Cell-free protein synthesis systems for vaccine design and production

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Highlights

- Cell-free protein synthesis (CFPS) systems allow for highly optimizable and rapid protein production without constraints of a cellular host.
- CFPS technology can be used to overcome limitations in current vaccine production and expand vaccine development.
- CFPS systems have been explored for the development of subunit, conjugate, virus-like particle, and membrane augmented vaccines as well as design tools.

Abstract

Vaccines are vital for protection against existing and emergent diseases. Current vaccine production strategies are limited by long production times, risky viral material, weak immunogenicity, and poor stability, ultimately restricting the safe or rapid production of vaccines for widespread utilization. Cell-free protein synthesis (CFPS) systems, which use extracted transcriptional and translational machinery from cells, are promising tools for vaccine production because they can rapidly produce proteins without the constraints of living cells, have a highly optimizable open system, and can be used for on-demand biomanufacturing. Here, we review how CFPS systems have been explored for the production of subunit, conjugate, virus-like particle, and membrane augmented vaccines along with as a tool in vaccine design. We also discuss efforts to address potential limitations with CFPS such as the presence of endotoxins, poor protein folding, reaction stability, and glycosylation to enable promising future vaccine design and production.

Graphical Abstract

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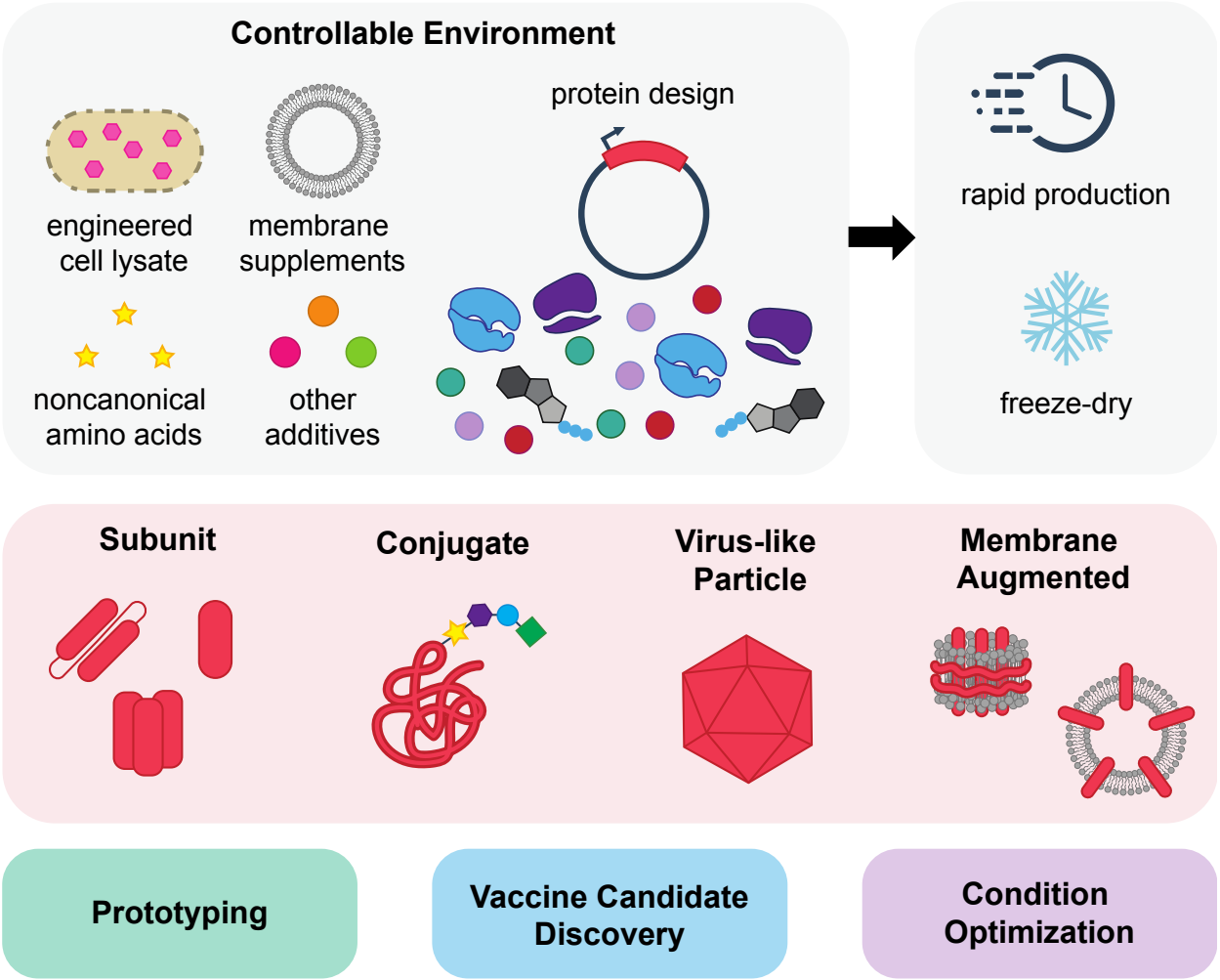

