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REVIEW

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Protein nanocages: A new frontier in mucosal vaccine delivery and immune activation

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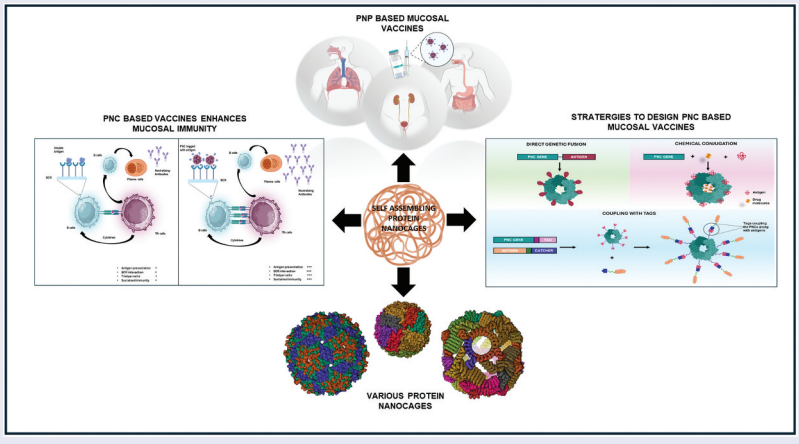
ABSTRACT

Mucosal infectious diseases represent a significant global health burden, impacting millions of people worldwide through pathogens that invade the respiratory, gastrointestinal, and urogenital tracts. Mucosal vaccines provide a promising strategy to combat these diseases by preventing pathogens from entering through the portals as well as within the systemic response compartment. However, challenges such as antigen instability, inefficient delivery, suboptimal immune activation, and the complex biology of mucosal barriers hinder their development. These limitations require integrating specialized adjuvants and delivery systems. Protein nanocages, self-assembling nanoscale structures that can be engineered, may provide an innovative solution for co-delivering antigens and adjuvants. With their remarkable stability, biocompatibility, and design versatility, protein nanocages can potentially overcome existing challenges in mucosal vaccine delivery and enhance protective immune responses. This review highlights the potential of protein nanocages to revolutionize mucosal vaccine development by addressing these challenges.



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Introduction

The global burden of mortality and morbidity caused by mucosal pathogens remains alarmingly high, underscoring the urgent need for innovative strategies to combat these diseases. The COVID-19 pandemic has starkly illustrated the devastating impact and ongoing threat posed by emerging mucosal infection.[1,2] To suppress the rapid spreading of pandemic mucosal pathogens, the infection should be checked at the portal of entry, per se, the mucosal epithelia, rather than inhibiting the multiplication of those pathogens inside the body. Injectable vaccines preferably generate immune responses in the systemic compartment, which is more effective in inhibiting pathogens that have already invaded the systemic compartment.[3–5] Antibodies in the circulation have limited efficacy in blocking the initial infectious process at the mucosae. This highlights the critical importance of developing mucosal vaccines that induce protective immune responses at both the portal of entries and the systemic compartment.

While conventional parenteral vaccines have effectively reduced severe illness and mortality by inducing protective systemic immune responses, they face significant challenges in eliciting antigen-specific immune responses at mucosal

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surfaces and preventing horizontal transmission of infectious diseases.[6–8] The inefficiency of parenteral vaccines to effectively block disease transmission highlights a critical gap in current vaccination strategies, underscoring the urgent need for effective mucosal vaccines that can address both disease severity and contagion. Mucosal vaccines, administered via oral, nasal, sublingual, or genital routes, offer a promising approach to addressing these limitations since they generate secretory IgA and local cellular immune responses that should block further dissemination of infecting pathogens and eliminate initial infection foci.[9–11] By targeting mucosal surfaces, these vaccines can stimulate localized and systemic protective immune responses, providing robust defense against a wide range of infections. Beyond pandemic mucosal pathogens, mucosal vaccines hold potential for combating major global health challenges, including *Mycobacterium tuberculosis*, HIV, tumors occurring in mucosal tissues such as cervical cancer, and other numerous intractable infectious agents.[3,7,12–14] Most currently available mucosal vaccines are based on conventional whole-cell live attenuated or inactivated vaccine platforms.[10,12,13] While conventional mucosal vaccines have demonstrated the capability to elicit robust immune responses, they often raise significant safety concerns. These include the potential reactivation of live attenuated pathogens, leading to virulence restoration, and the oncogenic risks posed by certain inactivated virus formulations.[15,16] Recently, traditional vaccines have been increasingly replaced by adjuvanted subunit, RNA, or DNA vaccines, which offer improved antigen specificity and safety. However, mucosal vaccines based on high-purity antigens face several challenges, including poor antigen stability, limited immunogenicity, and delivery inefficiencies. Addressing these issues requires incorporating effective vaccine delivery systems and potent adjuvants that could cope with barriers in mucosae, which should be requisites for the success of mucosal vaccines.[7,14,17–20]

The staggering advancement of mucosal vaccines can be attributed to two primary obstacles. First, these vaccines must overcome multiple mucosal barriers to effectively reach the mucosal immune system. The mucus layer that covers all mucosal surfaces serves as a physical obstruction, hindering antigen presentation. In case of intranasal administration, nasal hair and keratinized stratified squamous epithelia in the nostril, combined with ciliary movement and mucus in the respiratory system, can obstruct antigens from reaching their intended targets.[14] The gastrointestinal tract presents its own set of hurdles, including proteolytic enzymes, peristalsis and the presence of commensal microorganisms that may interfere with vaccine effectiveness.[21,22] Within the urogenital tract, the periodic changes in the vaginal environment and the existence of cervicovaginal mucus can impact vaccine stability and absorption.[23] Second, the development of mucosal vaccines is further constrained by the lack of potent and reliable mucosal adjuvants, which are essential for enhancing immune responses at mucosal sites.[15,24–26] As a result, a critical aspect of developing novel mucosal vaccines lies in designing and identifying effective delivery carriers and adjuvants. Recently, nanocarrier-based mucosal vaccines have shown promising efficacies in overcoming the challenges associated with traditional mucosal vaccine platforms, including delivery barriers and limited immunogenicity.[27–30] Advancements in nanotechnology, along with a deeper understanding of the mucosal immune system, have empowered researchers to develop a multitude of nano-vaccines with enhanced ability to target and activate the mucosal immune system.[31] Among the various nanocarrier-based delivery systems, protein nanocage (PNC)-based mucosal vaccines have also demonstrated remarkable effectiveness, eliciting potent protective immune responses against a wide range of infectious diseases. PNCs are multimeric proteins with a cage-like structure, serving storage units, protective shells, frameworks for enzyme complexes, and carriers for genetic material.[32,33] These structures spontaneously form core-shell configurations ranging from 8 nm to 100 nm in size, making them useful candidates for drug delivery. Their inherent characteristics include consistent size and shape, a high surface-to-volume ratio, structural robustness with highly organized architecture, monodispersibility, low toxicity, high stability, biocompatibility, biodegradability, and ease of modification using rather easy genetic and chemical techniques. Notably, PNCs are composed of repeating subunits, enabling multivalent incorporation of both antigens and potent adjuvants. This multifunctionality facilitates the co-delivery of antigens and adjuvants, making protein nanocages a utilitarian platform for vaccine development.[34–36] Moreover, the dimensions of protein nanocages facilitate their efficient accumulation in lymphoid organs, where antigen-presenting cells (APCs) are located. Within these organs, APCs present antigens to effector T cells and activate B cells by secreted cytokines, thereby jumpstarting robust immune responses. Due to these properties, there are a multitude of attempts to use protein nanocages as antigen carriers for developing vaccines against a wide range of infectious diseases and cancers.[35,37]

In this review, we provide an updated overview of various types of self-assembling protein nanoparticles used for vaccine development, taking into account the mucosal immune system's organization, routes of mucosal vaccination, and the major barriers to mucosal vaccine delivery. Additionally, we explore the design of protein nanocage-based vaccines to enhance mucosal vaccine delivery and discuss some of the most widely used protein nanocages in vaccine development.

Protein-based nanocarriers for vaccine development

PNCs are highly ordered, self-assembling nanostructures that have recently gained significant attention in the field of vaccinology due to their unique properties. These naturally occurring nanoparticles are ubiquitous across a wide spectrum of life, ranging from microorganisms such as viruses, bacteria, and archaea to more complex organisms, including plants, insects, and mammals.[38,39] Their inherent ability to self-assemble into precise geometric arrangements makes them excellent candidates for drug delivery applications. In particular, PNCs' repetitive and highly organized surface structures closely mimic natural pathogen-associated molecular patterns (PAMPs), facilitating enhanced recognition by the immune system. This property can be harnessed to elicit robust immune responses when used as a vaccine platform. Additionally, PNCs' modularity allows for the encapsulation

or surface display of antigens, adjuvants, or other bioactive molecules, offering customizable options to modulate specific immune pathways.

These self-assembling nanoscale architectures can be broadly classified into three main categories based on their origin: virus-like particles (VLPs), non-viral protein nanoparticles (NVPNs), and computationally designed nanoparticles (Figure 1). VLPs are self-assembling PNCs that closely mimic the structure of a virus but lack the core genetic material, rendering them noninfectious. These nanocages typically range in size from 20 to 200 nm.[40,41] These VLPs are derived from various sources, such as viruses and bacteriophages, and can be produced using a variety of expression systems, including bacteria, yeast, insect cells, and plants.[42] Bacteriophages such as AP205, Qβ, MS2, and HBsAg are prominent examples of VLPs.[43–47] On the other hand, NVPNs are naturally occurring self-assembling nanostructures derived from non-viral sources, including both prokaryotic and eukaryotic organisms. These nanocages are made of protein monomeric subunits that assemble into homogeneous, symmetric, and complex nanostructures. Their size ranges between 10 and 100 nm. NVPNs can be produced using various expression systems, such as bacteria, yeast, plants, insects, and mammalian cells.[35] Ferritin, encapsulin, vault, and lumazine synthase are notable non-viral PNCs that could be used for vaccine development.[48–52] In contrast, computationally designed protein nanocages are self-assembling nanostructures created using computational tools. These artificial protein nanostructures are engineered either by modifying existing protein scaffolds or by designing from scratch without any preexisting scaffold to achieve specific structural and functional characteristics properties. There are several methods for computationally designing nanocages, including hierarchical computational design and single peptide nanocage design,[53] hybrid computational method,[54] multi-component design,[37] and symmetry-based design.[55] These computational methodologies enable the creation of highly intricate nanostructures capable of assembling up to 960 monomeric units to form nanocages as large as 96 nm. Depending on the specific design requirements, these computationally designed nanocages can be expressed in various systems, such as bacteria, yeast, and insect cells. Table 1 provides detailed information concerning different types of protein nanocages, disclosing their sources, size ranges, and production systems.

