to nanoparticle scaffolds. As a part of natural post-translational modification, proteins undergo splicing reactions. During this procedure, intein, an internal protein domain, self-catalyzes its excision, resulting in the ligation of flanking external protein domains, also known as exteins.[139-141] Using this mechanism, inteins can be used to induce trans-splicing to ligate fragmented or two different proteins into a protein for vaccine assembly as demonstrated in Figure 10d. Tang et al. demonstrated intein-mediated fusion of enterovirus 71 (EV71) epitope SP70 to human heavy chain of ferritin (HFT) nanoparticles.[142] gp41-1 intein^{C} (int^{C}) was introduced to N-terminal of HFT while gb1 tag, B1 domain of streptococcal Protein G, was fused to int^{C} to improve the solubility and yield of HFT proteins and, thus, their expression efficiency. Two modules, gb1-int^{N} linked SP70 (gb1-int^{N}-SP70) and monovalent streptavidin, knowns as rhizavidin, (gb1-int^{N}-rhizavidin), were then reacted with gb1-int^{C} of HFT to be covalently fused to HFT while splicing out gb1-int^{N} and gb1-int^{C} as byproducts.

Then, the rhizavidin interacted with biotinylated CpG to carry both SP70 epitope and CpG adjuvant (SP70/CpG-HFT). As such, intein-mediated trans-splicing can not only assemble vaccine but also remove any unnecessary tag at the same time after purification. Mice were immunized with the SP70/CpG-HFT nanoparticles, which markedly enhanced immune responses against EV71 virus. Compared to empty HFT scaffold and HFT carrying only SP70 or CpG, co-delivery of SP70 and CpG by HFT nanoparticles resulted in stronger antibody immune responses and higher survival rate against challenge with EV71 virus. The same strategy was also employed to generate hepatitis B virus core protein (HBc) VLPs decorated with nucleocapsid protein (NP) of SARS-CoV-2.[143] The HBc VLPs conjugated with NP significantly improved NP-specific immune responses in mice. The HBc VLPs decorated with NP elicited stronger IgG, IgG2a, IgG2b, and IgG3 responses than soluble NP. Likewise, intein splicing is a useful tool to display antigens on various vaccine platforms for effective immune responses.

Enzymatic biotag coupling is an emerging site-specific method to conjugate antigens. Importantly, the biotag fused to antigen or scaffold is small enough to minimize the formation of misfolded protein. However, following reaction, enzyme or any byproducts, such as intein spliced products, must be removed and, therefore, require additional purification steps. Another challenge associated with enzymatic biotag coupling is a separate expression process to obtain enzyme, except for intein splicing. Despite the additional processes, it is undoubtedly an important tool as it provides fast, site-specific, and controlled conjugation of antigens.

Conclusions and Outlook

Subunit protein vaccine nanoparticles of various types are promising platforms thanks to their proven track record for safety and stability. However, of the different protein nanoparticle platforms developed, only VLPs have been approved for clinical use as vaccines. Due to their good safety profile, they are utilized in licensed vaccines including anti-malaria RTS,S/AS01[144, 145] and anti-HPV Gardasil-9[146-148]. RTS,S/AS01, also known as Mosquirix, is the first licensed malaria vaccine developed by Glaxo Smith Kline (GSK).[146-148] The subunit vaccine nanoparticle is based on VLP constructed from hepatitis B virus surface antigen, which is recombinantly fused to circumsporozoite protein domains including B and T cell epitopes, and adjuvanted with AS01. It has shown 32-36% efficacy in infants 5 to 17 months of age against malaria. Gardasil-9 is also a VLP based recombinant vaccine nanoparticle, from Merck & Co.[146-148] This HPV vaccine is designed to protect against HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58, and it is currently in use to curb both HPV infection and HPV-related cancers. Other licensed recombinant VLP vaccines include Recombivax HB by Merck & Co and Engerix-B by GSK against HBV, and Hecolin by Xiamen Innovax Biotech Co against hepatitis E virus (HEV).[149]All the licensed VLPs use recombinant fusion to present antigens on the surface.