Introduction

In 2018, the World Health Organization called for accelerated R&D on a list of priority diseases with no efficacious drugs or vaccines. The list includes numerous viral diseases that have the potential to cause future public health crises such as Nipah, Zika, severe acute respiratory syndrome (SARS), and Ebola diseases [1]. In addition, it has been difficult to generate vaccines for diseases caused by pathogens such as human immunodeficiency virus (HIV), dengue virus, influenza virus, and *Plasmodium* parasites. This difficulty is due to broad sequence diversity, potential exacerbation of disease in individuals previously never infected, antigenic drift, and the complexity of parasite life stages, respectively [2-4]. Current strategies to manufacture vaccines, such as live attenuated, inactivated, subunit, conjugate, and virus-like particle vaccines, have limitations that hinder development, efficacy, and widespread global use (Table 1). Major constraints include the risk associated with live pathogens, long production times, the need for cold chain manufacturing, or the requirement of living cellular hosts that ultimately limit the reach of vaccine distribution or increase the costs and time required to produce vaccines [5-8]. Without effective tools to develop vaccines to address the myriad of pathogenic threats, we are vulnerable to ongoing and future epidemics.

Table 1. Benefits and limitations of current vaccination strategies.

Vaccine Approach	Typical Production Method	Benefits	Limitations	Sources
Live attenuated	Perform serial passaging through a foreign host to mutate to a less pathogenic form	- Causes potent immune response since contains same components as natural pathogen presented in same manner - Often confers lifelong immunity with 1-2 doses	- Can take years to generate attenuated strain - Is risky: mutations can revert back to virulent pathogen - Cannot be given to immunocompromised individuals - Needs cold chain manufacturing and storage	[2,6,9]
Inactivated	Use heat, chemical (formaldehyde or β-propiolactane) or radiation to inactivate	- Cannot revert to infectious form - Can be stored and transported in freeze-dried form	- Produces weaker immune response compared to live attenuated vaccines, requiring multiple boosters - Is risky if inactivation is incomplete - Can contain unrelated antigens from pathogen	[6,8]
Subunit	Purify or recombinantly express antigens	- Is non-infectious - Is less risky than live attenuated pathogens since no chance of reversion	- Has poor immunogenicity by itself, requiring adjuvants and multiple boosters - Can be time consuming to identify best antigens to use	[2,6]
Conjugate	Attach polysaccharide antigen to carrier protein	- Is safe and effective in preventing numerous bacterial infections - Is cost-effective in high burden settings	- Needs to be linked to a carrier protein for stronger immune response - Typically uses chemical conjugation which can disrupt protective epitopes - Requires cold chain manufacturing and storage	[10-12]

Virus-like particles	Express viral structural proteins that self-assemble Bud from cells	- Mimics size and shape of native viruses - Displays repetitive subunits	- Is difficult to purify - Can be challenging to self-assemble	[8,13,14]

Traditional vaccines are typically produced in a recombinant host and can lead to many of the aforementioned issues. With advancements in synthetic biology, we can now produce proteins outside of a cell. Specifically, cell-free protein synthesis (CFPS) systems provide a platform to circumvent many of the issues associated with current vaccine production strategies. CFPS uses extracted transcriptional and translational machinery from cells to synthesize proteins of interest *in vitro*, without requiring a cellular host. CFPS systems are derived from either crude cell extracts or purified recombinant translation factors and supplemented with energy sources, salts, amino acids, nucleotides, cofactors, and additional reagents if necessary [15,16]. While CFPS systems have been predominantly used for the design of biosensors [17], high throughput prototyping [18], metabolic engineering [19], and therapeutics [20], its use for vaccine development and production is still an emerging area. CFPS features such as the lack of a cellular host, an open environment, and rapid protein production have the potential to overcome drawbacks in current production techniques and expand opportunities in vaccine development.

In this review, we highlight the advantages of CFPS systems for vaccine development and production. We discuss examples of how the valuable features of CFPS have been harnessed to generate a variety of vaccines as well as how the technology is beneficial as a tool in vaccine discovery. Lastly, we discuss existing challenges in cell-free vaccine production that need to be addressed in order to expand the use of this technology for development of potent vaccines.