Mucosal vaccine

The mucosal immune system, which is constituted of approximately 80% of the body's immune cells, differs significantly from the systemic immune system in both its organization and function.[117] This difference should be seriously taken into

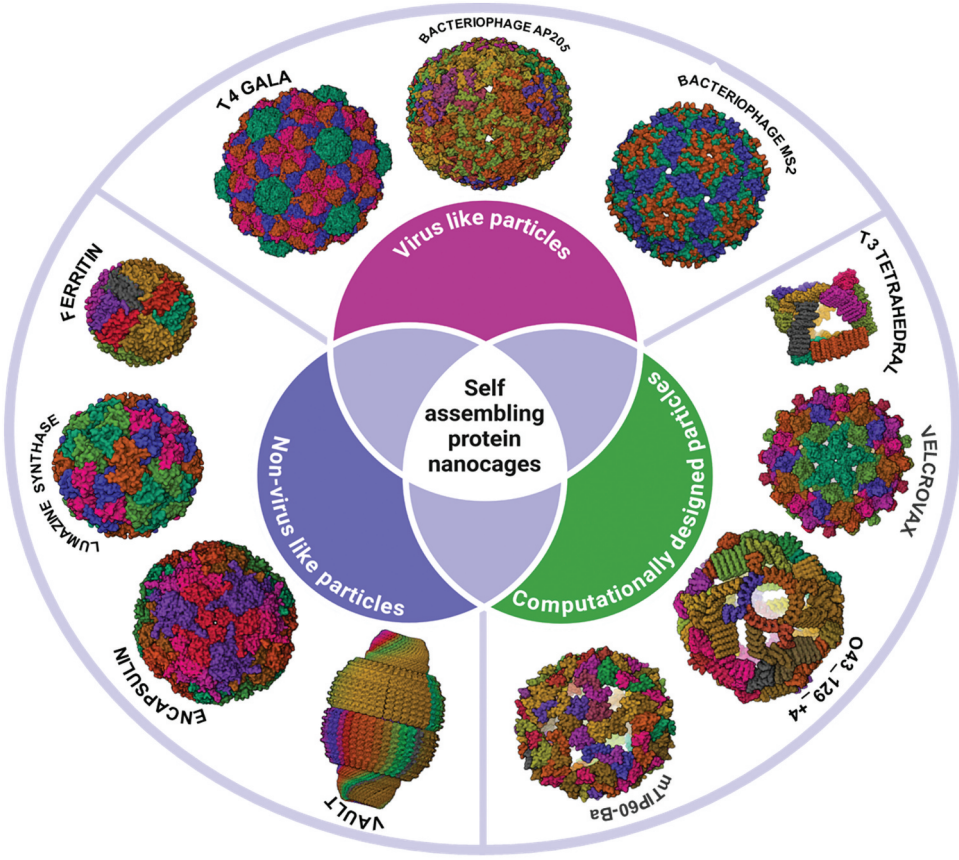

Figure 1. Different types of self-assembling protein nanocages (PNCs). Structures of representative PNCs. VLPs: (1) surface structure of T4 GALA PNC (PDB: 7MH2), (2) surface structure of bacteriophage AP205 VLP (PDB: 5LQP), (3) surface structure of bacteriophage MS2 VLP (PDB: 2MS2). Non-VLP: (1) surface structures of ferritin (PDB: 3BVE), (2) surface structures of lumazine synthase (PDB: 1HQK), (3) surface structures of encapsulin (PDB: 3DKT)), (4) surface structures of vault (PDB: 7PKZ). Computationally designed particles: (1) surface structures of T3 tetrahedral nanocage (PDB: 8TL7), (2) surface structures of VelcroVax tandem HBcAg with SUMO-Affimer inserted at MIR (PDB: 7ZQA), (3) surface structures of O43_129_+4 (PDB: 8V3B), (4) surface structures of mTIP60-ba (metal-ion induced TIP60 (K67E) complex with barium ions (PDB: 7XM1).

Table 1. Summary of various types of PNCs and structural properties.

Type of PNC	Protein Nanocages	Molecular Weight	Geometry	Expression Systems	Advantages	Disadvantages	Reference
Non-Viral Protein Nanocage	Ferritin	509 KDa	Octahedral	Prokaryotic, Mammalian, Insect	– Highly stable – Repetitive Antigen Display – Biocompatible and safe – Economic production – Feasible with mass production	– Rigid assembly – Challenges in Clinical Translation (due to limited human trails so far)	49,56–60
	Encapsulin	1.9 MDa	Icosahedral	Prokaryotic, Mammalian	–High stability and biocompatibility – Multivalent antigen display – Feasible with mass production – Cost effective in production	–Challenges in disassembly and reassembly – Nanocage specific immunogenicity triggered	59,61–68
	Vault	13 MDa	Dihedral-Barrel like structure	Bacterial, Insect, Mammalian	– High stability and biocompatibility – Non- immunogenic – Large-scale production is feasible	– Limited exposure of antigens on the surface and are contained within their cavity – Decrease in pH destabilizes vault structure	59,69–73
	Lumazine synthase	1 MDa	Icosahedral	Bacterial, Mammalian, Plant	– High stability even at high temperature of 95°C – High density antigen presentation	– Scaleup difficulties	35,52,74–77
	DNA binding protein from starved cells	216 KDa	Tetrahedral	Bacterial, Mammalian	– Low toxicity – High biocompatibility – High stability – High density antigen presentation	– Complex assembly requirements – Potential self-immunogenicity	78–80
	Dihydrolipoyl acetyltransferase	1.6 MDa	Icosahedral	Bacterial, Mammalian	– Highly stable – Biocompatibility –High stability	– Large scale production of pure and well-folded nanocage can be technically challenging	35,36,81,82
	Heat shock proteins (HSP)	396 KDa	Octahedral	Bacterial, Yeast, Plant, Mammalian	- High stability (up to 70°C)	–Challenges in scalability and cost effectiveness for mass production	35,83–86
Viral-like Protein Nanocage	HBcAg VLP	4 MDa	Icosahedral	*E. coli*, Yeast, Insect, Mammalian, Plant, *Xenopus* oocytes	–High safety and stability – Scalable and cost effective –Rapid high yield production in plant expression system	– Post translational modification challenges in some expression systems like baculovirus/ insect cell	87–90
	SARS-CoV-2 VLP	1 MDa	Spherical	Plant, Insect, Mammalian	–Multivalent antigen display – Safer and stable – Feasible for mass production	– High cost of production due to the need for specialized expression systems and purification methods	91–95
	HIV-1 Gag-eGFP VLP	84 KDa	Spherical	*E. coli*, Yeast, Insect, Mammalian	– Simple self-assembling process – Versatile antigen display	– Limited structural characterization and complex maturation pose challenges for clinical scale mass production	96,97
	HBcAg-wDIII VLP	96 KDa	Spherical	Plant	–Proper protein display – Safe and highly stable – High yield and scalable – Rapid production	– Initial production setup costs may be higher, especially for cell-culture based systems.	98
	Bacteriophage AP205 VLP	13.5 KDa	Icosahedral	Bacterial	– Arranges antigens in a highly repetitive and organized manner – High immunogenicity of presented antigens on surface – Feasible for mass production	– Sometimes expression in bacterial system can lead to insoluble aggregations – Structural constraints	43,99,100
	Bacteriophage MS2 virus-like particle	2.4 MDa	Icosahedral	Bacterial	– High stability – Ease of production – Safer	– Limited insertion capacity in the nanocage – Improper assembly or suboptimal buffer changes can lead to VLP aggregation, reducing yield and efficacy	101,102

(*Continued*)

Table 1. (Continued).

Type of PNC	Protein Nanocages	Molecular Weight	Geometry	Expression Systems	Advantages	Disadvantages	Reference
Computationally Designed Protein Nanocage	I3–01 nanocage	1.3 MDa	Icosahedral	*E. coli*	– Highly stable – Large internal cavity space, allowing more cargo capacity	– Secretion challenges in mammalian cells	37,103,104
	Prototype oxygen impermeable protein nanocage	NA	Icosahedral	Cell-free System, Bacterial	–Stable assembly – Biocompatible and ease to modify	– Complexity in production –Scalability challenges	35,86,105
	TIP60 nanocage	1.1 MDa	Icosahedral	Yeast, Mammalian	– Versatile modification possible	– Complex assembly – Smaller pore size limits entry of large molecule	106–108
	trp RNA-binding attenuation protein (TRAP) nanocage	2.2 MDa	Octahedral	Bacterial, Cell-free System, Mammalian	– Structural stability – RNA binding capability	– Size limitation – ryptophan dependence, its activity is regulated by tryptophan binding – Complexity in assembly	35,109
	His6-HuHF and His6 SF nanocage	514 KDa	Octahedral	Bacterial, Insect, Mammalian, Cell-free System	– Stable and biocompatible – Ease of functionalization – feasible for mass production	– During production or reassembly protein aggregation can occur.	35,110
	I53–50	1.1 MDa	Icosahedral	Mammalian	– Multivalent display – High stability – High yield	– Complex production – Size limitation	37,111–113
	T3 tetrahedral	939 KDa	tetrahedral	Mammalian, *E. coli*	– Structural stability – Exceptional cargo encapsulation	–Scalability challenges –Optimization needed for large scale production	114–116
	043_129_+4	939.04 KDa	tetrahedral	Bacterial, Yeast, Insect, Mammalian	– Computational design principles enable the nanocage to be expanded, contracted or reinforced by varying the number of modules	NA	37
	mTIP60-Ba (metal-ion induced TIP60 (K67E) complex with barium ions	1.074 MDa	Icosahedral	*E. coli*	– Structure stability – Efficient assembly	– Yield variability –Potential toxicity –Metal dependency	107

account in developing effective vaccines and delivery strategies against mucosal infections. The mucosal immune system comprises an integrated network of tissues and cells, collectively referred to as mucosa-associated lymphoid tissue (MALT). MALT includes gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), and nasopharynx-associated lymphoid tissue (NALT), forming the largest lymphoid organ system in the body.[118,119] This system plays a vital role in initiating and regulating immune responses at mucosal surfaces, making it a key focus for mucosal vaccine development. The mucosal immune system serves three primary functions: (1) detecting and inhibiting the initial entry of pathogens, (2) preventing the uptake of ingested or inhaled antigens, and (3) modulating immune responses through professional antigen-presenting cells (APCs). APCs are pivotal in initiating adaptive immune responses and mediating vaccine-induced immunity at mucosal sites.