While vaccine effectiveness of subunit protein vaccine nanoparticles without adjuvant generally tends to be lower than whole pathogen vaccines, numerous approaches have been established in an effort to enhance immune responses by incorporating multiple antigenic proteins on the surface of protein nanoparticle scaffolds. They also have been formulated

with adjuvants.[48, 64, 106, 117, 142, 144-147] Here, we have reviewed methods and techniques that enable incorporation of antigens into protein nanoparticle design. Chemical conjugation, including amine, thiol, and click chemistry and crosslinking, is an extensively used route that enables direct addition of antigens onto the surface of nanoparticles with high conjugation efficiency. Without the help of biotags, the approach allows separately expressed antigen and protein nanoparticles to be covalently linked to one another. Although it is often difficult to control orientation of antigen using crosslinkers or amine reactive chemistry due to a lack of site-specific conjugation, this can be addressed by using site-specific click chemistry with non-natural amino acids, dityrosine crosslinking, and even maleimide conjugation reaction to unpaired thiols, which are relatively uncommon in proteins. Furthermore, chemical conjugation raises a concern associated with antigen stability and immunogenicity as it can damage secondary structure and molecularly alters the antigen itself. Another challenge of chemical conjugation is that the methods often require an excess amount of reagents and additional separation steps to remove byproducts, catalysts, or reagents, some of which are toxic. All the challenges associated with chemical conjugation can be overcome using genetic fusion of proteins as they are site specific, achieve 100% “reaction”, and do not use chemical reagents. Recombinant antigens genetically fused to self-assembling motifs or VLPs can spontaneously form nanoparticles presenting multiple copies of antigens on the surface. However, genetic fusion requires genetic modification of each antigen, requiring time and resources. Furthermore, when antigens are genetically fused to proteins required to form nanoparticles, their expression and folding may not be as effective as separate expression of the antigens. Thus, since selection of fusion termini and linker design as well as expression of folded recombinant protein needs optimization, the generation of vaccines via genetic modification is generally slower than that via chemical modification, though chemical conjugation usually also requires optimization or reagent screening.

To facilitate more rapid development of vaccines, increasing attention has been directed toward the use of biotags that enable plug-and-display. Although biotags need to be fused to antigens or scaffold monomers, their size is usually small enough to prevent protein misfolding or reduce inefficient expression. Once a biotag coupling gene and method is established for conjugation of antigens to a particular nanoparticle scaffold, antigen replacement on that scaffold is very rapid as new antigens can be easily modified to include biotags. Additionally, precise arrangement of antigen can be achieved via its site-specific conjugation. Despite the advantages, the valency of tag coupled antigens on a nanoparticle can be lower than that of antigens genetically fused to a self-assembling nanoparticle or VLP because the yield of biotag mediated coupling of antigen with its counterpart is not 100%. For this reason, precise control of valency by genetic fusion is more achievable than that by tag coupling. Therefore, for maximizing valency or assessing an effect of valency on immune responses, genetic fusion can be more appropriate than both biotag and chemical modification. However, it is also worth noting that the genetic fusion is usually limited to the N- or C- termini of protein, whereas several biotags can be inserted at different sites within an antigen sequence. Thus, biotag coupling of antigen can be a better approach than genetic fusion for precise control of orientation. In addition to conjugation efficiency and control of valency and orientation, the size of protein linker also needs to be considered for correct

protein folding and minimizing off-target immunogenicity. Small peptide tags and catchers can minimize or circumvent protein misfolding for efficient protein expression as well as off-target immunogenicity. Often, this can be achieved by employing enzymatic biotag coupling system such as Snoopligase conjugation and intein-mediated protein trans-splicing. Also, conjugation of multiple different antigens onto nanoparticles can be accomplished by using the enzyme-mediated conjugation together with nonenzymatic biotag coupling method. Nevertheless, additional processes are necessary to produce enzyme and remove it conjugation, and if it needs to be purchased, it can be an expensive material.