The biochemical benefits of cell-free protein synthesis for vaccine development

CFPS systems offer many benefits that can address limitations in current vaccines as well as expand production techniques of existing vaccines. First, the system environment is highly controllable compared to complex living cells, allowing for allocation of resources for protein production rather than cell survival [21]. Along those lines, the lack of a cellular host enables production of proteins that have toxic substrates [22] or are harmful to cells [23]. Second, due to the open nature of cell-free reactions, they are amenable to the addition of noncanonical amino acids (ncAAs) which can be synthesized at precise sites to expand the functionality of proteins [24,25]. In a therapeutic context, ncAAs often contain biorthogonal functional groups that can partake in conjugation schemes such as for antibody-drug conjugates [24,26]. The components in cell-free systems can also be freeze-dried to extend outreach and on-site manufacturing [12,27]. Third, cell-free systems allow for rapid prototyping and production of proteins to meet demands since they can synthesize proteins at the hours timescale rather than the typical months to 3 years it can take for standard biomanufacturing methods [18,28-30]. Lastly, cell-free vaccine production is safer because it does not need harsh chemicals to inactivate live pathogens and does not contain risky virulent material [8,31]. Taken together, CFPS is a promising technology that can solve shortcomings in current vaccine development due to its highly optimizable nature, rapid protein production, and ability to be freeze-dried for on-demand biomanufacturing.

Different types of cellular extracts can be used for cell-free protein synthesis depending on the application. *E. coli* extracts are commonly employed because they are simple to prepare, are cost-effective, can produce high yields, are highly amenable to reaction optimization, are scalable, and have well established use in literature [32,33]. Notable disadvantages, however, include a

limited ability to include post-translational modifications on synthesized proteins, difficulties producing membrane proteins without membrane solubilization supplements, and the potential for endotoxin contamination [24]. Eukaryotic cell-free systems can be better than bacterial ones for the synthesis of complex proteins because they can contain endogenous microsomes from the endoplasmic reticulum or allow for exogenous addition for post translational modifications. However, they tend to have relatively lower protein yields, higher costs, and more labor intensive extract preparation than bacterial systems [24,33]. Eukaryotic cell-free systems that have been explored include insect (*Spodoptera frugiperda* 21), Chinese hamster ovary, rabbit reticulocyte, plant-based wheat germ, and yeast-based *Pichia pastoris* and *Saccharomyces cerevisiae* [24,32-34]. Overall, coupled with the biochemical benefits of CFPS systems, the variety of cell-free extracts facilitates the use of cell-free technologies for vaccine purposes.

Applications of cell-free protein synthesis for vaccine development

Using CFPS has the potential to circumvent time, storage, and labor-intensive production limitations required for vaccine production from existing cell-based methods [31] and has led to a various early demonstrations of vaccine design. Here, we highlight examples of vaccines produced using CFPS (Table 2) and how the technology has been applied in vaccine design.

Table 2. Overview of cell-free expressed proteins and their applications for vaccines.

Type of vaccine	Cell-free expressed component(s)	Application		Cell-free system	Sources
		Pathogen	Disease		
Subunit	Ovalbumin (model)	--	--	*E. coli*	[35]
Subunit	B-cell lymphoma idiotype Granulocyte macrophage colony-stimulating factor	B-cell lymphoma	B-cell lymphoma	*E. coli*	[36-38]
Subunit	Hemagglutinin	Influenza virus A (H1N1)	Influenza	*E. coli*	[39]
Subunit	Heavy chain proteins for botulinum toxins A, B, and E	*Clostridium botulinum*	Botulism	*E. coli*	[40]
Subunit	Nervous necrosis virus coat protein	Nervous necrosis virus	Viral nervous necrosis	*E. coli*	[41]
Subunit	Invasion plasmid antigens IpaB and c-terminal domain IpaH	*Shigella flexneri*	Shigella infection	*E. coli*	[42]
Subunit	Truncated Pfs230	*Plasmodium falciparum*	Malaria	Wheat germ	[43]
Subunit	FhSAP2	*Fasciola hepatica*	Fascioliasis	Rabbit reticulocyte	[44]
Subunit	Diphtheria toxoid DT5	*Corynebacterium diphtheriae*	Diphteria	Chinese hamster ovary	[45]
Conjugate	*E. coli* maltose-binding protein (MBP) *Haemophilus influenzae* protein D (PD)	*F. tularenis*	*F. tularensis* infection	*E. coli*	[12]

	Neisseria meningitidis porin protein (PorA) Detoxified *Corynebacterium diphtheriae* toxin (CRM197) Detoxified *Clostridium tetani* toxin fragment C (TTc) Detoxified *Clostridium tetani* toxin light chain (TTlight) *Pseudomonas aeruginosa* exotoxin A (EPA)				
Conjugate	Detoxified diphtheria CRM197	*Streptococcus pneumoniae*	Pneumococcal Disease	*E. coli*	[46,47]
Conjugate	Streptolysin O (SLO) toxoid	*Streptococcus pyogenes*	Group A streptococcal infection	*E. coli*	[48]
Conjugate	*Streptococcus pyogenes* adhesion and division protein (SpyAD)	*Streptococcus pyogenes*	Group A streptococcal infection	*E. coli*	[49]
Virus-like particle	MS2 bacteriophage coat protein C-terminally truncated Hepatitis B core protein	--	--	*E. coli*	[50,51]
Virus-like particle	VP1 capsid protein	Human norovirus GII.3 and GII.4	Viral gastroenteritis	*E. coli*	[52]
Virus-like particle	Tandem-core hepatitis B core antigen (VLP) Matrix 2 ectodomain protein Hemagglutinin stalk protein	Influenza virus A	Influenza	*E. coli*	[53]
Membrane augmented	Major outer-membrane protein (mMOMP)	*Chlamydia muridarum*	Chlamydia	*E. coli*	[54]
Membrane augmented	Outer membrane protein (OprF)	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* infection	*E. coli*	[55]