Mucosal vaccination offers additional benefits, including the ability to stimulate immune responses at locations distant from the administration site (such as stimulating genitourinary and systemic immune responses by intranasal immunization), providing widespread mucosal and systemic immunity. Although mucosal vaccines can be administered via various routes, such as oral, nasal, pulmonary, rectal, vaginal, ocular, sublingual, or transcutaneous, only the oral and nasal routes are currently used for approved mucosal vaccines[120] (Table 2). Traditional vaccination methods using injections primarily target the systemic immune system, often leading to weak mucosal immune responses. In contrast, direct mucosal administration of vaccines has been shown to stimulate both mucosal and systemic immunity effectively. However, several challenges hinder the successful induction of mucosal immunity. One major issue is the dilution of vaccine antigens within mucosal secretions, which can reduce their effective concentration in lymphoid organs and limit their deposition on mucosal epithelial surfaces, thereby compromising the immune response induction.[124] Additionally, antigens delivered mucosally are susceptible to being trapped within the mucus layer and subsequently degraded by proteases or nucleases.[124] This can significantly reduce antigen stability and availability for uptake by APCs, posing a major challenge for effective mucosal vaccine design. For oral immunization, the stomach's acidic environment presents an additional challenge by degrading vaccine antigens before they elicit immune responses. Furthermore, the abundant colonization of mucosal tissues by commensal microbes may interfere with or skew immune responses to vaccine antigens. While these microbes maintain mucosal homeostasis, they can also act as a barrier to optimal mucosal immunity by competing with vaccine antigens to access epithelial and immune cells.[125] Moreover, mucosal or oral tolerance poses a significant challenge in inducing protective immunity through mucosal immunization.[126]

Vaccine formulations containing peptides, proteins, DNA/RNA, or polysaccharides are vulnerable to degradation during mucosal passage. Because the degradation would lead to losing biological functionality, protective strategies, such as encapsulation within nanocage delivery systems or incorporating stabilizing agents, should ensure intact antigen delivery to target immune cells. Mucosal vaccines harboring the physicochemical properties of pathogens – particularly their shape, charge, and size – tended to be more effective. These properties enhance antigen uptake by mucosal antigen-presenting cells and promote immune responses by simulating the natural interactions between pathogens and the mucosal immune system.[124] Therefore, an effective vaccine design and delivery strategy for mucosal immunization should address three critical aspects: (1) overcoming mucosal barriers to ensure the stability and secure delivery of antigens, (2) targeting mucosal APCs to enable proper antigen processing, presentation, and subsequent *T*- or B-cell activation, and (3) modulating immunological milieu for more efficient and durable effector and memory responses. Properly engineered protein nanoparticle-based delivery systems would offer an opportunity to traverse physiological mucosal barriers, efficiently target immune cells, and precisely control antigen presentation.[127]

Protein nanocages for mucosal vaccines

Nanotechnology-based approaches offer promising solutions for the targeted delivery of vaccine antigens across mucosal surfaces. These approaches may provide a margin for tailoring antigen and adjuvant properties through engineering, such as solubility, stability, and surface characteristics to overcome mucosal barriers. This versatility made nanotechnology an attractive tool for innovating vaccinology to improve efficacy and delivery efficiency.[127] Several PNC-based vaccines have been approved for commercial human use, and many candidates are in clinical trial stages (Tables 3 and 4).

Nanoparticles can either encapsulate vaccine antigens or adsorb them onto their surface, protecting them from rapid degradation and enabling sustained immune responses. By covalently conjugating antigens to nanoparticles, we could mimic the natural presentation of pathogens to APCs, potentially eliciting a more robust and targeted immune response. Additionally, the nanoscale size of these particles offers a high surface area-to-volume ratio and enhanced diffusion rates, making them highly effective for delivering vaccine antigens, to various mucosal sites such as the eye, oral cavity, nasal passages, lung airways, and gut mucosa. Nanoparticle-based delivery systems offer several key advantages over conventional approaches in vaccine development (Figure 2). These include the capability for localized and targeted antigen delivery, enhancing the precision of immune response activation. They also improve antigen presentation and processing, leading to more efficient immune activation. Furthermore, nanoparticles can sustain a higher antigen concentration at mucosal surfaces, prolonging immune system exposure and facilitating more robust responses. They enhance the bioavailability of antigens, ensuring more effective utilization by the immune system. Additionally, nanoparticles may innately harness immunomodulatory capabilities, enabling fine-tuning of immune responses by either promoting stimulation through proinflammatory cytokines or suppression via anti-inflammatory cytokines, depending on the immunological context in the milieu. Various nanoparticle-based delivery systems have been explored for mucosal vaccine delivery, including VLPs, non-viral protein nanoparticles (NVPNs), and computationally

Table 2. Commercially available mucosal vaccines for Humans.[121–123]

Vaccine name	Targeted pathogen	Mucosal route	Formulation	Manufacturer	Approval year	Approval authority
Convidecia Air	SARS-CoV-2	Inhaled aerosol	Recombinant viral vector – used a replication-defective adenovirus type 5	CanSino Biologics Inc (CanSino BIO), Tianjin, China	2022	National Medical Products Administration of China
iNCOVACC	SARS-CoV-2	Intranasal drops	Recombinant adenoviral vector	Bharat Biotech International Ltd, Hyderabad, India in partnership with Washington University	2022	Central Drugs Standard Control Organization- India
Rotovac 5D	Rotavirus	Oral drop	Live attenuated	Bharat Biotech International Ltd, Hyderabad, India	2021	WHO
Pandemic Live Attenuated Vaccine	Influenza Type A (H5N1)	Nasal spray	Live attenuated	AstraZeneca Pharmaceuticals LP., Nijmegen, Netherlands	2020	European Medicines Agency (EMA)
Oral Poliomyelitis vaccines	Poliovirus	Oral drop	Live attenuated	PT Bio Farma (Persero), Bandung, Indonesia	2020	WHO
Rotasil Thermo	Rotavirus	Oral liquid	Live attenuated	Serum Institute of India Pvt. Ltd, Hadapsar, India	2020	WHO
Monovalent Oral Poliomyelitis Vaccine Type 2	Poliovirus	Oral drop	Live attenuated	PT Bio Farma (Persero), Bandung, Indonesia	2019	WHO
Rotasil	Rotavirus	Oral drop	Live attenuated	Serum Institute of India Pvt. Ltd, Hadapsar, India	2018	WHO
Rotavac	Rotavirus	Oral drop	Live attenuated	Bharat Biotech International Ltd, Hyderabad, India	2018	WHO
Bivalent OPV Type 1 and 3 Poliomyelitis Vaccine	Poliovirus	Oral drop	Live attenuated	Panacea Biotec Ltd., Malpur, India	2018	WHO
Biopolio B1/3	Poliovirus	Oral drop	Live attenuated	Bharat Biotech International Limited, Hyderabad, India	2017	WHO
Poliomyelitis Vaccine, Live attenuated, Type 1 and 3	Poliovirus	Oral drop	Live attenuated	Beijing Institute of Biological Products Co., Ltd., Beijing, China	2017	WHO
Vaxchora	*Vibrio cholerae*	Oral drink	Live attenuated	Emergent Travel Health, Redwood City, CA, USA	2016	FDA
Oral Monovalent Type 2 Poliomyelitis Vaccine (MOPV2)	Poliovirus	Oral drop	Live attenuated	Sanofi Pasteur, Lyon, France	2016	WHO
Euvichol	*Vibrio cholerae*	Oral drink	Inactivated	EuBiologics Co., Ltd Chuncheon-si South Korea	2015	WHO
Nasovac-S	Influenza Types A and B	Nasal spray	Live attenuated	Serum Institute of India Pvt. Ltd., Hadapsar, India	2015	WHO
BIOPOLIO	Poliovirus	Oral drop	Live attenuated	Bharat Biotech International Ltd, Hadapsar, India	2015	WHO
Shanchol	*Vibrio cholerae*	Oral liquid suspension	Inactivated	Sanofi Healthcare India Pvt Ltd, Medchal, India	2013	WHO
Poliomyelitis Vaccine, Bivalent Types 1 and 3	Poliovirus	Oral drop	Live attenuated	Serum Institute of India Pvt. Ltd., Hadapsar, India	2013	WHO
Nasovac	Influenza Type A (H1N1)	Nasal spray	Live attenuated	Serum Institute of India Pvt. Ltd., Hadapsar, India	2012	WHO
Adenovirus Vaccine (types 4 and 7)	Acute Ad4 and Ad7 respiratory disease	Oral-2 tablets	Live attenuated	Barr Labs, Inc. North Wales, PA, USA	2011	FDA (military use only)
Oral Bivalent Types 1 and 3 Poliomyelitis Vaccine	Poliovirus	Oral drop	Live attenuated	Sanofi Pasteur, Lyon, France	2011	WHO
Polio Sabin Mono Three (oral)	Poliovirus	Oral drop	Live attenuated	GlaxoSmithKline Biologicals SA, Rixensart, Belgium	2010	WHO
Bivalent Type 1 & 3 Oral Poliomyelitis Vaccine, IP (bOPV1 & 3)	Poliovirus	Oral drop	Live attenuated	Haffkine Bio Pharmaceutical Corporation Ltd., Mumbai, India	2010	WHO
Bivalent Oral Poliomyelitis Vaccine Type 1 & 3 (bOPV 1 & 3)	Poliovirus	Oral drop	Live attenuated	PT Bio Farma (Persero), Bandung, Indonesia	2010	WHO
Monovalent Oral Poliomyelitis Vaccine Type 1 (mOPV1)	Poliovirus	Oral drop	Live attenuated	PT Bio Farma (Persero), Bandung, Indonesia	2009	WHO
Monovalent type 1 Oral Poliomyelitis Vaccine, IP (mOPV1)	Poliovirus	Oral drop	Live attenuated	Haffkine Bio Pharmaceutical Corporation Ltd., Mumbai, India	2009	WHO
Polio Sabin Mono T1	Poliovirus	Oral drop	Live attenuated	GlaxoSmithKline Biologicals SA, Rixensart, Belgium	2009	WHO
Rotarix	Rotavirus	Oral drop	Live attenuated	GlaxoSmithKline Biologicals Rixensart, Belgium	2008	FDA
RotaTeq	Rotavirus	Oral drop	Live attenuated	Merck Sharp & Dohme Corp Whitehouse Station, NJ, USA	2006	FDA
Poliomyelitis vaccine IP	Poliovirus	Oral drop	Live attenuated	Haffkine Bio Pharmaceutical Corporation Ltd., Mumbai, India	2006	WHO
FluMist	Seasonal Influenza	Nasal spray	Live attenuated	MedImmune, LLC Gaithersburg, MD, USA	2003	FDA
Influenza A (H1N1) 2009 Monovalent Vaccine	Influenza Type A (H1N1)	Nasal spray	Live attenuated	MedImmune, LLC Gaithersburg, MD, USA	2003	FDA