In light of the advantages and disadvantages, each different method should be carefully evaluated depending on the goal of both the vaccine design and the questions being addressed in the vaccine study. Certainly, chemical modification and biotag coupling can expedite the process of vaccine generation, which can be quite valuable in a pandemic situation. However, different conjugation methods have not been directly compared extensively within the context of immunology. Comparison of different conjugation methods needs to be explored for optimal protective efficacy of protein vaccine nanoparticles by considering various factors including orientation, structure, arrangement, and valency of antigens on nanoparticles. To be more specific, chemical conjugation can be appropriate for conjugation of smaller antigen peptides onto nanoparticles because peptide is less sensitive to damage by chemicals than structural antigens and have such short sequences that even amine conjugation may be site specific, or nearly so.[150] Genetic fusion or biotag coupling may be the best for structural antigens as these methods not only ensure proper orientation of antigens on nanoparticles but also keep the structure of antigen intact for optimal antibody immune responses. In parallel, establishment of more conjugation methods will be of great importance in the rapidly evolving field of subunit nanovaccines to combat existing pathogens and emerging future pandemics. Although this review mainly focused on antigen conjugation to protein scaffolds for enhanced vaccine effectiveness, there still remain some more general challenges associated with protein scaffolds to address. Immune responses can vary significantly depending on the physical properties of protein scaffolds, such as the density of conjugation sites for optimal antigen spacing and valency and the size of scaffolds for improved antigen uptake by antigen presenting cells.[24] These physical features of protein scaffolds need to be comprehensively explored and optimized for improving vaccine effectiveness. The components of protein scaffolds are also important factors to be considered for maximizing immune responses against antigens of interest. Proteins, or even glycans, of protein scaffolds can induce irrelevant immune responses, thus misdirecting antigen-specific immune responses.[151-153] To minimize off-target responses, scaffolds should be carefully designed using components with low immunogenicity or reducing all non-antigen components. Therefore, a strategic approach to take advantage of synergistic effects from effective antigen conjugation methods and optimized properties of protein scaffolds will be important for improving immune responses against various pathogens.

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Biographies

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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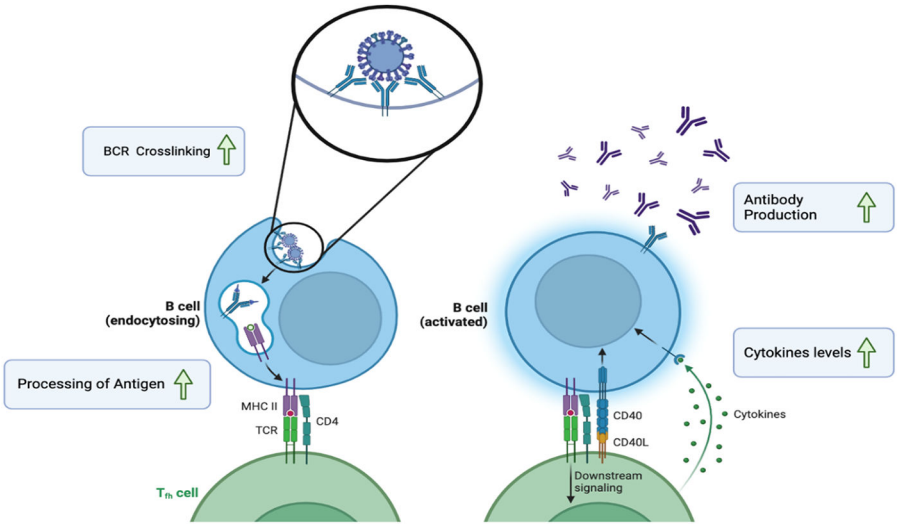

Figure 1.

Scheme of impact of improved antigen valency on vaccine carrier, enhancing BCR crosslinking allowing for antigen processing and T cell activation. (Illustrated by BioRender)

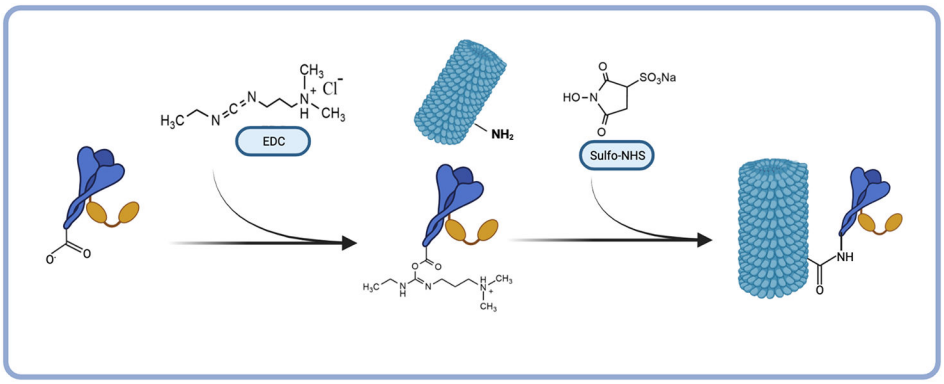

Figure 2.