Subunit vaccines

Subunit vaccines consist of purified or recombinantly expressed proteins from viral or bacterial pathogens that act as vaccine antigens. Since they typically just contain the antigenic protein and no pathogen-associated molecular patterns, they tend to be weakly immunogenic, requiring co-administration with adjuvants and boosters [2,9]. One way CFPS can be utilized to enhance the efficacy of subunit vaccines is by directly conjugating the antigen to an adjuvant. To demonstrate this potential of enhancing antigen immunogenicity through adjuvant conjugation, a model antigen ovalbumin was synthesized with p-azidomethyl-L-phenylalanine (pAMF) for copper-free click chemistry conjugation to TLR9 agonist CpG1018 [35]. Here, CFPS allowed for a quick method of generating an antigen with site-specific conjugation of adjuvant that led to improved antigen presentation *in vitro* and higher antigen-specific CD8+ T cell production *in vivo* with less CpG1018 needed. Moving forward, this strategy could improve the protective responses of a variety of subunit vaccines.

CFPS can also be used to rapidly produce personalized therapeutic vaccines such as for B-cell lymphoma. Using *E. coli* based cell-free systems, the Swartz group created single-chain variable fragment (scFv) fusion proteins consisting of a B-cell lymphoma idiotype (Id) protein antigen with granulocyte-macrophage colony-stimulating factor (GM-CSF) or an immunostimulatory peptide from interleukin-1β [36,37]. In their work, they tuned reaction conditions to promote disulfide bond formation and the arrangement of the variable heavy chain (VH), variable light chain (VL), and GM-CSF [36]. This optimization ultimately led to the generation of an effective lymphoma vaccine that conferred protection in mice studies against a tumor challenge compared to a more traditional mammalian immunoglobulin chemically conjugated to immunogenic keyhole limpet hemocyanin (KLH) [37]. Next, to improve the poor immunogenicity of Id vaccines, thought to be due to chemical conjugation strategies that damage antigenic determinants, a diabody vaccine was produced [38]. This therapeutic vaccine consisted of a lymphoma Id and B-cell targeting molecule αCD19 to bind to noncognate B cells for antigen presentation. In this study, CFPS presented a strategy to rapidly generate B cell-targeting fusion vaccines without damage from chemical conjugation schemes. Compared to the months it may take to optimize and produce these vaccines in transfected mammalian or insect cell lines, CFPS offered quick production times and demonstrates promise in the area of personalized vaccines [37].

Beyond the aforementioned studies, a variety of subunit vaccines have been developed, primarily using *E. coli* based CFPS systems. These include: hemagglutinin (HA) stem trimer with correct immunogenic conformation for influenza [39]; heavy chain proteins for botulinum toxins A, B, and E that protected mice against high-dose toxin challenges [40]; nervous necrosis virus (NNV) coat protein that improved sevenband grouper mortality rates [41]; and invasion plasmid antigens (Ipa) IpaB and C-terminal domain of IpaH for Shigella protection [42]. Beyond *E. coli* based cell-free systems, other lysates have been explored to produce subunit vaccines such as wheat germ lysate for fragments of Pfs230, a gametocyte/gamete surface protein in *Plasmodium falciparum*, that induced transmission-blocking antibodies for malaria [43]; rabbit reticulocyte lysate for SAP2 of *Fasciola hepatica* with correct conformation for fascioliasis disease [44]; and Chinese hamster ovary lysate for diphtheria toxoid DT5 for diphtheria-tetanus applications in a point of care protein expression and purification device [45]. Taken together, these examples demonstrate the capacity of cell-free systems to generate diverse antigens for vaccines.

Conjugate vaccines

Conjugate vaccines consist of polysaccharide antigens from bacterial surfaces attached to an immunostimulatory carrier protein. By conjugating weak bacterial antigens to carrier proteins such as tetanus toxoid, diphtheria toxoid, or detoxified diphtheria toxoid mutant, a strong immune response for the antigen can be generated [10]. Showcasing how CFPS can be used to generate

portable, on-demand conjugate vaccines, the iVAX (in vitro conjugate vaccine expression) platform was developed by Stark and Jaroentomeechai et al. [12]. iVAX utilizes freeze-dried reactions containing lysates with pathogen-specific polysaccharide antigens and a bacterial oligosaccharyltransferase enzyme as well as an immunostimulatory carrier protein plasmid [12]. This platform is modular, allowing the carrier protein and bacterial O-antigen polysaccharide to be exchanged. For instance, antigens from *F. tularensis* subsp. *tularensis* (type A) Schu S4, ETEC *E. coli* O78, and UPEC *E. coli* O7 were interchanged. Ultimately, this platform produced vaccines that could protect mice from a lethal *F. tularenis* challenge by conjugating *Ft*O-PS to carrier proteins $MBP^{4xDQNAT}$, $PD^{4xDQNAT}$, or $EPA^{DNNNS\text{-}DQNRT}$. By using CFPS, the authors were able to address limitations in cold chain manufacturing to promote wide-spread access to conjugate vaccines.