(*Continued*)

Table 2. (Continued).

Vaccine name	Targeted pathogen	Mucosal route	Formulation	Manufacturer	Approval year	Approval authority
FluMist Quadrivalent	Influenza Types A and B	Nasal spray	Live attenuated	MedImmune, LLC Gaithersburg, MD, USA	2003	FDA
Dukoral	*Vibrio cholerae*	Oral drink	Inactivated (with recombinant cholera toxin subunit B)	Valneva Sweden AB, Stockholm, Sweden	2001	WHO
Vivotif	*Salmonella typhi*	Oral capsule	Live attenuated	Berna Biotech, Ltd. Berne, Switzerland	1989	FDA
Bivalent oral polio vaccine (b0PV)	Poliovirus	Oral drop	Live attenuated	Dr. Albert Sabin	1961	FDA

designed NVPNs. Developing an effective and safe delivery system requires a comprehensive understanding of the biomaterial used, the cargo (antigen and adjuvant), the target cells or tissues, and the desired immune responses.[133] Nanoparticles must be designed to be safe, pure, non-reactogenic, and biocompatible to ensure their suitability for vaccine delivery.[134] Nanoparticles should provide optimal encapsulation or conjugation of antigens and adjuvants, ensuring their protection from degradation and enhancing their delivery efficiency.[133] Protecting the antigen from harsh pH conditions and enzymatic activities in the mucosa is crucial to prevent its degradation. Nanoparticles must also efficiently deliver antigens to the appropriate APCs to ensure effective immune response activation.[135] In designing an effective nanoparticle carrier, antigen uptake and processing, release kinetics, and the mechanisms involved in generating mucosal immunity should be considered. Their versatility, efficiency, and ability to address these complexities make nanoparticle-based systems a highly valuable in advancing vaccine development and enhancing efficacy.[135]

Protein nanocage vaccines inducing enhanced humoral and cellular immune responses

Vaccine based on protein nanocages have shown great potential as a platform for eliciting stronger humoral and cellular immune responses. PNC-based vaccines generate robust B-cell IgG responses through two key features: (1) the coupling of antigens to a larger nanocage framework, which enhances antigen uptake by antigen-presenting cells (APCs) and promotes retention within lymphoid follicles, and (2) the repetitive and highly organized arrangement of antigens on the nanocage surface, facilitating efficient cross-linking and activation of multiple B-cell receptors (Figure 3). The attachment of antigens to nanoparticles increases their overall size to an optimal range for effective uptake by APCs. This enhanced uptake improves antigen processing and presentation by APCs to T-helper cells, thereby promoting a more robust and efficacious immune response.[136,137] Larger nanocages are more efficiently opsonized with complement, facilitating their binding to the follicular dendritic cells (FDCs) surface. This process prolongs antigen retention within lymphoid follicles and enhances antigen presentation to B cells, ultimately leading to a more robust and sustained humoral immune response.[138] Particles displaying multivalent antigens can enhance B-cell activation by efficiently crosslinking multiple B-cell receptors (BCRs).[139] This cross-linking promotes stronger signal transduction, leading to robust B-cell activation, proliferation, and differentiation into antibody-secreting plasma cells and memory B cells. A study examining the impact of antigen density on memory response supports this claim.[140] High-density conjugation of a model peptide antigen to a VLP robustly triggered a specific IgG antibody response. In contrast, low-density conjugation failed to elicit such a response despite an increased total antigen quantity. This finding suggests that antigen density plays a critical role in immune activation, with effects extending beyond the mere amount of antigen present.

Protein nanocage based vaccines also enhance robust cellular immune responses. These self-assembling nano

Table 3. FDA-Approved PNC-based vaccines in the market.

Vaccine Name	Company	Type of PNC	Target Pathogen	Reference
Engerix®	GlaxoSmithKline	VLP	HBV	[128]
Recombivax®	Merck & Co.	VLP	HBV	[129]
Cervarix®	GlaxoSmithKline	VLP	HPV	[130]
Gardasil®	Merck & Co.	VLP	HPV	[131]
Gardasil 9®	Merck Sharp & Dohme LLC	VLP	HPV	[132]

structures effectively display antigens on their surface, which are robustly taken up by the antigen presenting cells, leading to improved processing and presentation of these antigens resulting in enhanced stimulation of T-cells. Some studies have proven the aforementioned statement, for instance in a study conducted by Zipeng et al., has shown the protein nanocage enhanced cytotoxic T cells responses by selectively targeting and eliminating carcinoma-associated fibroblasts (CAFs).[141] Another research done by Qiang Zhang et al, has also shown nanocage facilitated efficient delivery of CpG to antigens presenting cells leading to robust dendritic cell activation, antigen presentation and subsequent expansion of tumor specific cytotoxic T cells.[142] Recent studies have also demonstrated that protein nanocage based vaccine significantly increases both $CD8^+$ and $CD4^+$ T cell responses. For example, research on a ferritin based multivalent SARS-CoV-2 vaccine has shown its capacity to trigger both B cells and T cell mediated immune responses. The vaccine induced strong activation of $CD4^+$ T cells particularly those producing interferon-gamma (IFN1) and increased levels of $CD8^+$ T cells activation. Additionally, the vaccine substantially increased memory B cell population in lymph nodes, which is essential for long term immunity. Overall, the vaccine has promoted a balanced Th1/Th2 immune responses.[34]

The immune responses can be triggered by engaging both damage-associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). PAMPs, derived from microbial components, activate innate immunity through pathways like Toll-like receptors (TLRs), RIG-1-like Receptors (RLRs), NOD-like Receptors (NLRs), C-type Lectin Receptors (CLRs), AIM2-like Receptors (ALRs), Inflammasome activation driving antigen presentation and adaptive immune activation.[143,144] Meanwhile, DAMPs (like HMGB1, heat shock proteins and ATP), are released during cell stress or death, thereby complementing this response by signaling tissue damage and enhancing antigen- presentation cell maturation. For instance, in a recent research, ferritin nanocages have been shown to amplify immune responses via DAMP release during immunogenic cell death, synergizing with PAMP- mediated pathways to improve vaccine efficacy. This dual mechanism allows protein nanocages to reprogram the immune microenvironment, promoting robust humoral and cellular immunity while enhancing memory T-cell populations for long-term protection.[145]

The development of vaccines for mucosal pathogens that have a propensity for evading immune responses, such as HIV, influenza, and SARS-CoV, can be significantly advanced through protein nanocage-based vaccines.[146] A phenomenon known as antibody-dependent enhancement (ADE), observed in HIV and other viral infections, occurs when non-neutralizing antibodies bind to the virus and inadvertently promote its entry into immune cells.[147] To address this issue, researchers have employed a strategy called epitope focusing to design antigens that direct antibody responses specifically toward neutralizing epitopes. This approach involves isolating neutralizing epitopes from antigens that are often poorly immunogenic on their own. However, when these epitopes are conjugated to a PNC platform in multivalency, their repetitive and organized presentation should enhance their immunogenicity, resulting in strong and targeted humoral immune responses toward the neutralizing epitope.[148] In addition to epitope focusing, the activation of a cell-mediated immune response can be achieved by incorporating T-cell epitopes into the interior of the protein nanocage platform.[149,150] The incorporation of universal $CD4^+$ T cell epitopes would also contribute to a robust humoral response by recruiting helper T cells to assist B cells in antibody production. Meanwhile, incorporating $CD8^+$ T cell epitopes can produce pathogen-specific cytotoxic T cells, boosting the cellular immune response that targets and eliminates infected cells. Integrating these strategies into PNC-based platforms may enable the development of successful vaccines against challenging pathogens by eliciting both targeted humoral and cellular immunity.