Scheme of EDC/Sulfo-NHS conjugation on TMV. EDC activates carboxyl groups on RBD fused to the Fc domain of a human IgG, forming of an O-acylisourea active ester intermediate. Sulfo-NHS addition makes a semi-stable amine reactive NHS-Ester amenable to the conjugation of primary amine source from TMV carrier.[42] (Illustrated by BioRender)

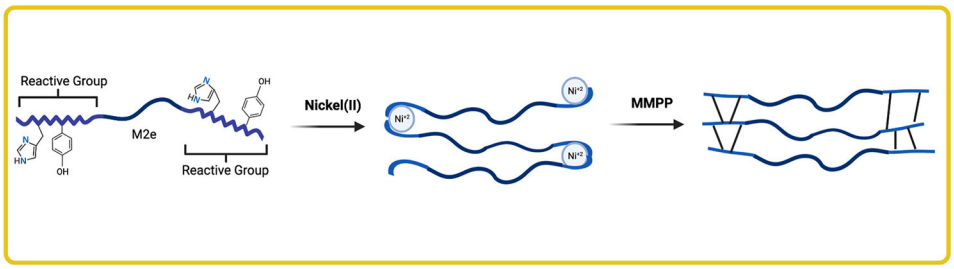

Figure 3.

Scheme of dityrosine crosslinking methods for M2e peptide into nanocluster. Binding of $H_6(GY)_2$ reactive groups with nickel brings tyrosines in proximity to react under oxidizing conditions, achieved with magnesium monoperoxyphthalic acid (MMPP).[47] (Illustrated by BioRender)

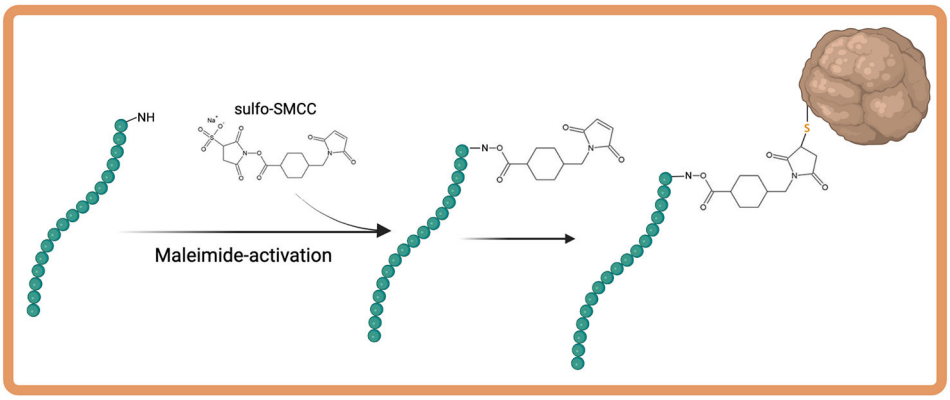

Figure 4.

Peptide modification with maleimide via amine site to enable specific reaction using Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) to cysteine sulfhydryl group on protein scaffold at pH 6.5-7.5. (Illustrated by BioRender)