A major benefit of CFPS is the ability to incorporate ncAAs into proteins for site-specific conjugation of molecules. This is particularly beneficial for generating conjugate vaccines with precise placement of polysaccharides on carrier proteins. For example, towards a highly multivalent vaccine for pneumococcal diseases, an enhanced carrier protein (eCRM) based on detoxified diphtheria CRM 197 was developed that enables site-specific click chemistry conjugation of 24 distinct polysaccharides [46]. The designed cell-free produced carrier contained ncAAs away from primary T-cell epitope sites to improve immune responses. The VAX-24 vaccine candidate includes antigens against all pneumococcal serotypes in licensed pneumococcal vaccines Prevnar13 (PCV13) and Pneumovax23 (PPSV23), and it demonstrated comparable immune responses to conjugate vaccine Prevnar 13 and higher responses than the leading high coverage polysaccharide vaccine Pneumovax 23. Separately, Bautista and Pill-Pepe et al. used eCRM and linked four clinically relevant *S. pneumoniae* serotypes [47]. In their study, they demonstrated that the reaction kinetics and conjugation efficiency of copper-free click chemistry reactions could be improved by the addition of the zwitterionic surfactant lauryldimethylamine N-oxide (LDAO).

Conventional methods of creating conjugate vaccines using reductive amination chemistry can lead to shielding of epitopes and reduced functional antibody responses [48]. By using CFPS, however, ncAAs can be precisely added while retaining immunogenic epitopes of the carrier proteins. For instance, Kapoor et al. conjugated a truncated streptolysin O (SLO) toxoid produced by CFPS to a conserved group A cell wall carbohydrate (GAC) to protect against *Streptococcus* [48]. Another example of precise, site-specific protein conjugation enabled by ncAAs includes cell-free expression of the carrier protein SpyAD, which is a conserved group A surface protein on *Streptococcus* that has both B-cell and T-cell epitopes that also act as antigens for the pathogen [49]. In this study, the authors conjugated SpyAD to a modified GAC (GAC^{PR}) that lacks a suspected cross-reactive epitope to human heart tissue. In a mice immunization study of SpyAD-GAC^{PR} along with other vaccine antigens SLO and C5a peptidase, the cell-free vaccine was protective against *Streptococcus* challenges in systemic and skin infection models. Altogether, CFPS enables more precise generation of conjugate vaccines that better retain protein structure and immunogenicity and have efficient antigen presentation.

Virus-like particle vaccines

Virus-like particle (VLP) vaccines are composed of self-assembling viral structural proteins that form 20 to 200 nm particles [13]. Since they do not contain genetic material, they are non-infectious, yet they retain virus size and the ability to display repetitive subunits on their surfaces to provide more immunogenicity compared to subunit vaccines alone [14]. VLPs have typically been generated in cellular hosts and are featured in licensed vaccines for hepatitis B (Recombivax HB and Engerix-B), human papillomavirus (Gardasil, Cervarix, and Gardasil-9), and hepatitis E (Hecolin for HEV) [13]. To overcome initial limitations in VLP production such as low yields and

poor scalability, the Swartz group demonstrated the first instance of generating VLPs using a prokaryote-based cell-free system in 2007 [50]. Here, they expressed the bacteriophage MS2 coat protein and produced 14 times more VLPs compared to the previously best published yield from cellular systems. Similar high yields were also generated for a C-terminally truncated Hepatitis B core protein VLP. Expanding on these foundations to contain surface modifications, bacteriophage MS2 and bacteriophage Qβ VLPs were expressed with global replacement of methionine groups with analogues that have terminal azide and alkyne groups to enable click chemistry [51]. Specifically, ncAA sites were placed at exposed loop regions to allow for conjugation of ligands such as proteins, nucleic acids, and small molecules to the surface while minimally disrupting protein folding. To demonstrate the potential of using cell-free generated VLPs as a tumor-specific vaccine for B cell lymphoma, an idiotypic antibody fragment antigen, GM-CSF cytokine, and CpG DNA immunostimulant were co-attached to the surface of VLPs in a single click-chemistry reaction [51]. In these cases, it was demonstrated that *E. coli* based cell-free systems can produce high yields of VLPs and in addition, could be rapidly modified post assembly to contain surface modifications to address a diverse range of diseases.

