Polyester as antigen carrier toward particulate vaccines

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ABSTRACT: Spherical polyhydroxyalkanoate (PHA) inclusions are naturally self-assembled inside bacteria. These PHA beads are shell-core structures composed of a hydrophobic PHA core surrounded by proteins, such as the PHA synthase (PhaC). PhaC is covalently attached and served as anchor protein for foreign protein such vaccine candidate antigens. PHA beads displaying single and multiple antigens showed enhanced immunological properties when compared to respective soluble vaccines. This review highlights the unique design space of the PHA bead-based vaccines toward development of safe and synthetic particulate vaccines. The PHA bead technology will be compared with chemically synthesized polyesters, such as polylactic acids, formulated to deliver vaccine antigens. The performance of PHA bead vaccine candidates to induce specific immune responses and protective immunity against bacterial and viral pathogens in animal trials will be summarized. We propose that the PHA bead technology offers a versatile vaccine platform for design and cost-effective manufacture of synthetic multivalent vaccines.

KEYWORDS

Subunit vaccines, particulate vaccines, polyester, polyhydroxyalkanoate, self-assembly

1. INTRODUCTION

The concept of vaccines emerged in 1796 based on the discovery of the small pox vaccine by Edward Jenner. Since this discovery, the implementation of vaccines to prevent infectious diseases has been one of the most successful approaches in medical sciences. The story began when James Phipps, a smallpox sick boy, received a mixed fluid from a cowpox blister resulting in a fast recovery. A few months after he received an inoculum prepared from smallpox substances, symptoms of the disease were not detected and a long-lasting immunity was observed.[1] The vaccination theory was then developed in 1879 by Luis Pasteur with his Anthrax vaccine.[2] These pieces of evidence led to extensive use of vaccination to prevent numerous infectious diseases (**Table 1**). A vaccine formulation needs to be safe and generate immunological memory, additionally, it cannot elicit an autoimmune response and involve complicated administration routes.[1] Through history, vaccine development has resulted in different vaccine forms, including live/attenuated and inactivated whole pathogen vaccines as well as defined subunit and particulate vaccines (**Table 1**).

Table 1. Selected vaccines used in animals or humans against bacterial and viral infections.

Vaccine type	Disease	Pathogen	Vaccine characteristics	Ref
Live/Attenuated Bacteria and viruses	Smallpox	Variola major and variola minor viruses	Calf skin preparation of cowpox infected	[3]
	Tuberculosis	*M. tuberculosis*	*M. bovis* BCG*	[4]

		Yellow fever / Gastroenteritis	Flavivirus / Rotavirus	Attenuated virus*	5-6
Inactivated bacteria and viruses		Typhoid enteric fever	*S. typhi*	Inactivated whole bacteria	6
		Influenza	Influenza virus	Inactivated virus	
Subunit vaccine	Purified	Pneumonia	*S. pneumoniae*	Purified polysaccharide*	7
		Meningitis	*N. meningitidis*	Purified polysaccharide*	8
		Tetanus	*C. tetani*	Toxoid*	9
	Conjugated	Pneumonia / Meningitis	*S. pneumoniae / N. meningitidis*	Carbohydrate-protein conjugate*	10,11
	Recombinant (purified)	Hepatitis B	Hepatitis B virus	Recombinant HBsAg-VLP*	12
		Meningitis	*N. meningitidis*	Recombinant proteins*	13,14
		Nosocomial infections	*P. aeruginosa*	Recombinant proteins/DNA	15
Particulate vaccines	Virus-like particles	Warts in skin or vaginal mucosa	Herpes papilloma's virus	Capsid proteins*	16-17

	Synthetic polyester particles	Sexual infection	*C. trachomatis*	Protein encapsulation	[17-18]
	Bioengineered polyester particles	Tuberculosis / Pneumonia / Meningitis	*M. tuberculosis / S. pneumoniae / N. meningitidis*	Protein and carbohydrates coated polyhydroxyalkanoate (PHA) beads	[17, 19-21]

Note: *C. tetani = Clostridium tetani. C. trachomatis = Chlamydia trachomatis. M. bovis = Mycobacterium bovis. M. tuberculosis = Mycobacterium tuberculosis. N. meningitidis = Neisseria meningitidis. S. typhi = Salmonella typhi. S. pneumoniae = Streptococcus pneumoniae. P. aeruginosa = Pseudomonas aeruginosa*. * Licensed vaccine

Attenuated live vaccines, i.e. non-virulent live pathogens, are still replicating but unlikely to cause the disease.[1, 22-23] In contrast, inactivated pathogens cannot replicate and are considered as safer option.[1, 22-23] Attenuated/inactivated whole pathogen vaccines are in general highly immunogenic and contain not only the targeted antigens but also other bacterial or viral components that effectively activate the host immune system. Some components are highly reactogenic leading to adverse effects that are not desirable for vaccines. To eliminate this reactogenicity and to create safer vaccines, subunit vaccines offer an attractive alternative.

Subunit vaccines usually comprise selected soluble antigens such as proteins, peptides, carbohydrates and DNA from disease-causing pathogens.[1, 3] Although subunit vaccines are safer than conventional vaccines, they show a significant reduction in immunogenicity. Hence,

immunostimulatory adjuvants are used in commercial vaccine formulations in order to enhance immune responses. The addition of adjuvants adds to production cost and often reduces safety of vaccine formulations. To further improve subunit vaccines, antigens have been formulated into immunogenic polymer particles by using encapsulation, chemical conjugation or self-assembly.[16-21] These particulate vaccines hold the promise to serve as a potent antigen delivery system overcoming the low immunogenicity of soluble subunit vaccines.[24-25] This review focuses on the use of various polyester particles as antigen delivery systems. We will compare chemically synthesised polyesters (e.g., poly (lactic acid) (PLA)/poly (lactic-co-glycolic acid (PLGA)) with biologically synthesized polyesters (e.g., polyhydroxyalkanoate (PHA)) with respect to their design, manufacture and performance as particulate vaccines.

The adaptative immune response after infection or vaccination starts when antigen presenting cells (APCs) such as macrophages (M φ) or dendritic cells (DCs) internalise the pathogen or vaccine and after processing presents respective epitopes in the lymph nodes to immune cells.[26-27] The antigen presentation phenomenon implicates the interaction between MHC molecules and T cell receptors (TCR), the co-stimulatory molecules (CD40-CD40L) and cytokines production (**Figure 1**). Processed antigenic peptides from extracellular or intracellular pathogens are presented on MHC class II or I, respectively. Antigen presentation by MHC II results in differentiation of the naïve CD4+ T cells toward Th2 phenotype (antibody response) while antigen presentation by MHC class I results in the Th1 phenotype (cell-mediated immune response). The cross-presentation phenomenon, i.e. simultaneous display of epitopes on MHC I and MHC II, was also described for antigens from extracellular pathogens and mediated induction of Th1 and Th2 responses. The Th2 cells orchestrate the humoral immune response through cytokine production (e.g., interleukin-2 (IL2), IL4, IL5, IL10, and IL13), prompting B lymphocyte activation,

proliferation and differentiation into plasma (antibodies producer) and memory cells. On the other hand, the Th1 cells produce cytokines (e.g., IL2, tumour necrosis factor-alpha (TNFα), and interferon-gamma (IFNγ)) that contribute to proliferation and differentiation of CD8+ T cells toward formation of cytotoxic T cells.[28-30]

Particulate vaccines possess promising immunological advantages that can circumvent the low immunogenicity of the soluble subunit vaccines. The internalization mechanism and subsequent antigen processing pathways for soluble subunit and particulate vaccines show significant differences as outlined in **Figure 1**.