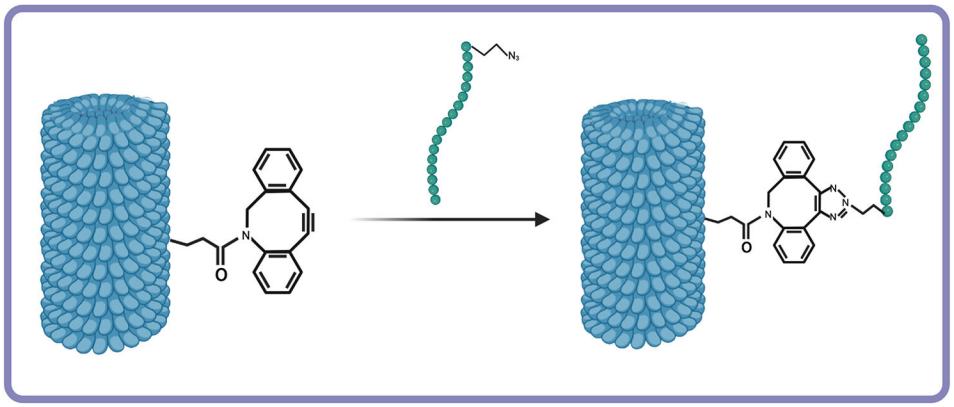

Figure 5.

Dibenzocyclooctyne (DBCO) on cucumber mosaic virus (CMV) conjugated with accessible azides on p33 peptide via strain-promoted 1,3-dipolar cycloaddition.[64] (Illustrated by BioRender)

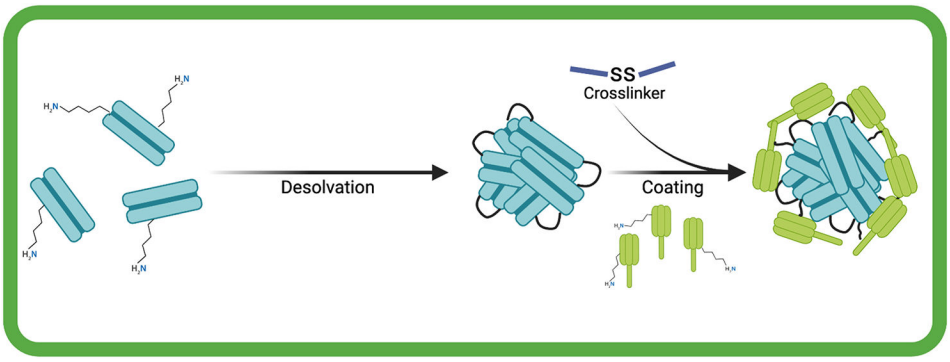

Figure 6.

Formulation of M2e nanoparticles via desolvation with organic solvent is followed by adsorption of HA stalk on the surface and stabilization with DTSSP crosslinker, which links accessible lysines and terminal amines through NHS-ester conjugation.[70] (Illustrated by BioRender)

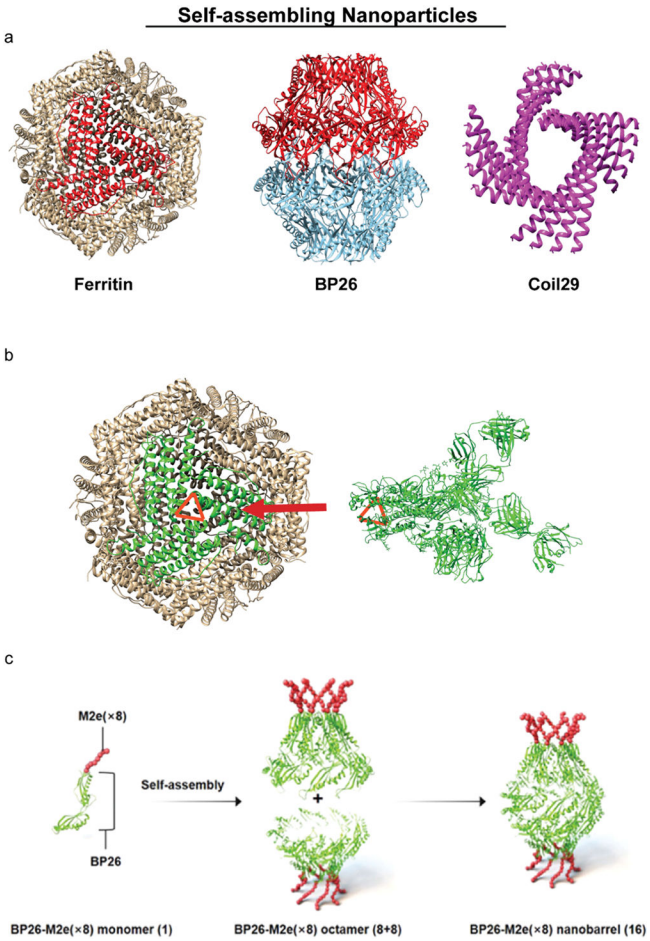

Figure 7.