As for mucosal vaccines, PNC-based vaccine platforms tend to significantly enhance mucosal immune responses by improving antigen stability and protection, enhancing antigen delivery and uptake, stimulating both systemic and mucosal immunity, increasing mucosal antibody production, enhancing cellular immunity, exhibiting adjuvant-like properties, allowing versatile administration routes, providing potential cross-protection, and improving germinal center reactions for better B-cell activation and antibody affinity.[151]

Protein nanocage (PNC)-based mucosal vaccine design strategies

Engineering protein nanocages through modifications at distinct interfaces

Protein nanocages feature three distinct surfaces that can be engineered: the outer surface, the inner surface, and the interface between the outer and inner surfaces (Figure 4). These surfaces provide various options for genetic and chemical modifications, enabling the development of diverse vaccine design applications for specific immunological goals.

Inner surface modification

PNCs provide an optimal environment for encapsulating molecular cargo within their internal cavities. The nanocage interior modifications make it possible to improve encapsulation efficiency, fine-tune the release profile, and strengthen binding affinity. Genetic and chemical modifications can be

Table 4. PNC-based vaccines in active clinical trials.

Type of PNCs	VLPs	NCT No.	Target Disease	Adjuvant	Immunogens	Phase	Sponsor
VLP Based Vaccine	VLPs made of HA and NA proteins	NCT01897701	Influenza	ISCOMATRIX	Hemagglutinin (HA) protein	Phase 1	Novavax
	Norovirus GI.1/GII.4 VLP	NCT02475278	Norovirus (GII.4)		Norovirus VLPs	Phase 2	Takeda
	VLPs made of L1 proteins of HPV	NCT01984697	HPV-related cancers	Aluminum	9-valent HPV L1 VLPs	Phase 3	Merck Sharp & Dohme LLC
	Virus-like particles (VLPs) composed of the spike protein of SARS-CoV-2	NCT04962893	COVID-19		SARS-CoV-2 VLPs harboring M, N, E, and HexaPro S antigens	Phase 2	Ihsan Gursel, PhD
	VLPs made of HA and NA proteins	NCT04622592	Influenza	AS03	Quadrivalent VLP influenza vaccine	Phase 1 Phase 2	Medicago
	VLPs from plant-produced SARS-CoV-2 spike protein.	NCT04636697	COVID-19	AS03	CoVLP vaccine (coronavirus-like particles)	Phase 2 Phase 3	Medicago
	M2e-VLPs	NCT00819013	Influenza A	Aluminum hydroxide	Influenza A M2e	Phase 1	Sanofi
	VLP (Bacteriophage AP205)	NCT05329220	SARS-CoV-2	Addavax	RBD of SARS-CoV-2	Phase 3	Bavarian Nordic
Non-VLP Based Vaccine	Ferritin	NCT04645147	Epstein-Barr virus (EBV)	Matrix-M1	EBV gp350	Phase 1	National Institute of Allergy and Infectious Diseases (NIAID)
	Ferritin	NCT03186781 NCT04579250	Influenza		Haemagglutinin (HA) stem domain	Phase 1	National Institute of Allergy and Infectious Diseases (NIAID)
		NCT03814720	Influenza		H1ssF	Phase 1	National Institute of Allergy and Infectious Diseases (NIAID)
		NCT04784767	COVID-19	Army liposomal formulation QS21 (ALFQ)	Spike protein of SARS-CoV-2	Phase 1	U.S. Army Medical Research and Development Command
		NCT04579250	Influenza		H10ssF	Phase 1	National Institute of Allergy and Infectious Diseases (NIAID)
		NCT05903339	HIV-1		V3G CH848 Pr-NP1	Phase 1	National Institute of Allergy and Infectious Diseases (NIAID)
	Lumazine synthase	NCT03547245	Human immunodeficiency virus (HIV)	AS01B/DPBS sucrose/ IM	eOD-GT8	Phase 1	International AIDS Vaccine Initiative
Computationally Designed Vaccine	I53–50	NCT05007951	COVID-19	A squalene-in-water emulsion (AS03)	RBD of SARS-CoV-2	Phase 3	SK Bioscience Co., Ltd.
	I3–01	NCT05125926 NCT05137444 NCT05664932	COVID-19		RBD of S protein	Phase 1 Phase 2/ 3 Phase 3	Yantai Patronus Biotech Co., Ltd.
	I53-dn5	NCT04896086	Influenza vaccine	A squalene-in-water emulsion (AS03)	Hemagglutinin HA antigen	Phase 1	National Institute of Allergy and Infectious Diseases (NIAID)

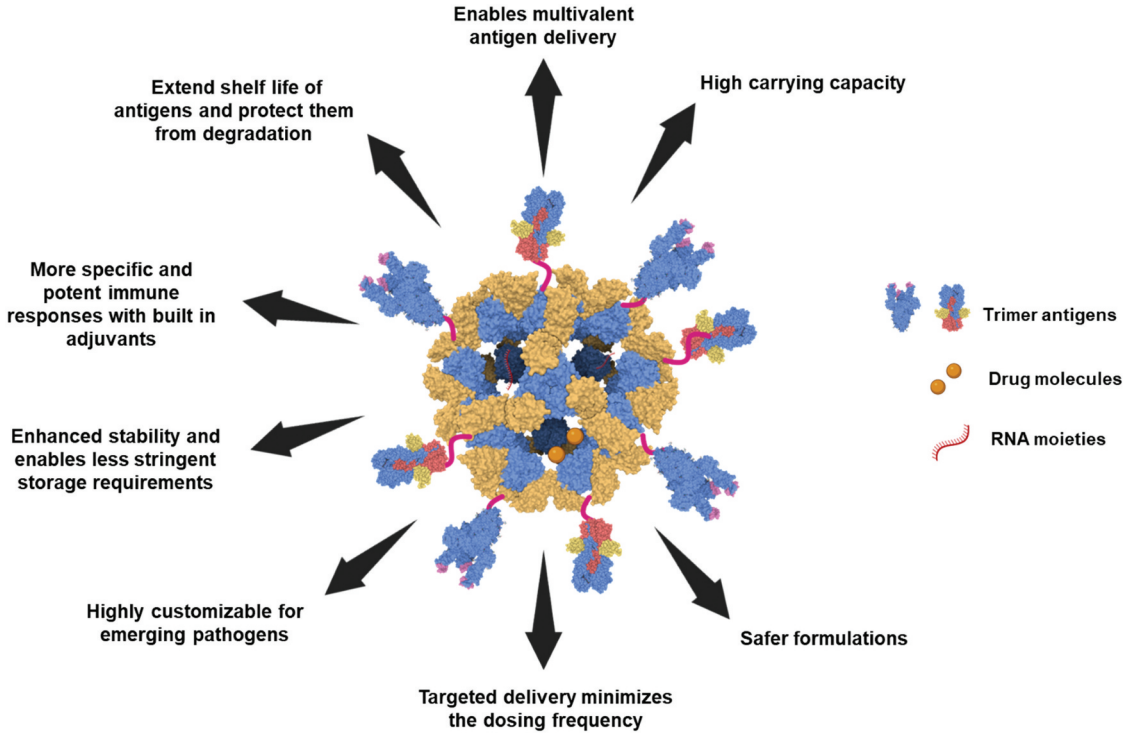

Figure 2. Advantages of PNC-Based vaccines. PNCs offer several unique advantages for vaccine delivery. They can present antigens in a highly organized, multivalent manner, allow ease in modification with functional elements like adjuvants and targeting moieties, co-deliver both antigens and other immunostimulatory molecules, and potentially enhance immune responses.

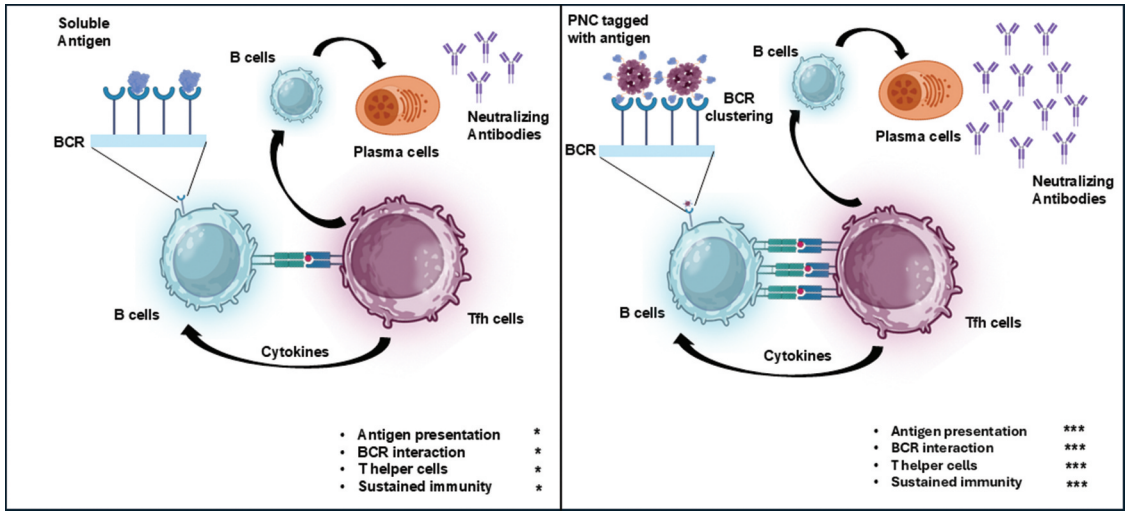

Figure 3. Enhanced humoral immune response in antigen displayed with PNCs compared to antigen alone. The humoral immune responses triggered by soluble antigens interacting with B-cell receptors (BCRs) are less effective and shorter in duration compared to the responses elicited by protein nanocages (PNCs) that display an organized arrangement of antigens. In contrast to soluble antigens, PNCs present multiple antigen copies, enabling concurrent engagement with numerous BCRs, a process known as BCR clustering. This results in robust and long-lasting antigen recognition by B cells, which initiates intracellular signaling cascades, antigen internalization, and processing of MHC class II presentation to T follicular helper (tfh) cells. This sequence of events stimulates tfh cells to release regulatory cytokines, facilitating the differentiation of B cells into plasma cells that produce antigen-specific neutralizing antibodies. The intensity of these responses is indicated as follows: high - ***, low - *.