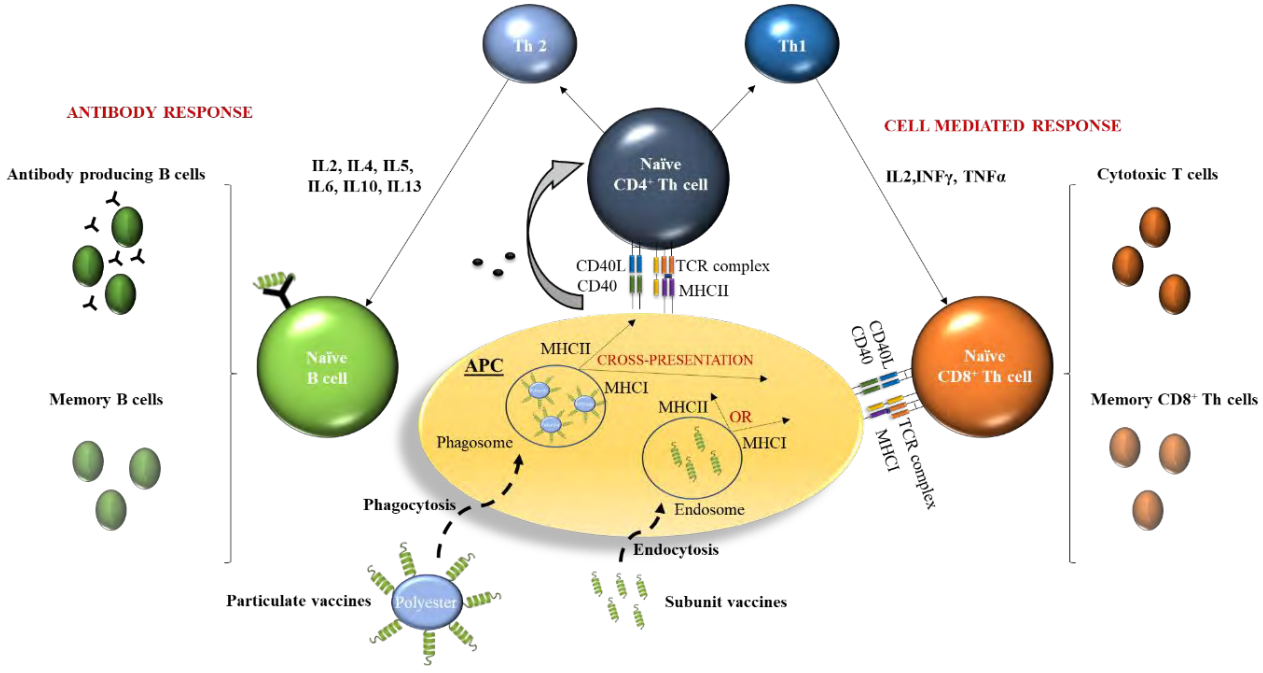

Figure 1. Differences in antigen uptake, processing and presentation between subunit and particulate vaccines. Antigen-presenting cell (APC), T helper lymphocyte (Th cell), major histocompatibility complex (MHC), T cell receptor complex (TCR complex), B lymphocyte (B cell), Interleukin (IL).

Subunit vaccines, i.e. purified soluble antigens, are captured and internalized by APCs through receptor-mediated endocytosis (**Figure 1**). Resulting endosomes preferentially mediate presentation of epitopes on MHC class II resulting in Th2 mediated antibody responses. In contrast, particulate vaccines exhibit a similar size to pathogens and are taken up by APCs through receptor-mediated phagocytosis (**Figure 1**). The resulting phagosome is a specialized organelle that promotes cross-presentation resulting in a mixed Th1/Th2 immune response that is advantageous toward protection against various infectious diseases (**Figure 1)**.[16-17, 27, 31]

Various APCs differ in regard to their preference for certain particle sizes such as dendritic cells (DCs) prefer particle diameters between 0.02–0.2 μm (e.g. viruses), while macrophages (M φ) prefer uptake of particles with sizes between 0.5–5 μm (e.g. bacteria).[27]

Incorporation of antigens into a particulate carrier system can confer antigen stability.[32] While particulate vaccines are efficiently taken up by APC for induction of an immune response, they might also directly drain to the lymph nodes if smaller than 200 nm enabling direct stimulation of B cells.[17] Display of multiple copies of antigenic epitopes on a particulate carrier system can be advantageous as it might promote multiple antigen-receptor interactions at the surface of immature B cells mediating activation of antibody production at lower antigen concentration.[17, 27] In addition, particles displaying repetitive copies of antigens can induce a more sustained antigenic presentation via APCs, i.e. enhanced immunogenicty, when compared to soluble subunit vaccines.[33-34] In the following paragraphs, the design of particluate vaccines using either chemically synthesized or bioenginered polyester particles as antigen carrier systems will be desrcribed and discussed.

2. SYNTHETIC POLYESTER-BASED PARTICULATE VACCINE

In the past decades, polyester-based nano- and micro-particles have been employed as carriers to deliver antigens owing to their biodegradability and biocompatibility as well as available economic manufacturing methods. Their physicochemical properties, including size, hydrophobicity, and surface chemistry can be controlled during synthesis imparting better control over release kinetics of loaded antigens, hence impacting immune responses. Furthermore, these polyester-based particulate vaccines can be administered *via* different routes, ranging from intranasal, oral, intradermal and intramuscular to subcutaneous, providing further means to improve immune responses against a broad range of bacterial and viral pathogens. Typical methods to produce those carriers are mainly based on chemical and genetic engineering approaches. This section focuses on the chemical methods, along with engineering characteristics of the carriers and formulations to enhance immune responses.

2.1 Antigen Incorporation. Poly (lactic-co-glycolic acid) (PLGA) and poly (lactic acid) (PLA) are the two most common polyester-based materials used to generate nano- and micro-particles for vaccine delivery (**Table 2**). To achieve this, the double emulsion method is frequently used (**Figure 2A**). Briefly, an aqueous solution containing antigen cargo (w_1) is added to an organic solution (e.g., dichloromethane or methylene chloride) containing polymers (o) and mixed under homogenization to form water droplets dispersed in the organic phase (i.e. water-in-oil (w_1/o) emulsion). This emulsion is then mixed with a second water phase (w_2) containing a stabilizer, such as poly (vinyl alcohol) (PVA), and homogenization results in external droplets encapsulating the internal droplets (w_1/o) dispersed in the water phase (i.e., $w_1/o/w_2$ emulsion). Evaporation or extraction of the organic solvent within the droplets led to the solidification of the droplets, i.e. formation of polymeric particles. This method facilitates cargo encapsulation within the polymer

matrix (in w_1/o emulsion) before polymer hardening (i.e., pre-loading), thus minimizing cargo leakag[35] (**Figure 2B** (i)). Additionally, the double-emulsion method enables co-encapsulation of antigens with, for example, immune-stimulating adjuvants to enhance immune responses[36] or excipients, such as sugars, proteins, salts, amino acids and combinations thereof, to stabilize cargoes that are sensitive to certain environments (e.g., pH or temperature).[37] Alternative ways to load antigens are by either physical adsorption[38] (**Figure 2B** (ii)) or chemical conjugation[39] (**Figure 2B** (iii)) to the particle surfaces. The former is based on electrostatic or hydrophobic interactions between the antigen and the particle surface, while the latter is based on specific affinity depending on the compatibility of the functional groups between those chemical entities. Another important aspect related to the production method is the sterilization of the resultant particles. Sterilization process, like steam (high steam pressure, 120–135°C), dry-heat (160–190°C), or radiation (ionizing or γ) are not suitable as they deform the particles or crosslink/break the polymer chains.[40] Alternative sterilization processes without compromising the integrity of the polymer matrix of the particles can be conducted based on gas sterilization using ethylene oxide or filtration.