Construction of multivalent vaccine nanoparticles using self-assembling motifs. (a) Self-assembling ferritin (PDB: 4V6B), BP26 (PDB: 4HVZ), and Coil29 (PDB: 3J89). (b) Trimeric HA (PDB: 3SM5) can be genetically fused to helices at threefold axis represented by a red triangle.[80] (c) M2e was fused to the C-terminus of BP26 monomer, presenting a total of 16 M2e monomers on a BP26 nanobarrel. Adapted with permission from [93]; Copyright 2021 American Chemical Society.

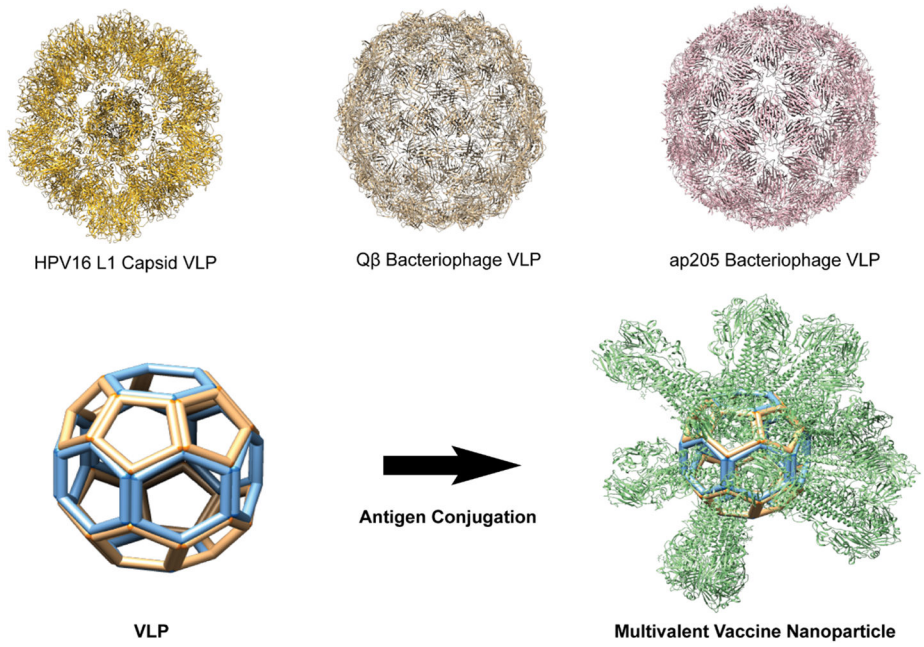

Figure 8.

Construction of multivalent vaccine nanoparticles using VLPs. VLPs including HPV16 L1 capsid (PDB: 1DZL), Qβ bacteriophage (PDB: 5VLY), and ap205 bacteriophage (PDB: 5LQP) can be decorated with antigens.