employed to control molecular nucleation and attachment. Various molecular cargos, including small molecules, peptides, protein-based drugs, and RNA/DNA therapeutics, can be successfully encapsulated and released from protein nanocage interiors. Larger nanocages, such as vault, can accommodate larger cargos like antibodies. These inner surface modifications would open new avenues for more sophisticated vaccine design, enabling precise control over cargo delivery and immune activation.[152]

Outer surface modification

PNCs' exterior surfaces are most frequently targeted in the vaccine development. Interactions between the nanocarriers and immune cells are primarily mediated by the outer surface

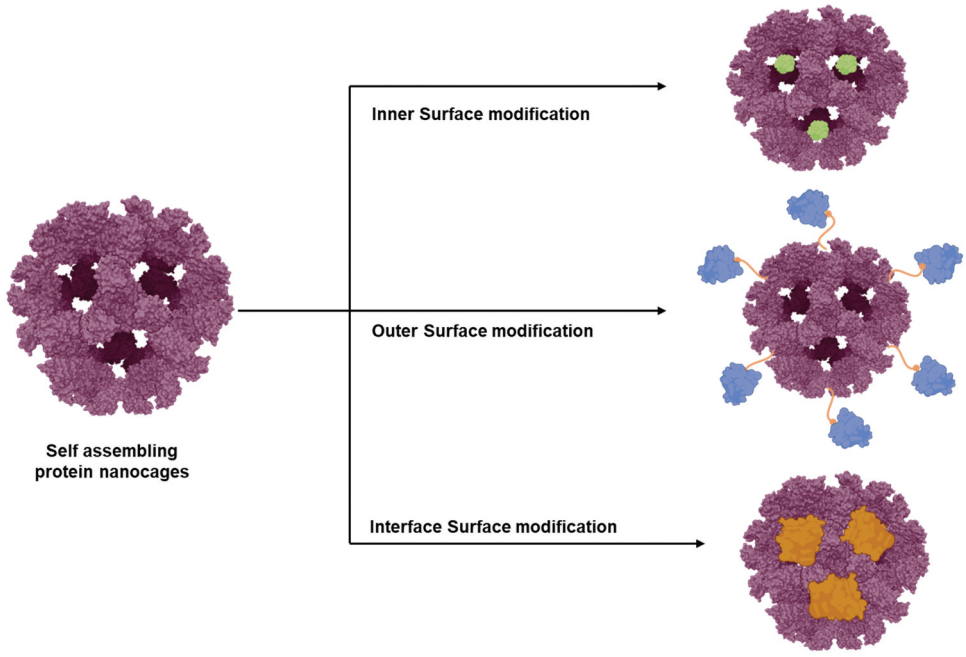

Figure 4. Modification of PNCs at different interfaces. Self-assembling PNCs can be modified at different interfaces, namely, the exterior surface, the interior surface, and the interface region between the exterior and interior surfaces.

of the protein nanocages. Modifications to the outer surface aim to enhance surface characteristics by integrating molecular recognition domains (e.g., receptor-targeting domains or pathogen-derived antigens) and altering the physiological properties of the nanocage carrier. Common methods for modifying the outer surface include genetic fusion, surface point mutations, loop insertions, chemical conjugation, and coupling the exterior with functional tags.[153–159] These engineered alterations are designed to enhance several key properties, including prolonged circulation in the bloodstream, improved accumulation at targeted lymphoid tissues, increased circulatory half-life, and enhanced specific cellular responses. Additionally, it is possible to introduce post-translational modifications, such as glycosylation, which has been shown to improve pharmacokinetics, such as germinal center delivery.[160,161]

Modification at the interface between outer and inner surfaces

When modifying protein nanocages at their interfaces, a high degree of cooperativity in protein nanocage assembly is essential.[162–164] The interface engineering primarily relies on the hydrophobic packing of interfacing residues.[165] Protein nanocages can efficiently self-assemble with a high degree of cooperativity, even at relatively weak interfaces. However, if the interactions between interface subunits are too strong, it can lead to kinetic trapping, resulting in partial or off-target nanocage assemblies. The right balance in interfacing strength is crucial to ensure proper assembly, structural accuracy, and functional performance of the nanocages. The solubility of expressed nanocages, along with the attached entities at the interface region, can be adversely affected if there is significant hydrophobicity between the protein nanocage monomer subunits. For addressing this limitation, designing hydrophilic interfaces is a key strategy, as it promotes better solubility, reduces aggregation, and facilitates efficient assembly of the nanocages while maintaining their structural and functional integrity.[166] There are two primary approaches to modifying the interface regions in protein nanocages: (1) utilizing existing protein-protein interfaces and strongly fusing them to the nanocage subunits, or (2) designing *de novo* new interfaces computationally. These modifications would facilitate directly attaching natural proteins, such as antibodies, enabling advanced functionalization and expanding their applications in vaccine design.[167] Alterations in the interfaces of nanocages can also enable controlled assembly and disassembly of monomer subunits in response to specific environmental conditions. Protein nanocages that are sensitive to metal ions, pH changes, or ionic strength have been utilized for various applications, including efficient packing and selective drug delivery. These environmentally responsive designs provide flexibility and precision, making them highly suitable for highly effective vaccine delivery system developments.[168–170]

Strategies to present antigens and adjuvants on the PNCs

There are three primary approaches used to attach antigens and potent adjuvants to protein nanocages (PNCs): (1) Direct genetic fusion, where antigens or adjuvants are genetically encoded as part of the nanocage subunits; (2) Chemical conjugation, which involves covalent attachment of antigens or adjuvants to the nanocage surface using chemical linkers; and (3) Coupling with tags, where molecular tags facilitate targeted binding of antigens or adjuvants to the nanocage (Figure 5). These technologies enable the creation of versatile platforms capable of displaying multiple antigens and adjuvants, significantly enhancing both the diversity and quantity of antigen/adjuvant presentation.

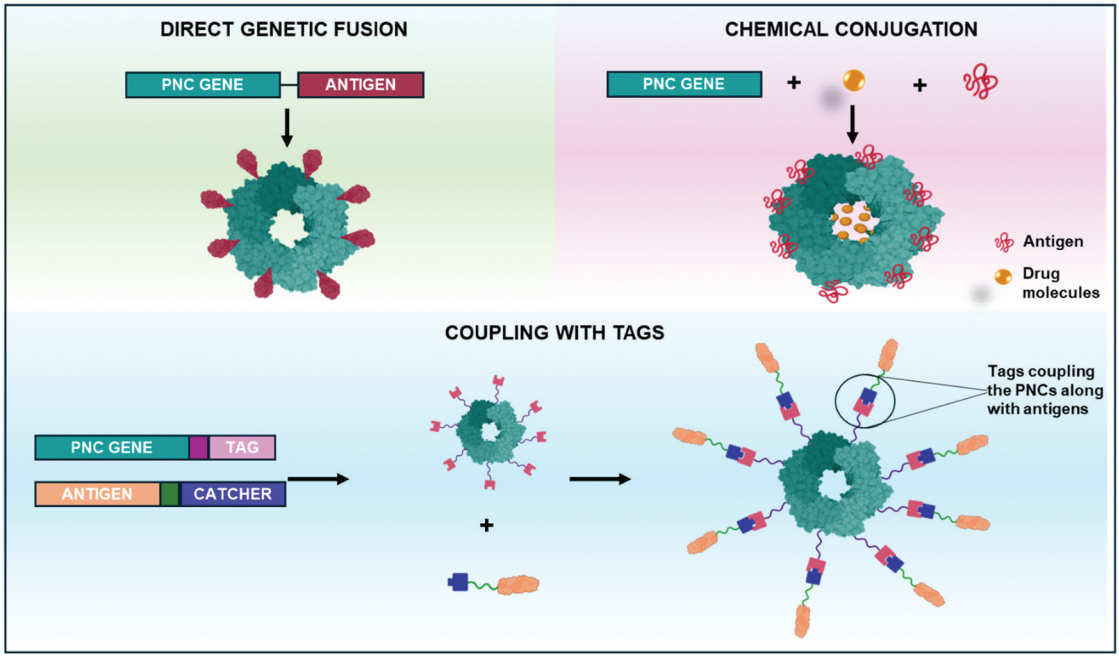

Figure 5. Strategies to conjugate antigens on PNPs. Three major strategies exist for presenting antigens: (1) genetic fusion: involves the direct fusion of antigen with the corresponding PNC subunit and expressed genetically (2) chemical conjugation: involves chemical crosslinking agents to form irreversible bonds between the chemically active amino acid side chains of both PNCs and antigens or drug moieties (3) coupling with tags: involve genetically fusing the catcher to one entity and tag to another, thereby it results in strong affinity interaction between catcher and tag system forming PNCs displaying antigens on to their surface in an orderly manner.

Direct genetic fusion

Direct genetic fusion is one of the most fundamental and widely employed approaches for conjugating antigens to PNC platforms. Since PNCs self-assemble from numerous identical protein subunits, many of which possess readily accessible carboxyl and amino terminals, it is relatively straightforward to genetically incorporate antigens at either terminal. Choosing the right terminal is essential, as it guarantees the antigen is available and properly oriented for immune recognition. However, challenges can arise with direct fusion construction, such as improper expression or misfolding of the fused antigen along with the PNC. For example, with the ferritin PNC, the carboxyl-terminal is oriented toward the inner cavity of the nanocage, making it less suitable for antigen fusion, while the amino-terminal is well exposed on the outer surface, making it ideal for antigen conjugation.[171,172] To improve assembly efficiency and proper folding of PNCs with fused antigens, a flexible linker can be introduced between the antigen and the nanocage subunit. To address challenges related to low or absent expression, computational screening platforms such as SPEEDesign (Stabiliser for Protein Expression and Epitope Design), ProteinMPNN, and Rosetta Diffusion modeling can be employed.[173–175] These tools aid in optimizing fusion constructs, enhancing protein stability, and ensuring proper folding, thereby improving the overall performance of the genetically fused antigen-PNC platforms.