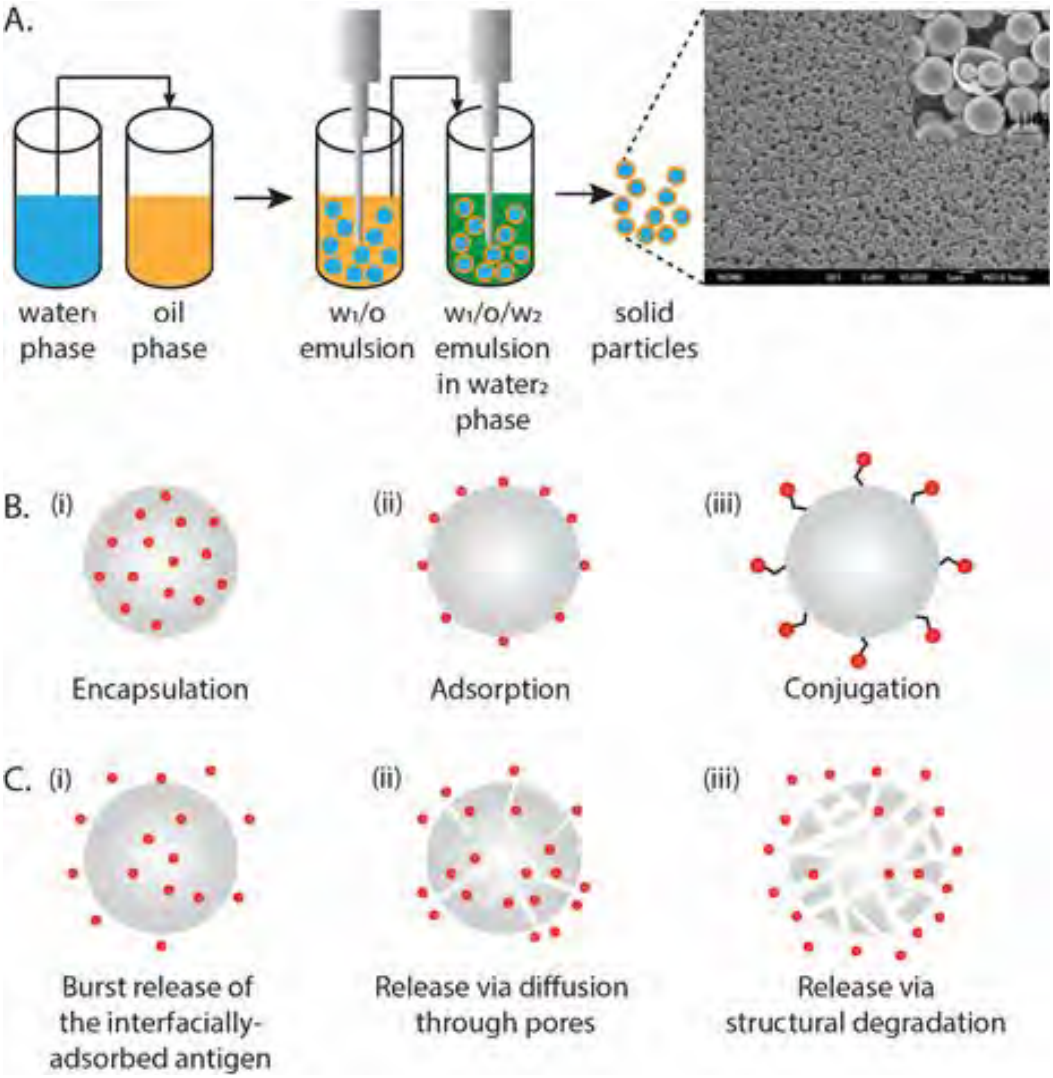

Figure 2. Synthetic polyester-based carrier systems as particulate vaccines. (A) Double emulsion method for the synthesis of nano- and microparticles. (B) Methods to load antigen in/on to the particles. (C) Mechanisms of antigen release. Red dots represent antigen. The electron microscopy image shows PLGA nanoparticles reproduced with permission from Liu *et al.*[41] Copyright 2015 American Chemical Society.

Physicochemical properties of the resultant PLGA/PLA nano- and micro-particles have influenced the antigen release kinetics as well as their biodistribution, which in turn affects immune responses.[42-44] To control these properties, various experimental conditions can be varied during particle formation. For example, increasing the shearing energy during the second emulsification step from high-speed stirring-based homogenization to ultrasound-based sonication significantly decreased the particle size from micro- to nano-meter ranges.[45-47] Decreasing the polymer molecular weight can also decrease the size of the resultant polymer particles.[45] Increasing the lactic acid to glycolic acid ratio in the PLGA composition increases the particle hydrophobicity due to the presence of methyl side groups in the lactic acid co-polymers. In aqueous solution such as water, PLGA particles with increased hydrophobicity levels (high lactic acid to glycolic acid ratio) absorb less water hence are less prone to undergo hydrolysis when compared to less hydrophobic PLGA particles, which in turn lowers the degradation rate of those particles hence impacting release kinetics.[48] To modify the surface characteristics of the particles, macromolecules, such as glycol chitosan, chitosan,[38, 47, 49-50] or polyethyleneimine[51] can be added in the water phase during the first emulsification step. The resultant modified particles exhibited a positive Zeta potential (i.e. a positive surface charge) as opposed to the negatively charged PLGA/PLA particles synthesized using common ingredients. Positively charged PLGA/PLA particles have been used to adsorb negatively-charged cargo such as DNA on the particle surfaces[51] or else to improve phagocytic uptake by the APCs.[44] Inclusion of mucoadhesive polymers like chitosan and its derivatives on the nanoparticles is beneficial for use as mucosal vaccine as it increased the nasal cell internalization.[38] Stealth polymers, like poly(ethylene glycol) (PEG),[18] or targeting molecules,[50, 52] such as CKS9 peptide,[50] have also been conjugated on the PLA/PLGA

particles surfaces in order to enhance *in vivo* stability and local biodistribution to the targeted cells (**Table 2**).