Compared to cell-based methods of VLP production, cell-free methods can produce similar or more amounts of VLP protein at much shorter time scales. Human norovirus (HuNoV) VLPs were created by expressing the two capsid proteins, VP1 HuNoV genotype GII.3 and genotype GII.4 in an *E. coli* based CFPS system [52]. In just 4 hours, 0.62 g/L and 0.57 g/L of VP1-GII.3 and VP1-GII.4 VLPs were produced, which are similar yields to a cell-based method for VLP production in *P. pastoris* but in a fraction of the time. Lastly, CFPS can act as a screening tool for optimizing VLP assembly and yields [53]. Using CFPS systems, the authors were able to explore multiple plasmid backbones, arginine-rich region modifications, and peptide linker lengths in order to optimize tandem-core hepatitis B core antigen (HBcAg) VLPs. Ultimately, they were able to quickly analyze and produce two influenza candidates containing influenza A matrix 2 protein ectodomain (M2e) and hemagglutinin stalk globule (HA2). All in all, CFPS can enable modifications of VLPs, improve production time, and allow screening of assembly conditions.

Membrane augmented vaccines

Many vaccine targets for enveloped viruses and gram-negative bacteria are membrane proteins, which are difficult to produce in solution with CFPS due to the hydrophobic nature of membrane proteins [56]. Because most CFPS systems to date lack cellular membranes, membrane proteins expressed in these solutions are prone to aggregation and misfolding. By supplementing cell-free reactions with lipid structures such as liposomes—bilayer vesicles containing phospholipids, and nanodiscs—phospholipid bilayers stabilized by scaffold proteins, production of membrane proteins can be improved by reintroducing amphiphilic structures that support membrane protein folding and structure. Specifically, it has been shown that the presence of membranes can benefit cell-free production of membrane proteins through co-translational integration of proteins into the lipid scaffolds [57,58]. Liposomes are favorable vehicles for therapies and vaccines because they are largely biocompatible, can encapsulate drugs or biomolecules, are amenable to surface modifications with ligands and polymers, and can contain co-localized antigens and adjuvants [59-62]. Features such as particle size, charge, fluidity, and phospholipid makeup can be tuned to influence vaccine activity [60,63-65]. For example, nanodiscs were used to develop a *Chlamydia* vaccine by using an *E. coli* based cell-free system to co-express *Chlamydia muridarum* major outer-membrane protein (mMOMP) with truncated Δ49apolipoprotein A1 as a structural protein in the presence of telodendrimer nanolipoprotein particles (tNLPs) [54,66]. These mMOMP-tNLP vaccines allowed inclusion of CpG oligodeoxynucleotide 1826 adjuvant, leading to improved humoral immune responses in mice vaccination compared to no adjuvant and buffer controls. More recently, a cell-free liposome-based vaccine for *Pseudomonas aeruginosa* was created by expressing outer membrane protein OprF, a conserved porin, in liposomes [55].

Properly oriented and folded OprF was integrated into the liposomes and assembled into a vaccine that provided a 90% protection rate against a lethal dose of mucoid CF isolate CHA strain. By supplementing CFPS reactions with membranes, membrane proteins that serve as antigens can be more easily produced, expanding the diversity of vaccines that will be possible to generate with CFPS.

CFPS as a tool for vaccine development

Beyond using CFPS to produce proteins for a vaccine, the technology can aid in vaccine design. For instance, when designing a malaria vaccine, it is important to identify malaria-specific proteins that interact with patient antibodies to find potential proteins that could be used as vaccines. However, screening a library of *P. falciparum* proteins is difficult to achieve in bacterial expression systems due to improper folding [67]. To screen for malaria vaccine candidates, Kanoi et al. used a wheat germ extract to cell-free express a library of 1,827 proteins from *P. falciparum*. This library was first evaluated for interactions with antibodies from the sera of indigenous residents in a malaria endemic part of Northern Uganda [67]. Here, 53 proteins were identified as potential targets for vaccine candidates. The library was also evaluated for reactivity to blood samples from malaria-exposed Malians and correlated to growth inhibition assay results [68]. As a result, the study identified protein LSA3 as a blood-stage vaccine candidate. Moreover, CFPS allows for rapid prototyping and high throughput screening of assembly conditions for multi-component vaccines. For example, virus-like particles can be difficult to assemble and manufacture for vaccine applications. Towards making more robust particles, a *Pichia pastoris* based cell-free system was used to develop a fast hepatitis B core (HBc) VLP assembly and purification scheme that has potential use for prototyping protein variants for vaccines [69]. In addition, an *E. coli* based system was utilized to screen how features of a surface spike region in HBc VLPs affect stable particle assembly [70]. These features included artificial disulfide bonds, surface charge, and transplantation. Overall, these studies demonstrate the potential of CFPS to streamline the vaccine candidate discovery process, which can be coupled with current cell-free and cell-based vaccine production strategies.