Chemical conjugation

Antigen attachment to the surface of a protein nanocage (PNP) through chemical conjugation involves the use of crosslinking agents to form highly stable and permanent covalent bonds between the antigen and PNC.[176] Various crosslinking agents target exposed functional groups, such as aspartates, lysines, cysteines, and glutamates, on both PNCs and antigens.[177] However, this method lacks selectivity, which can potentially damage the structure of the antigen or PNPs and may result in uneven antigen distribution, ultimately influencing immune responses. To address these limitations, click chemistry has gained significant popularity in recent.[178] Click chemistry offers high specificity, efficiency, and biocompatibility, enabling precise antigen conjugation to PNCs without compromising their structural integrity, thereby improving the consistency and efficacy of immune responses. Multiple chemical conjugation methods are used for vaccine development, one of which is EDC/NHS conjugation. This method employs the bioconjugation agents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) to activate carboxylic acid groups on biomolecules.[179,180] The EDC/Sulfo-NHS coupling method has been widely applied in developing peptide nanostructures and conjugating protein antigens to PNC surfaces, such as VLPs.[181–184] Maleimide, a chemical substance with chemoselectivity toward thiols, forms stable bonds with sulfhydryl groups in cysteine residues, a technique commonly employed in developing protein nanoparticle-based vaccines.[185,186] While these reactions are rapid, highly selective, and efficient, they require additional steps to introduce reactive functional groups into both the antigens and PNCs. This is accomplished by incorporating amino acid analogs and unnatural amino acids into the proteins, enabling precise and stable conjugation.[187]

Coupling with conjugating tags

The tag-coupling method involves attaching a tag to the antigen and a catcher to the PNC (vice versa), with each component being separately produced and purified before being combined to create PNCs displaying the antigens. Typically,

the tag is genetically linked to one end of the one protein, while the catcher, designed to specifically bind the tag, is attached to one end of the other to be expressed on the exposed surface. Following independent expression, purification, and mixing, the catcher binds strongly to the tag, resulting in a PNC that displays the antigen on its surface for immune activation.

The CnaB2 adhesin domain from *Streptococcus pyogenes* fibronectin-binding protein, FbaB, is naturally stabilized by an isopeptide bond. This bond forms through a spontaneous amidation reaction between the side chain of an aspartic acid in a 13-residue peptide (SpyTag) and a lysine residue in its 116-residue protein partner (SpyCatcher), both derived from the adhesin domain. This reaction forms a covalent isopeptide bond, enabling stable and specific conjugation of two polypeptides. This characteristic has been utilized as a simple yet highly selective and robust method for conjugating antigens to PNCs. The SpyTag and SpyCatcher sequences (Table 5) can be attached to either the end of the PNC or the antigen sequences using simple techniques such as PCR, enabling flexible design options. Introducing an improved SpyTag003-SpyCatcher003 system, which offers higher affinity and faster reaction kinetics, has made this method one of the most versatile and frequently used approaches for attaching antigens to PNC platforms.

Other protein/peptide-based tag-coupling methodologies suitable for linking antigens to PNC platforms include SnoopTag/SnoopCatcher,[191] sortase,[196] and Barnase – Barstar[197] systems. The SnoopTag-SnoopCatcher system also relies on isopeptide bond formation through a transamidation reaction between a lysine in a 12-residue peptide tag (SnoopTag) and an asparagine in a 112-residue cognate protein partner (SnoopCatcher), derived from a *Streptococcus pneumoniae* adhesin molecule. The sortase A system involves peptide bond formation through a transpeptidation reaction between the sortase A recognition motif, LPXTG (X representing any amino acid), and the oligo-glycine sequence[196] at a protein's N-terminus, mediated by the *Staphylococcus aureus* enzyme sortase A. The Barnase-Barnstar system depends on the strong non-covalent interaction between dimerization domains of barnase (a 110-residue ribonuclease) and barnstar (an 89-residue barnase inhibitor) from *Bacillus amyloliquefaciens*. Furthermore, the carboxyl- and amino terminals of both barnase and barnstar are accessible for protein/peptide fusion, as they are not part of their dimerization domains.[198]

PNC-based delivery systems for mucosal vaccines

Various self-assembling PNCs have been used in recent years to generate effective mucosal vaccines (Table 6).

Virus-like particles (VLPs)

VLPs have emerged as a promising platform for mucosal vaccine delivery, offering several advantages over traditional vaccine formulations (Figure 1). In recent decades, they have significantly contributed to the advancement of vaccine development.[207,208] VLPs are structures that mimic live viruses but lack their genetic material. These particles comprise viral structural proteins that spontaneously assemble, presenting viral antigens in their natural form and eliciting strong immune responses. VLPs typically range from 15–400 nm in size, allowing for efficient uptake by antigen-presenting cells

Table 5. Summary of various coupling systems.

Tags	Catchers	Tag Sequence	Description	Reference
SpyTag	SpyCatcher	AHIVMVDAYKPTK	Original Catcher-Tag technology.	188
SpyTag	SpyLigase	AHIVMVDAYKPTK	Rationally engineered system for ligating two peptides.	189
KTag		ATHIKFSKRD		
SpyTag	SpyCatcher ΔN1ΔC1	AHIVMVDAYKPTK	Minimal SpyCatcher construct that still binds efficiently to SpyTag.	190
SnoopTag	SnoopCatcher	KLGDIEFIKVNK	Orthogonal technology to SpyCatcher	191
SpyTag002	SpyCatcher002	VPTIVMVDAYKRYK	Improved SpyCatcher-SpyTag system with a faster reaction rate.	192
SnoopTagJr	SnoopLigase	KLGSIEFIKVNK	Rationally engineered system for ligating two peptides	193
DogTag		DIPATYEFTDGKHYITNEPIPPK		
SpyTag002	SpyDock	VPTIVMVDAYKRYK	Protein affinity purification system (Spy&Go) based on SpyCatcher.	194
SpyTag003	SpyCatcher003	RGVPHIVMVDAYKRYK	Efficient protein coupling tool for irreversible peptide-protein ligation	195

Table 6. Studies on PNC based mucosal vaccines.

Type of PNCs	PNC	Targetted Pathogen	Antigen	Adjuvant	Route of Administration	Reference
VLP Based Vaccine	SVA VLP	Senecavirus A (SVA)	Capsid proteins VP0, VP1 and VP3 of SVA	ISA 201	Intra Nasal	199
	$CUMV_{TT}$ VL	SARS-CoV-2	RBD	–	Intra Nasal	200
	Qβ VLP	Influenza	M2	–	Intra Nasal	45
Non-VLP Based	Ferritin	Influenza	Hemagglutinin (HA)/ Ectodomain of HA	–	Intra Nasal	201
	Ferritin	Influenza	HA, M2e, NA HCA-2/transmembrane protein M2 (M2e)	–	Intra Nasal	202,203
	Ferritin	*Pseudomonas aeruginosa*	PcrV and OprI	–	Intra Nasal	204
	Encapsulin	*Streptococcus pneumoniae*	heat-killed *S. pneumoniae (HKSP)*	–	Intra Nasal	205
Computationally Designed	I3–01	SARS-CoV-2	RBD	–	Intra Nasal	206
	I53–50	SARS-CoV-2	Spike protein	MPLA liposomes	Intra Nasal	113

and transport across mucosal barriers.[209] Their particulate nature and ability to display multiple epitopes in a highly organized manner contribute to the robust activation of both humoral and cellular immune responses.[210] When administered via mucosal routes, VLP – based vaccines can induce strong local IgA production and systemic antibody responses, providing protection at the site of pathogen entry.[211] The versatility of VLPs allows for the incorporation of foreign antigens and adjuvants, enabling the development of chimeric particles with enhanced immunogenicity.[209] Recent studies have modified VLPs to improve the penetration through mucus barriers: PEGylation enhanced diffusion and stability in mucosal environment.[14] Additionally, incorporating mucoadhesive components like chitosan has been investigated to increase retention time and improve antigen uptake at mucosal surfaces.[212] Recent research on Virus-like particle (VLP) vaccinations for Senecavirus A (SVA) in 2020 demonstrated strong mucosal immune responses in pigs, including the production of SVA-specific IgA antibodies on mucosal surfaces. These antibodies have a key role in preventing viral entry and multiplication at the site of infection, which helps to explain the strong immune responses that are protective.[199] Additionally, the respiratory system produces neutralizing antibodies when virus-like particle (VLP) vaccinations against SARS-CoV-2 are administered nasally, triggering strong mucosal immune responses. By preventing the virus at its first point of entry, mucosal immunity – which is typified by IgA and IgG antibodies on nasal surfaces – provides enhanced protection against SARS-CoV-2 and its worrisome variants.[200] VLP-based vaccines have shown promise against various pathogens, including influenza, norovirus, and human papillomavirus, with some formulations already commercialized like Recombivax for hepatitis B, Gardasil for human papillomavirus and Hecolin for hepatitis E.[40,207,213] The potential of VLPs to induce balanced local and systemic immune responses makes them an attractive platform for combating mucosal pathogens and addressing the limitations of traditional vaccine approaches.