2.2 Immunological Properties. Table 2 summarizes information showing that antigens carried by PLGA/PLA particles may well induce stronger immune responses than antigens and/or adjuvants alone in solution. This might be due to the ability to sustain the antigen release for prolonged periods of time (**Figure 2C**), hence, extending the antigen exposure time to the immune systems.[18, 37, 41, 46, 48, 53-54] This controlled release property mainly relies on the size, polymer composition, particle porosity, antigen density and the antigen loading method. For example, Langer and Jaklenec *et al.*[37, 48] recently reported the use of PLGA microspheres for single-injection vaccination. To achieve this, the particles were designed to enable a triphasic pulsatile release profile *in vitro*, that is, an initial burst release attributed to the antigen release from the microparticle surfaces (**Figure 2C** (i)), followed by a sustained release of the encapsulated antigen through diffusion (**Figure 2C** (ii)) and finally antigen release dictated by polymer erosion (**Figure 2C** (iii)). The resultant antibody titers *in vivo* were statistically similar to bolus controls, eliminating the need for boosters.[48] Liu *et al.*[41] synthesized PLGA nanoparticles co-encapsulating ovalbumin as antigen and ammonium bicarbonate as pH-responsive antigen-release promoter, respectively. The antigen release can be triggered by the low pH within the phagosome after uptake by DCs. The low pH induced formation of NH_3 and CO_2 from NH_4CO_3 within the nanoparticles and resulted in nanoparticle degradation. Compared to antigen alone, alum salts, and standard PLGA nanoparticles *in vivo*, the pH-responsive PLGA nanoparticles induced immune responses in an orchestrated event and resulted in strongest humoral and cellular immune responses.

In addition to the controlled release properties aforementioned, there are further strategies to enhance and tune immune responses of antigen-encapsulating PLGA/PLA carriers against specific

diseases (**Table 2**). Such an approach is the co-delivery[52, 54] or co-administration[51, 55-56] of pattern recognition receptors (PRRs) ligands, such as binding Toll-like receptors (TLRs), with the particulate vaccine. Whether co-delivery (antigen and PRR ligands are in the same particle) or co-administered (antigen and PRR ligands are in different particles) should be considered with respect to the targeted infectious disease. Zhu *et al.*[52] utilized PLGA nanoparticles to co-encapsulate macrophage-activating lipoprotein (MALP-2) (TLR2 ligand), polyinosine-polycytidylic acid (poly(I:C)) (TLR3 ligand) and cytosine phosphorothioate-guanine (CpG)-oligodeoxynucleotides (ODN) (TLR9 ligand). The nanoparticles were then surface-engineered with methacrylate-based Eudragit FS30D to enable particle transport under low pH and the presence of degrading enzymes in the intestine. The oral co-delivery of the nanoparticles in mice induced colorectal immunity and protected against rectal or vaginal viral challenge with the vaccinia virus vPE-1. Kasturi *et al.*[56] demonstrated that co-administration of PLGA nanoparticles containing ovalbumin, PLGA (OVA), and PLGA nanoparticles containing both MPL (TLR4 ligand) and R837 (TLR7 ligand), PLGA (MPL+R837), can induce stronger IgG1 and IgG2c production in mice than co-delivery using PLGA nanoparticles encapsulating OVA, MPL and R837 altogether, PLGA(OVA+MPL+R837). In addition to OVA, the PLGA nanoparticles encapsulated the protective antigen (PA) from *Bacillus anthracis* or hemagglutinin (HA) from avian influenza H5N1. The co-administration of PLGA (MPLA+R837) plus PLGA (OVA), PLGA (PA) or PLGA (HA) also induced substantial synergistic enhancement of antigen-specific antibody responses. In turn, these antibodies protected mice against lethal avian and swine influenza virus strains and promoted immunity in rhesus macaques against pandemic H1N1 influenza.

Despite the rapid development of polyester-based particulate vaccines, there are limiting factors for their widespread use. The pH of degrading PLGA to lactic acid and glycolic acid within

the PLGA particles can decrease significantly. The local acidification is compounded when microparticles are used, reaching the pH of 1.5,[57] as compared to nanoparticles due to their larger volume and deeper inner core.[58] Such a low pH poses an adverse effect on the structural integrity of the encapsulated antigens, leading to denaturation and aggregation,[32] which in turn lowers the uptake levels of antigen taken up by APCs, hence impairs immune responses.[59] In addition, degradation of the encapsulated antigens can occur during their exposure to organic solvent during polyester particle formation, high temperatures during organic-solvent removal, and/or incompatibility with excipients.

Furthermore, it is challenging to synthesize particles with a high degree of uniformity and batch-to-batch reproducibility considering the multiple production steps (**Figure 2A**), especially when producing particles with multiple surface functionalities as well as when scaling-up the production from lab- to industrial scale. It should also be noted that the inclusion of toxic organic compounds during the chemical syntheses of PLA/PLGA particles requires tedious removal steps down to the levels that are considered to be medically safe. Manufacturing is further complicated by the need to independently produce the individual antigens to be incorporated into the PLA/PLGA particles. To overcome these limitations biologically assembled polyester particles have been considered as particulate vaccine platform. Polyhydroxyalkanoate (PHA) beads produced by engineered bacteria are one of the promising vaccine development approaches for next generation particulate vaccines as discussed below.

Table 2. Synthetic polyester-based nano- and micro-particles for vaccine delivery.

Polyester material	Surface function	Antigen	Disease	Immune responses	Ref
PLA	PEG	Encapsulated M278 peptide	*Chlamydia trachomatis*	Cellular and humoral immune responses were elicited in BALB/c mice. The immune serum inhibited *C. trachomatis* infectivity of macrophages.	18
PLA	–	Encapsulated Vi polysaccharide	*Salmonella typhi*	Immunization of BALB/c mice with the 0.33 μm-particles elicited a stronger anti-Vi IgG responses and higher memory antibody responses than their micron-sized counterparts.	46

PLA	Glycol-chitosan (GCS) or Oleic acid (OA)	Encapsulated recombinant SeM protein, or *S. equi* enzymatic extract protein	*Streptococcus equi*	Mucosal and humoral immune responses were elicited in BALB/c mice. The antigen-loaded particles induced not only IgG and IgG2a sub-types levels, but also IL-2 and IFN-γ cytokines titers.	38, 49
PLA or PLGA	–	Encapsulated tetanus toxoid (TT) protein	Tetanus	Immunization of Wistar rats with physical mixtures of different size particles resulted in enhanced anti-TT antibody titers.	45
PLA	–	Encapsulated tetanus toxoid	Tetanus	Production of anti-tetanus toxoid IgG titres in BALB/c mice from single dose of the PLA microparticle vaccine was comparable to that of the three doses of alum vaccine.	61

PLA	PEG	Encapsulated tetanus toxoid (TT)	Tetanus	The PLA-PEG nanoparticle vaccine mixed with aluminium phosphate adjuvant containing TT showed 80% survival rate in the challenge studies in Swiss albino mice.	62
PLA or PLGA	PEG	Encapsulated inactivated whole-cell *Vibrio cholerae*	*Vibrio cholerae*	Highest IgG, IgA and IgM serum were produced in mice after vaccination with PLA-PEG microparticles.	63
PLA	–	Adsorbed influenza virus split vaccine (A/Anhui/1/2005(H5N1)	H5N1 influenza	PLA microparticle vaccine elicited higher levels of IgM and hemagglutinitation inhibition titers, and induced strong Th1 polarization and antigen-specific $CD8^+$ T cell response.	64