Challenges and progress for cell-free vaccines

While CFPS systems demonstrate great potential for producing vaccines to protect people from existing and emerging infectious diseases, there are a few considerations that need to be addressed for this technology to be employed for large-scale vaccine production. In particular, *E. coli* based systems are commonly used because of relatively simple preparation, high yield protein synthesis within hours, ease of scalability, and cost-effective performance [24,71]. However, several challenges with CFPS remain, such as the potential presence of endotoxins in bacterial extracts, difficulty with disulfide bond formation, on-demand biomanufacturing, and difficulty glycosylating proteins. Overcoming these challenges will be critical to drive advancements in cell-free vaccine development.

First, the presence of lipopolysaccharides (LPS) or endotoxins, which are components in the outer membrane of gram-negative bacteria, can lead to septic shock. To combat this, a detergent Triton X-114 can be used for endotoxin extraction [72]. In addition, lysates can be formed from genetically engineered strains that modify the LPS structure to reduce endotoxin toxicity such as ClearColi BL21 DE3 (ΔgutQ, ΔkdsD, ΔlpxM, ΔpagP, ΔlpxP, ΔeptA) [72] or CLM24 ΔlpxM [12]. Next, despite the ease of use of prokaryote-based systems, disulfide bonds that are present in more complex antigens and antibody fragments of interest are difficult to fold in *E. coli* based environments due to lack of an endoplasmic reticulum and associated chaperones. Efforts have been made in modulating the redox potential of the CFPS solution with reduced and oxidized glutathione, adding protein disulfide isomerase (PDI) or disulfide bond C (DsbC) to help with disulfide bond formation, or utilizing an engineered strain, SHuffle, that has a more oxidizing

environment and expresses cytoplasmic DsbC [73-75]. Another consideration is the lack of post-translational modifications in *E. coli* based systems, such as glycosylation which is present in viral membrane proteins and conjugate vaccines. Eukaryotic cell-free systems, as highlighted before, are capable of such modifications and enhanced folding, but are not yet feasible to use for large-scale protein production due to low yields, high costs, and complex extract preparation [24]. Towards protein glycosylation in *E. coli* based systems, progress in glycoengineering includes engineering orthogonal *O*-glycoprotein biosynthesis in *E. coli* to create enriched extracts capable of cell-free glycoprotein construction [76], identifying processing methods to enrich extracts with membrane-bound glycosylation machinery PglB or PglO for improved glycoprotein titers [71], and redesigning difficult-to-express membrane-bound glycotransferases to improve soluble expression for use in one-pot cell-free glycoprotein production [75]. Finally, beyond designing and producing vaccines, it is important to consider vaccine stability in order to enable their widespread distribution. Towards on-demand biomanufacturing, lyoprotectants have been explored to enhance the stability of cell-free reactions for therapeutics [77] and low-cost conjugate vaccines [78]. Altogether, these advancements will pave the way forward in vaccine design and development.

Conclusion and Outlook

Cell-free protein synthesis systems are promising tools for vaccine production and development and can overcome challenges present in many current vaccine production strategies (i.e. attenuated, inactivated, subunit, conjugate, VLP) such as the use of harsh chemicals, the risk of live viral materials, long production times, weak immunogenicity, and poor stability. Harnessing the open nature, rapid synthesis, and highly optimizable features of these systems has led to the generation of subunit, conjugate, virus-like particle, and membrane augmented vaccines as well as screens for vaccine candidates and assembly conditions. Cell-free technologies are especially powerful because the abilities to lyophilize reactions for on-demand protein production, produce membrane proteins with membrane supplements, and precisely add noncanonical amino acids for antigen or adjuvant conjugation unlock new possibilities for vaccine design and manufacturing. Efforts on addressing endotoxins, poor protein folding, reaction stability for extended outreach, and glycosylation through strain engineering, rational protein design, and optimization of processing and reaction conditions have lowered the barrier of cell-free produced vaccines to date. While there is still room to improve protein folding and large-scale yield with these systems, advancements over the past years will allow for more high throughput vaccine development and an expanded collection of vaccines to meet the critical demand generated by emerging and existing diseases.

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Annotated references

at least 10% (8) of the references should be selected and annotated as being recent papers of special interest (·) or outstanding interest (··)

1. ** Stark JC, Jaroentomeechai T, Moeller TD, Hershewe JM, Warfel KF, Moricz BS, Martini AM, Dubner RS, Hsu KJ, Stevenson TC: On-demand biomanufacturing of protective conjugate vaccines. Science Advances 2021, 7:eabe9444.

The authors describe their *in vitro* conjugate vaccine expression (iVAX) system for on-demand production of conjugate vaccines. This system is modular, allowing antigens and protein carriers to be interchanged, inexpensive at ~$6 a dose, portable, cold-chain independent, and able to produce effective glycosylated vaccines.

2. * Weiss AM, Ajit J, Albin TJ, Kapoor N, Maroju S, Berges A, Pill L, Fairman J, Esser-Kahn AP: Site-specific antigen-adjuvant conjugation using cell-free protein synthesis enhances antigen presentation and CD8+ T-cell response. Scientific reports 2021, 11:1-9.