Ferritin

Ferritin, an essential protein for iron storage, functions as a detoxifier and reservoir. In its highly conserved three-dimensional structure, ferritin subunits symmetrically arrange 24-mer PNC to form a hollow shell with an 80 Å diameter cavity (Figure 1).[214,215] These self-assembled nanoparticles can effectively present antigens in a highly organized manner, mimicking viral structures resulting in enhanced immunogenicity.[58,204] The hollow cavity of ferritin nanocages allows the encapsulation of various antigens or drug substances.[58] Importantly, ferritin-based vaccines have demonstrated superior immunogenicity compared to conventional approaches in humans and experimental animals, inducing robust humoral and cellular immune responses in both systemic and mucosal compartments.[204,216] Recent studies have demonstrated that ferritin nanocage vaccines can provoke robust specific antibody responses, including mucosal IgA, and offer protection against viral challenges in animal models.[48,204] The biocompatibility and low toxicity of ferritin make it an excellent carrier system for vaccine development, addressing safety concerns associated with other delivery systems.[215] Ferritin-based vaccines have shown promise in eliciting broad protection antibody responses against multiple pathogens, including influenza, Epstein-Barr virus, HIV, and SARS-CoV-2.[48] According to a previous study, intranasal immunization with HMP-NPs, which are composed of the H3N2 virus's ectodomain of hemagglutinin and three tandem highly conserved influenza M1 epitopes fused with the universal helper T-cell epitope PADRE and ferritin nanocage without any adjuvant, conferred complete protection against the H3N2 virus, as well as partial protection against the H1N1 and H9N2 viruses, and significantly decreased lung viral loads.[201] The sequential immunization approach, which combines oral administration of Salmonella followed by an intranasal boost using ferritin-based nanoparticles, significantly enhanced mucosal immune responses against the H1N1 influenza virus. This method resulted in robust secretory IgA production in the respiratory system, offering improved protection at the virus's entry point and potentially providing broader immunity against various influenza strains.[202,217] The versatility of ferritin nanocages allows the development of mosaic vaccines, which can deliver a cocktail of antigens to provide broader immune protection against different viral variants.[48] As research results accumulate, ferritin-based mucosal vaccines show promise in combating a wide range of infectious diseases, including respiratory pathogens, and are currently being explored in multiple phase I clinical trials.[48,216] The increasing evidence of strong cellular and robust, durable humoral immune responses induced by ferritin-based vaccines compared to conventional approaches further underscores their potential as an effective strategy for future mucosal vaccine development.[48]

Encapsulin

Encapsulins are self-assembling, microbial proteinaceous nanocarriers found mainly in bacterial and archaeal species. They typically form ~30 nm sized 60-mer nanocages (Figure 1).[67,151] They can sequester functional protein cargos within their luminal spaces, making them ideal for antigen presentation and targeting effector immune cells. Recent studies have demonstrated the potential of encapsulin for vaccine development, particularly against respiratory pathogens like SARS-CoV-2 and influenza.[50,218] Encapsulin-based vaccines have shown the ability to induce robust humoral and cellular immune responses in both systemic and mucosal compartments when administered intranasally. A study conducted in 2024, explored the use of engineered encapsulin scaffolds in combination with PP7 VLPs for heterologous prime-boost vaccination strategies, demonstrating the potential to fine-tune epitope-focused antibody responses.[219] This approach led to developing selective antibody responses against SARS-CoV-2 RBD point mutants. Furthermore, encapsulin-based vaccines have shown extraordinarily high titers and broad anti-SARS-CoV-2 neutralization capabilities, even without co-administered adjuvants. Another research conducted in 2013, nanoparticles encapsulating heat-killed *Streptococcus pneumoniae* (NP-HKSP) were more likely than empty

nanoparticle to remain in the lungs for 11 days after intranasal injection. When compared to HKSP treatment alone, immunization with NPHKSP resulted in a notable resistance against *S. pneumoniae* infection. A notable rise in the antigen-specific Th1-associated IFN-γ cytokine response by pulmonary lymphocytes was linked with enhanced protection.[205] The biocompatibility, controlled release capabilities and targeted delivery potential of encapsulation make them an attractive option for mucosal vaccine development.

I3–01 nanocage

The I3–01 nanocage is a computationally designed protein nanocage derived from trimeric aldolase, consisting of 60 subunits that self-assemble into a stable icosahedral structure with a diameter of ~25 nm (Figure 1).[37,103] These nanocages are highly thermostable, can withstand up to 80 °C, and tolerate denaturing agents like 6.7 M guanidium hydrochloride. The I3–01 nanocarrier has a large internal cavity (~3000 nm^3), which can encapsulate antigens or other biomolecules, making it an excellent platform for antigen presentation. The I3–01 nanocage-based delivery systems have emerged as a promising platform for mucosal vaccines, particularly for respiratory pathogens like SARS-CoV-2 and influenza. This self-assembling protein nanoparticle is designed to enhance antigen presentation and boost immune responses. Recent studies have shown its effectiveness in displaying various antigens, including the receptor binding domain (RBD) of SARS-CoV-2 when coupled with a flexible SpyCatcher domain (SpyCage).[206] In a study done in 2024, the I3–01-based platform was evaluated in Syrian golden hamsters, where it was shown to induce robust IgG antibody responses upon intranasal vaccination. The study highlighted that hamsters vaccinated with the RBD+SpyCage exhibited improved viral clearance and reduced lung pathology following the SARS-CoV-2 challenge compared to unvaccinated controls. The study validated how mucosal vaccine strategies are able to augment local immunity to respiratory diseases such as SARS-CoV-2. Perhaps most significantly, the covalent interaction between the RBD and the scaffold was the factor that elicited an immunological response through the intranasal route.[206] This nanocage has also been explored for vaccines against other viral and parasitic pathogens. This new computationally designed innovative nanocage could be utilized to generate more effective mucosal vaccines against a wide range of mucosal infectious agents.

T3 tetrahedral nanocage

The T3 tetrahedral nanocage is a computationally designed protein nanostructure that self-assembles into tetrahedral geometry (Figure 1). It is composed of protein subunits engineered to form highly stable and symmetric structures. The T3 nanocage design allows standardized protein building blocks and inter-block interactions to create a stable scaffold capable of encapsulating or displaying antigens. Its tetrahedral structure enhances its ability to present antigens in a multivalent and organized manner, which is crucial for stimulating robust immune responses. The modularity of the T3 nanocage allows for customization, including the incorporation of multiple antigens and adjuvants, making it an ideal carrier for mucosal vaccine delivery systems targeting respiratory pathogens like SARS-CoV-2 and influenza.[220] Recent advancements in protein engineering software like Rosetta have led to the creation of single component tetrahedrons.[114,221] The tetrahedral architecture not only enhances stability and bioavailability but also promotes efficient uptake by APCs at mucosal sites. T3 nanocage, given its structural uniqueness, would occupy a specialized niche in developing future mucosal vaccines by harnessing specific targeting capabilities.

Safety concerns of PNCs

Despite the potential of protein nanocages as nanocarriers for vaccine delivery, there are numerous safety issues and possible negative effects to consider.[222] One concern is immunogenicity, where multiple doses may result in increased immunoglobulin IgG levels and B cell counts, potentially rendering the carriers ineffective.[223] Another issue is the "burst effect," an unregular release of any cargo loaded inside (for eg: drug), that may lead to adverse reactions.[224,225] Virus capsid protein derived protein nanocages present specific safety concerns, including the potential to revert to infectious forms.[90] Though the PNCs are potent and have many vital advantages for them to be used as the best vaccine delivery system, these safety concerns must also be considered while designing. These safety issues highlight the necessity for careful engineering, alteration and testing of protein nanocages to guarantee their efficacy and safety in biomedical applications.

Conclusion and future perspectives

Compared to traditional vaccines using live or inactivated pathogens, protein nanocage-based drug delivery systems offer unique opportunities for developing novel subunit mucosal vaccines and immunotherapies. Though many PNC-based mucosal vaccines are under development in their clinical and preclinical stages, no PNC-based mucosal vaccines have been approved for commercial use yet. Various protein nanoparticle delivery systems have been devised for mucosal vaccination, each with distinct strengths and limitations. The next generation of mucosal vaccines requires not only effective delivery systems that could overcome harsh conditions in the mucosae but also potent adjuvants that would overwhelm the tolerogenic mucosal immune system. PNCs could be employed in designing more effective mucosal vaccines because of their innate stability and constitutional multivalency. Despite significant progress in developing protein nanocages for antigen delivery through mucosal surfaces, challenges and unmet needs persist. It should be explored why different nanocages bring up different immune responses further to accelerate the development of better PNC-based mucosal vaccines. Technological tricks could enable displaying both antigen and adjuvant on a single PNC, resulting in more efficient activation of APCs to present antigens to T lymphocytes. The all-in-one PNC mucosal vaccine displaying both built-in adjuvant and focused antigens (inducing more potent neutralizing immune responses) will certainly serve as an effective

preventive and spread-inhibiting vaccines against future emerging infections such as Disease X. Future research should also evaluate how the physicochemical properties of nanocages influence specific immune pathways in the mucosa in more mechanistic ways. Notably, the immunogenicity of the protein nanocages themselves remains an underexplored area; greater focus is needed on minimizing or eliminating nanocage-specific immune responses to optimize efficacy after repeated immunizations and reduce reactogenicity. Alternatively, a prime-boost strategy employing different PNC platforms sequentially could be utilized until sufficiently deimmunized PNCs become available. Collectively, the nanocage platform offers a versatile approach to overcoming many challenges faced by current vaccines, particularly mucosal vaccines.

Author contributions

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Disclosure statement

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Notes on contributor

Joon Haeng Rhee and Shee Eun Lee have been working as team over 25 years. They have been working on molecular microbial pathogenesis and vaccine biology. For the molecular microbial pathogenesis studies, their team has been observing the *V. vulnificus*-host interactions using various molecular and cellular microbiological tools. They are the first reporter of the whole genome sequence of *V. vulnificus*, which became one of the most widely used standard strains in the *Vibrio* research field. Vaccine study was first started aiming the high mortality *V. vulnificus* infections. During the vaccine research, the team came across the finding that a flagellin protein of *V. vulnificus* has an excellent mucosal adjuvant effect in late 1990s, which was later proved by his group and others to be mediated by the TLR5 signaling. Since then, they are studying the basic science and applications related to the flagellin-TLR5-mediated immune modulation. Now flagellin is applied to the development of effective vaccines and immunotherapeutics against diverse diseases such as cancers, allergies, and Alzheimer's disease. They have co-authored more than 60 papers. Currently they are working on the development of mucosal vaccines employing protein nanocages displaying flagellin adjuvant and antigens, part of which was presented at the 2024 ISV Annual Congress in Seoul.

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Ethical statement

Relevant ethical approval does not apply to this study.

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