PLA	PEG	Encapsulated recombinant HBsAg protein	HBV	The nanoparticle vaccine based on copolymers PEG-PLA-PEG elicited mucosal secretory antibody (sIgA) and Th1 immune responses in mice as well as Th1/Th2 response with good IgG2a/IgG1 ratio.	65-66
PLA	PEG	Encapsulated CpG from HBV genome (HBV-CpG)	HBV	BALB/c and C57BL/6 mice immunized with the combination of rHBsAg + PLA nanoparticle vaccine induced highest anti-HBs IgG, IgG1 and IgG2a. Also, the formulation induced IFN-α in HBV-carrier mice.	67
PLA	Alginate	Encapsulated recombinant HBsAg protein	HBV	The single-shot microcapsule vaccine exhibited higher cytokine levels of IL-2, IFN-γ-	68

				TNF-α and granzyme B in mice as compared to three-shot alum based vaccine.	
PLA or PLGA	–	Encapsulated recombinant HBsAg protein	HBV	Mucosal vaccination of rats with the PLGA microparticle vaccines elicited higher sIgA, IL-2 and IFN-γ levels than that with the PLA microparticle vaccines.	69
PLA	Chitosan	Encapsulated or adsorbed recombinant HBsAg protein	HBV	PLA-chitosan microparticle with encapsulated HBsAg elicited high levels of IgG, IL-4, IL-6 and IFN-γ in immunized BALB/c mice.	70
PLA	Chitosan (CS), chitosan chloride (CSC), or polyethyle	Adsorbed recombinant HBsAg protein	HBV	The PLA microparticle vaccines elicited high levels of IL-1β, IL-6, TNF-α and IL-12 as compared to alum vaccine and free antigen in mice,	71

	neimine (PEI)			also comparable IgG and IgM levels with the alum vaccine.	
PLA	–	Adsorbed recombinant HIV-1 p24 protein	HIV	The PLA nanoparticle vaccines induced strong antibody levels in mice, rabbits and macaques. Strong cytokine T cells (CTL) responses and a Th1-biased cytokine release (IFN-γ and IL-2) were elicited in mice. Th1 responses were induced in macaques as indicated by high levels of IFN-γ-producing $CD4^+$ T cells and $CD8^+$ T cells.	[72-74]
PLA	–	Adsorbed HIV antigens: p24gag, wild-type Tat or	HIV	All of the PLA nanoparticle vaccines	[75]

		STLA Tat proteins		induced high levels IgG titers in rabbit.	
PLA	–	Co-adsorbed HIV-1 p24 and gp120 proteins	HIV	The PLA nanoparticles containing either individual or both antigens can induce similar levels of antibody titers in mice.	[76]
PLA	–	Conjugated α-GalCer	HIV	The PLA nanoparticle vaccine in mice stimulated NKT cells without inducing anergy and presented by mouse CD11c+ and CD11b+ containing dendritic cells and macrophages, respectively, suitable for use as immunomodulator.	[77]
PLA	–	Adsorbed HIV-1 p24 and encapsulated NOD1 or NOD2 ligands	HIV	The PLA nanoparticle vaccines significantly enhanced p24-specific systemic and mucosal immune responses in mice,	[78-79]

				as well as inducing proliferation of HIV-specific T cells from HIV^+ individuals.	
PLA or PLGA	–	Adsorbed JEV	JEV	Either PLA or PLGA microparticle vaccines induced high survival rate (80%) in JEV challenge in mice.	[80]
PLGA	*Dermatophagoides pteronyssinus*-2 (Der p2)	Encapsulated CpG	House dust mite induced allergy	The PLGA microparticle vaccine enhanced Th1 immunity in mice.	[81]
PLGA	–	Encapsulated a fusion protein ESAT-6 and Ag85B	Tuberculosis	The PLGA microparticle vaccines induced IgG1, IgG2b and IFN-γ titers in mice, but its levels were lower than that of liposome formulations.	[82]
PLGA	–	Encapsulated Spf66 peptide	Malaria	The PLGA microparticles vaccines induced mixed	[83-84]

				Th1/Th2-type immunity: IFN-γ, IgG2a, IgE and IgG1.	
PLGA	Anionic sodium dodecyl sulfate	Adsorbed recombinant p55 gag protein from HIV-1 to the anionic particle surface	HIV	The PLGA microparticle vaccines induced cytotoxic T cell responses following intramuscular immunization in mice.	85
PLGA	gp120 protein from HIV Or Antigen from *Neisseria meningitides* serotype B	Encapsulated monophosphoryl lipid A and synthetic lipopolysaccharide RC529	HIV or *Neisseria meningitides* serotype B	The PLGA microparticle vaccines induced high IgG titer in mice following intraperitonial immunization.	86
PLGA	–	Encapsulated chimeric	*Toxoplasma gondii*	The PLGA microparticle vaccines induced high humoral (IgG) and cellular	87

		recombinant rSAG1/2 protein		(IFN-γ) immune responses in BALB/c mice following intraperitonial immunization. The mice survival rate after challenge study was above 80%.	
PLGA	–	Encapsulated SBm7462 peptide	*Boophilus microplus*	The PLGA microparticle vaccines elicited high IgG titers in BALB/c mice after subcutaneous immunization.	88
PLGA	–	Encapsulated MVFMF2 peptide	–	The PLGA microparticle vaccines induced humoral immunity in rabbits.	89
PLGA	–	Encapsulated tetanus toxoid	Tetanus	The PLGA nano- and microparticle vaccines elicited high IgG titers in rats and guinea pigs following subcutaneous injections.	90-91

PLGA	–	Encapsulated recombinant staphylococcal enterotoxin A	*Staphylococcus aureus*	The PLGA microparticles induced strong humoral responses in mice and increased %survival rate of mice after challenge.	92
PLGA	–	Encapsulated H1N1 influenza virus derived conserved peptide	Swine H1N1 influenza virus	The PLGA nanoparticle vaccines induced virus specific T cell responses and reduced the challenged heterologous load in the airways of pigs.	93
PLGA	–	Encapsulated ovalbumin	–	The PLGA microparticle vaccines induced high IgG1 titer as compared to IgG2a titer – an indicative of Th2 dominated immune response.	94
PLGA	–	Encapsulated recombinant PopB and Pcrh proteins	*Pseudomonas aeroginosa*	The PLGA nanoparticle vaccines can elicit high Th17 responses both in	95

				the lung and in the spleen of mice after intranasal immunization. The challenge studies showed significantly lower bacterial counts in the lungs and improved survival.	
PLGA	–	Encapsulated recombinant HBsAg	HBV	The single-dose PLGA microparticle mixed with aluminium-based vaccines elicited comparable humoral immune response in mice to multiple injection of the aluminium-formulated vaccines.	96-98
PLGA	–	Encapsulated diphtheria toxoid	Diphtheria	The single-shot PLGA microparticle vaccines combined with aluminium-based vaccines induced	99