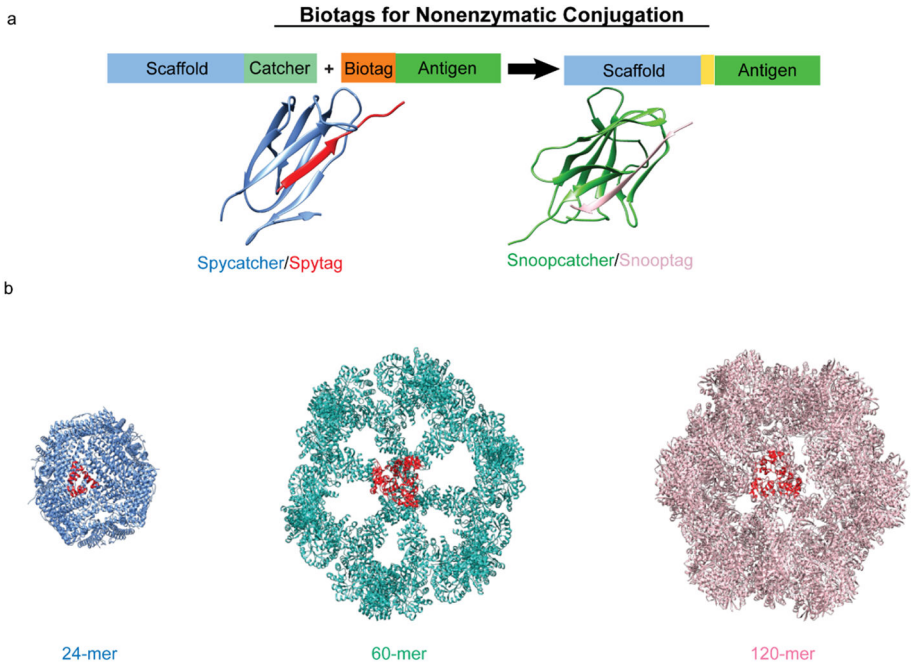

Figure 9.

Nonenzymatic Biotag coupling of antigens to protein nanoparticle scaffolds. (a) Conjugation by Spycatcher/Spytag (PDB: 4MLI) and Snoopcatcher/Snootag (PDB: 2WW8). (b) One of the sites for Spycatcher/Spytag-mediated conjugation of trimeric RBD onto Ferritin (PDB: 4V6B), mi3 (PDB: 7B3Y), and I53-50 (PDB: 7SGE) nanocages is colored in red.[121]

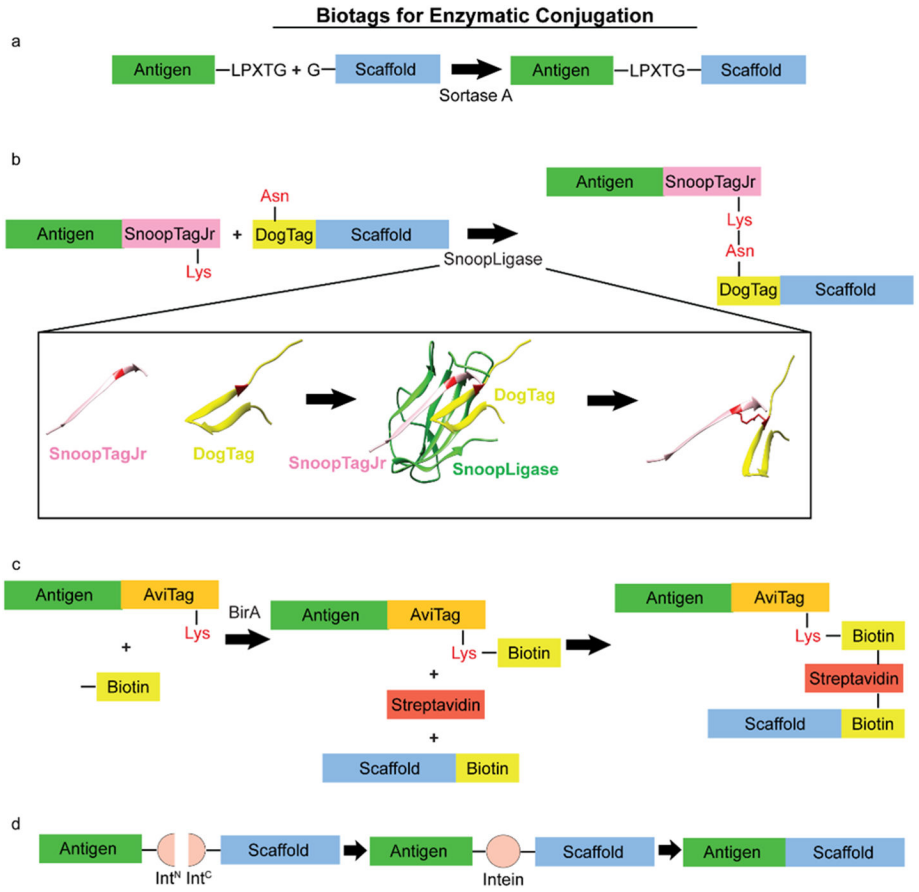

Figure 10.

Enzymatic Biotag coupling of antigens to protein nanoparticle scaffolds. Conjugation by (a) sortase transpeptidation, (b) Snoopligase platform (PDB: 2WW8), (c) BirA activation of Avitag with Biotin/Streptavidin conjugation platform, and (d) intein-mediated trans-splicing.