This article highlights a method of site-specific adjuvant conjugation of CpG oligodeoxynucleotide to model antigen ovalbumin using copper-free click chemistry to non-native amino acids. This strategy improved *in vitro* antigen presentation and CD8+ T-cell responses.

3. ** Kapoor N, Ndungo E, Pill L, Desalegn G, Berges A, Oaks EV, Fairman J, Pasetti MF: Efficient production of immunologically active Shigella invasion plasmid antigens IpaB and IpaH using a cell-free expression system. Applied Microbiology and Biotechnology 2022, 106:401-414.

The authors cell-free expressed functional invasion plasmid antigens (Ipa) B and C-terminal H proteins at efficient yields of 200 mg/L towards a *Shigella* vaccine.

4. ** Fairman J, Agarwal P, Barbanel S, Behrens C, Berges A, Burky J, Davey P, Fernsten P, Grainger C, Guo S: Non-clinical immunological comparison of a Next-Generation 24-valent pneumococcal conjugate vaccine (VAX-24) using site-specific carrier protein conjugation to the current standard of care (PCV13 and PPV23). Vaccine 2021, 39:3197-3206.

This article describes their work on creating 24-valent pneumococcal conjugate vaccine by site-specific conjugation of 24 serotype conjugates to an enhanced carrier protein, eCRM. The carrier has non-native amino acid para-azidomethyl-L-phenylalanine (pAMF) at selected lysines to enable copper-free click chemistry.

5. * Gao NJ, Uchiyama S, Pill L, Dahesh S, Olson J, Bautista L, Maroju S, Berges A, Liu JZ, Zurich RH: Site-specific conjugation of cell wall polyrhamnose to protein SpyAD envisioning a safe universal group A streptococcal vaccine. Infectious Microbes & Diseases 2021, 3:87-100.

This work uses CFPS to produce SpyD, a group A *Streptococcus* (GAS) surface protein, as a carrier protein conjugated with site-specific click chemistry to the polyrhamnose core of group A cell wall carbohydrate (GAC^{PR}) as a protective vaccine against a GAS challenge.

6. ** He W, Felderman M, Evans AC, Geng J, Homan D, Bourguet F, Fischer NO, Li Y, Lam KS, Noy A: Cell-free production of a functional oligomeric form of a Chlamydia major outer-membrane protein (MOMP) for vaccine development. Journal of Biological Chemistry 2017, 292:15121-15132.

The authors explore the use of telodendrimer nanolipoprotein particles, or nanodisc structures, to assemble vaccines for Chlamydia with membrane protein major outer-membrane protein (MOMP).

7. ** Mayeux G, Gayet L, Liguori L, Odier M, Martin DK, Cortès S, Schaack B, Lenormand J-L: Cell-free expression of the outer membrane protein OprF of Pseudomonas aeruginosa for vaccine purposes. Life science alliance 2021, 4.

The authors cell-free expressed outer membrane protein OprF in liposomes to generate a functional vaccine against *Pseudomonas aeruginosa*.

8. * Wilding KM, Hunt JP, Wilkerson JW, Funk PJ, Swensen RL, Carver WC, Christian ML, Bundy BC: Endotoxin-free E. coli-based cell-free protein synthesis: Pre-expression endotoxin removal approaches for on-demand cancer therapeutic production. Biotechnology journal 2019, 14:1800271.

The authors explored three strategies (Triton X-114 two phase extraction, polylysine affinity chromatography, and cell-free extract generated from engineered endotoxin-free ClearColi cells) to reduce endotoxin in *E. coli*-based cell-free systems for use in cancer therapeutics.

9. * Hershewe JM, Warfel KF, Iyer SM, Peruzzi JA, Sullivan CJ, Roth EW, DeLisa MP, Kamat NP, Jewett MC: Improving cell-free glycoprotein synthesis by characterizing and enriching native membrane vesicles. Nature communications 2021, 12:1-12.

This work investigates how extract processing methods and increasing membrane vesicle concentration can improve membrane-associated activities in *E. coli* cell-free systems such as glycoprotein production.

10. * Natarajan A, Jaroentomeechai T, Cabrera-Sánchez M, Mohammed JC, Cox EC, Young O, Shajahan A, Vilkhovoy M, Vadhin S, Varner JD: Engineering orthogonal human O-linked glycoprotein biosynthesis in bacteria. Nature chemical biology 2020, 16:1062-1070.

This work demonstrates that *E. coli* can be engineered with human-like *O*-glycosylation pathways and used in cell-free reactions for site-specific *O*-glycosylation.

11. * Jaroentomeechai T, Kwon YH, Liu Y, Young O, Bhawal R, Wilson JD, Li M, Chapla DG, Moremen KW, Jewett MC: A universal glycoenzyme biosynthesis pipeline that enables efficient cell-free remodeling of glycans. Nature communications 2022, 13:1-17.

This article discusses a strategy of redesigning nearly 100 membrane-bound glycosyltransferases to be water-soluble as a tool for glycoprotein production.