				high antibody levels in Sprague Dawley rats. Intradermal challenge in rabbits showed comparable neutralization titers to the multiple injections of the aluminium formulations.	
PLGA	–	Encapsulated antigen (ovalbumin, protective antigen from *Bacillus anthracis*, or haemagglutinin), or Toll-like receptor ligands (MPLA and R837)	Avian and swine influenza	Immunization of C57BL/6 or BALB/c mice with a mixture of particles containing antigen and particles containing TLR ligands elicited enhanced antigen-specific, neutralizing antibody responses. Immunization of rhesus macaques with those particles also induced	56

				robust humoral immune responses.	
PLGA	–	Co-encapsulated MPLA and α-GalCer	–	Immunization of BALB/c mice with the particles developed high release of IgG with a balanced IgG_1 and IgG_{2A} responses. Combination of MPLA and α-GalCer within the particles enhanced cellular immune responses.	[36]
PLGA	–	Encapsulated recombinant SAG1	*Toxoplasma gondii* tachyzoite infection	Immunization of BALB/c mice with the particles enhanced and maintained protective SAG1-specific humoral and cell-mediated immune responses.	[53]
PLGA	Chitosan (C) or	Encapsulated recombinant HBsAG	HBV	Immunization of rabbits with GC-PLGA nanoparticles elicited relatively stronger both	[47]

	Glycol-chitosan (GC)			cell-mediated and humoral immune response as compared to that of C-PLGA and PLGA nanoparticles.	
PLGA	–	Encapsulated ovalbumin and CpG	–	C57BL/6 mice vaccinated with the particles elicited high T_H1-associated IgG2b and IgG2c antibody titers, and also T_H2-associated IgG1 antibody titers.	54
PLGA	–	Encapsulated CpG	–	C57BL/6 mice vaccinated with the particles and added MPLA (TLR-4 agonist) in the injectate elicited substantial IFN-γ.	55
PLGA	Lipid bilayer and PEG	Surface-conjugated ovalbumin	–	The lipid bilayer conjugated with PEG and ovalbumin was budded from the PLGA core and formed delaminated-lipid	39

				vesicles with the diameter of 176 nm. BALB/c mice were immunized with the particles and added TLR-4 agonist, MPLA, in the injectate enhanced IgG, IgG_1 and IgG_{2A} titers.	
PLGA	Chitosan conjugated with M cell homing peptide (CKS9)	Encapsulated membrane protein B of *Brachyspira hyodysenteriae* (BmpB)	Swine dysentery	Immunization of BALB/c mice elevated secretory IgA responses in the mucosal tissues and systemic IgG antibody responses, as it induced both T_H1- and T_H2-type responses based on elevated IgG1 and IgG2a titers.	50
PLGA	PEI	Adsorbed DNA-VP2/Eth82, or DNA-chIL-2	IBDV	Vaccination of chickens with the particles containing DNA-VP2	51

				demonstrated immunogenicity for specific-pathogen-free chickens and induced an antibody response. The delayed addition of chIL-2 loaded microparticles improved challenge virus clearance as demonstrated by lower neutralizing antibody titers and reduced IL-4 and IFN-α mRNA expression.	
PLGA	–	Encapsulated inactivated polio vaccine (IPV)	Polio	Single-injection of the particles induced humoral immune responses in Wistar rats.	[37]
PLGA	–	Encapsulated ovalbumin	–	Immunization of C57BL/6 mice with the particles induced greater lymphocyte activation,	[41]

				more antigen-specific $CD8^+$ T cells, stronger IFN-γ and granzyme B capacity, enhanced IgG antibodies, and higher serum IgG2a/IgG1, as well as the improved generation of memory T cells.	

Abbreviations: PLA = poly (lactic acid). PLGA = poly (lactic-co-glycolic acid). PEG = poly (ethylene glycol). PEI = poly(ethyleneimine). GCS = glycolchitosan. OA = oleic acid. CpG = single-stranded DNA that contain cytosine triphosphate deoxynucleotide linked with a guanine triphosphate deoxynucleotide by phosphodiester. STLA Tat = mutant of Tat protein that differs from the wild-type one by four mutations (C27 → S, K51 → T, R55 → L and G79 → A). M278 peptide = derivative of a recombinant *Chlamydia trachomatis* major outer membrane protein. MPLA = derivate of lipid A of the lipopolysaccharide which exerts its adjuvant activity through Toll-Like Receptor (TLR)-4. α-GalCer = a glycolipid presented by CD1d molecules of antigen presenting cells (APCs) which activates iNKT8 cells. ESAT-6 = immunodominant antigens 6-kDa early secretory antigenic target. Ag85B =

antigen 85B. HBV = hepatitis B virus. HIV = human immunodeficiency virus. HPV = human papilloma virus. JEV = Japanese encephalitis virus. HTLV-1 = human T-lymphotrophic virus type 1. IBDV = Infectious bursal disease virus. HBsAg = hepatitis B surface antigen. SAG1 = tachyzoite surface antigen 1. NKT = natural killer T cells. IL = interleukin. IFN = interferon. IgG = immunoglobulin. NOD = nucleotide-binding oligomerization domain (NOD).”

3. POLYHYDROXYALKANOATES (PHAs) AS ANTIGEN CARRIER

3.1 Occurrence and physicochemical properties. PHAs are natural polyesters comprised of (*R*)-3-hydroxyfatty acids monomers[19, 100] (**Figure 3A**) and are synthesized by various bacteria and some archaea as reserve material.[101] PHAs are deposited as inclusion bodies (PHA beads) in the cytoplasm and are composed of a hydrophobic PHA core surrounded by a protein shell (**Figure 3B**).[19, 102] The size of PHA beads varies and generally ranges between 100–500 nm.[19, 103] Poly (3-hydroxybutyric acid) (PHB) is the most common form of PHA.[101, 104] Generally, bacterial cells can produce up to 10 PHA beads in individual cells,[105] and PHA can contribute up to 90% of cellular dry weight.[106-108]

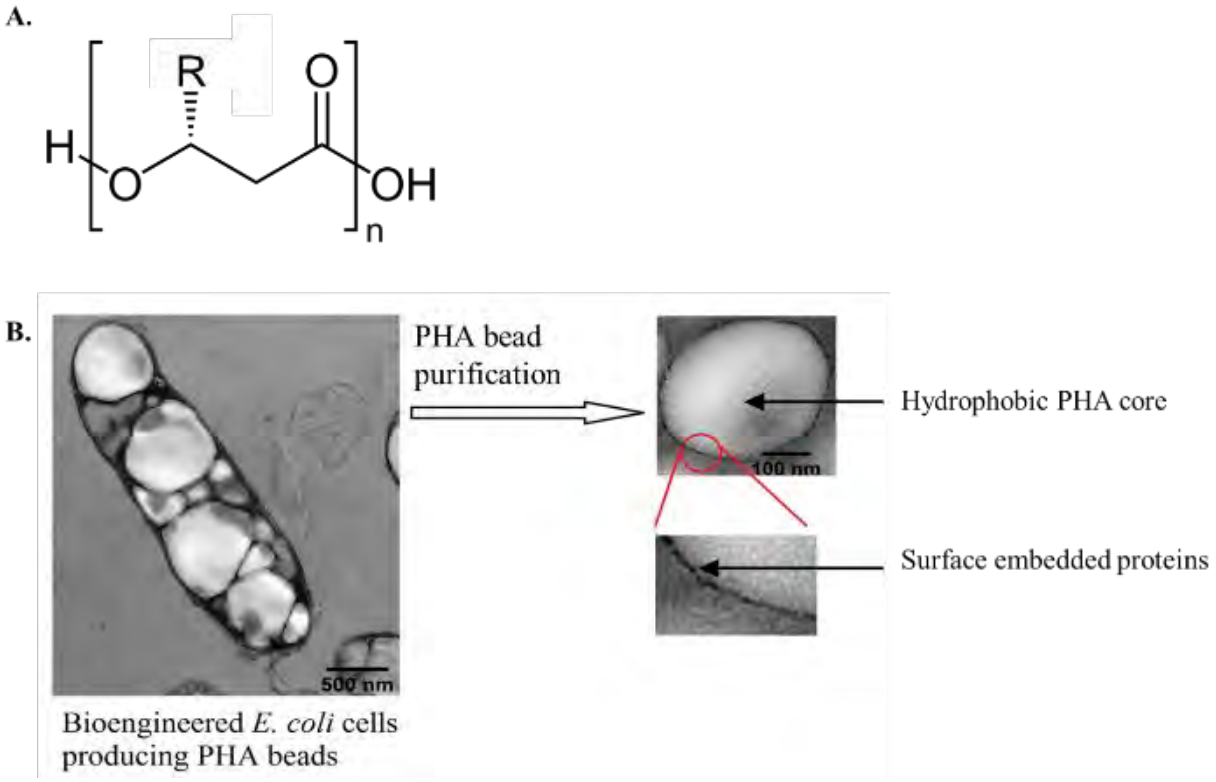

Figure 3. Composition and architecture of PHA beads. **A.** (*R*)-3-hydroxyfatty acid chemical structure. **B.** Bioengineered *E. coli* cell produces PHB beads as visualised by transmission electron microscopy. Note: *n* = number of monomers; R = alkyl side chain.

There are over 150 different building blocks of PHAs known.[102, 109-110] The alkyl side chain (designated as R, **Figure 3A**) determines the identity of the monomer unit of PHAs. The value of *n* varies from 100 to 30,000 and is dependent on the PHA-producing microorganisms and the alkyl side chains (**Figure 3A**).[102, 108-109, 111] PHAs can be classified into three main classes based on the chain length of the fatty acid monomers (**Table 3**): short chain length PHAs (scl-PHAs) which contain 3–5 carbon atoms, medium chain length PHAs (mcl-PHAs) which contain 6–14 carbon atoms, and long chain length PHAs (lcl-PHAs) which contains more than 14 carbon atoms.[108, 112-113] The constituents of PHAs could be homopolymers or copolymers of different hydroxy fatty

acids. Their monomeric composition and the hydroxyl fatty acids chain length greatly determine the material properties.[102, 109-110, 114] For example, scl-PHAs are hard and brittle due to their high degree of crystallinity. However, mcl-PHAs are more elastomeric with low melting temperatures and degree of crystallinity.[19, 108, 115]

Table 3. Monomer units of polyhydroxyalkanoates (PHAs).

X groups	R groups	Type of PHAs	Abbreviations	References
1	-H	Poly(3-hydroxypropionate)	PHP	108, 113
1	-CH3	Poly(3-hydroxybutyrate)	PHB	108, 113
1	-C3H7	Poly(3-hydroxyhexanoate)	PHHx	108
1	-C5H11	Poly(3-hydroxyoctanoate)	PHO	108, 113
1	-C7H15	Poly(3-hydroxydecanoate)	PHD	108, 113
1	-C11H23	Poly(3-hydroxytetradecanoate)	PHTD	108
1	-C15H31	Poly(3-hydroxyoctadecanoate)	PHOD	108
2	-H	Poly(4-hydroxybutyrate)	P4HB	108 113
3	-H	Poly(5-hydroxyvalerate)	P5HV	108, 113

Note: The molecular formula of hydroxy fatty acid monomer is designated as -[CO-(CH2) $_X$-CR-O]-. R and X are alkyl side chains and number of -CH2- respectively.

3.2 Biosynthesis of PHA and self-assembly of PHA beads. The biosynthesis of PHAs involves specific enzymes that catalyse diversion of metabolites such as acetyl-coenzyme A or intermediates of fatty acid metabolism toward synthesis of activated precursors, hydroxy fatty acyl-coenzyme As, as reviewed elsewhere.[19, 116] These activated precursors are polymerized to

PHA by the dimeric PHA synthase (PhaC).[117-118] The synthesized PHA chains impart amphipathic properties mediating self-assemble into PHA beads.[109, 119] The exact self-assembly mechanism of PHA beads is still unclear. However, micelle and budding models are the most widely accepted models for self-assembly of PHA beads (**Figure 4**).[109]

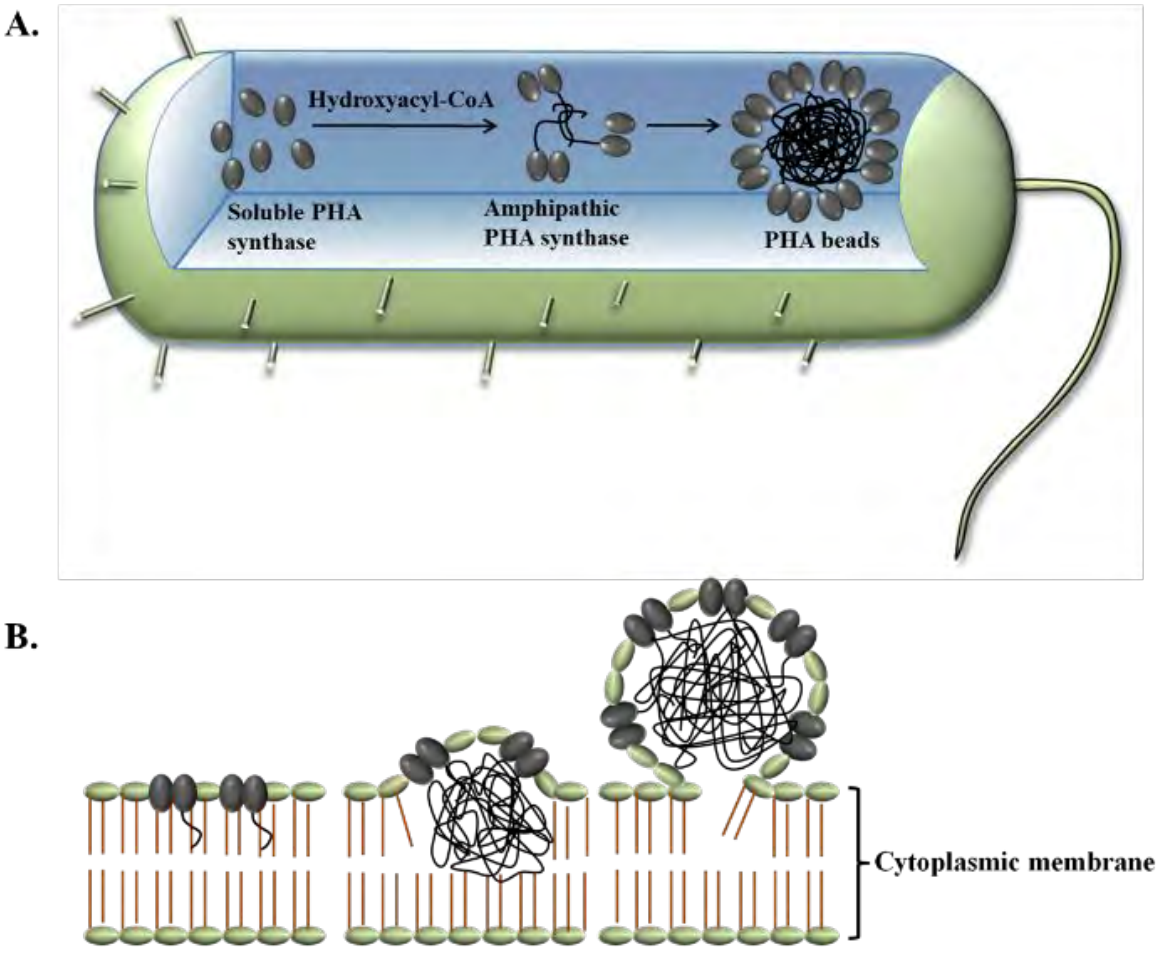

Figure 4. Proposed models of PHA bead self-assembly. **A**. Micelle model. **B**. Budding model.

The exact molecular composition of the outer layer of the PHA beads is not fully understood.[120] It is generally agreed that PHA bead surface contains embedded and PHA associated proteins, such as PHA synthase.[19, 121] However, it is contradictory whether the surface

layer also possesses a phospholipid monolayer.[120, 122] Electron microscopy depicted that PHA beads isolated from *Ralstonia eutropha* were coated with a 4 nm layer,[123] which may indicate it is the phospholipid monolayer as it is about the half size of the cytoplasmic membrane.[120] PHA beads extracted from *Bacillus megaterium* were composed of approximately 97.5% PHAs, 2% proteins, and 0.5% phospholipid.[124] The surface layers of PHA beads isolated from *B. megaterium* and *Bacillus cereus* suggested the presence of a membrane with a thickness of 15 – 20 nm.[125] These observations suggested that phospholipid layers might contribute to the composition of the PHA bead surface.[100, 126] However, the involvement of phospholipid layers is based on *in vitro* findings i.e. could be due to preparation artefacts. Recent studies investigated the formation of phospholipid layers *in vivo*.[120, 127-128] Particularly, electron cryotomography[122] and fluorescence microscopy[120] studies suggested that phospholipid layers are not present. Hence, the previously proposed phospholipid layers could be based on experimental artefacts.

The natural PHA bead formation process was harnessed by engineering bacteria to assembly PHA beads displaying foreign protein functions. This was achieved by protein engineering using PHA bead-associated proteins as anchoring domains for attachment of proteins of interest such as antigens. Besides protein antigen display, chemical conjugation of isolated PHA beads also enabled display of carbohydrate antigens (**Figure 5**).[100, 121] PhaC is of particular interest as anchoring protein because it is covalently bound to the PHA core of the beads; thereby avoiding leakage. Engineered PHA beads have been designed displaying various different protein functions toward medical and industrial applications.[100]

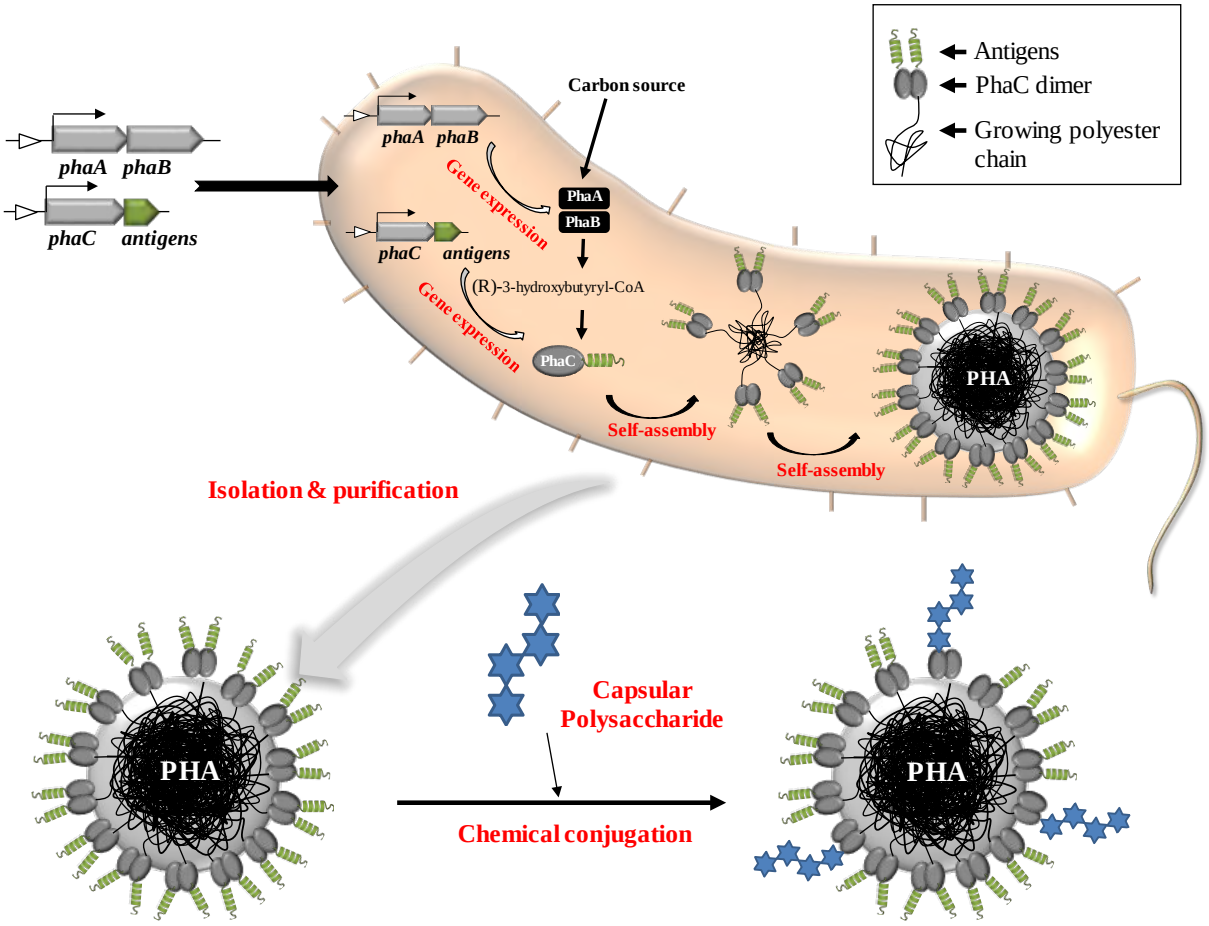

Figure 5. Schematic representation for production of PHA beads displaying foreign and biologically relevant molecules such as antigens.

3.3 Application of PHA beads as particulate vaccines. The utility of engineered PHA beads has been demonstrated for applications in medicine and industry (**Figure 6**) including protein purification, enzyme immobilization, diagnostics and imaging.[100, 121, 129] This review will focus on applications of PHA beads in vaccine development.[17]

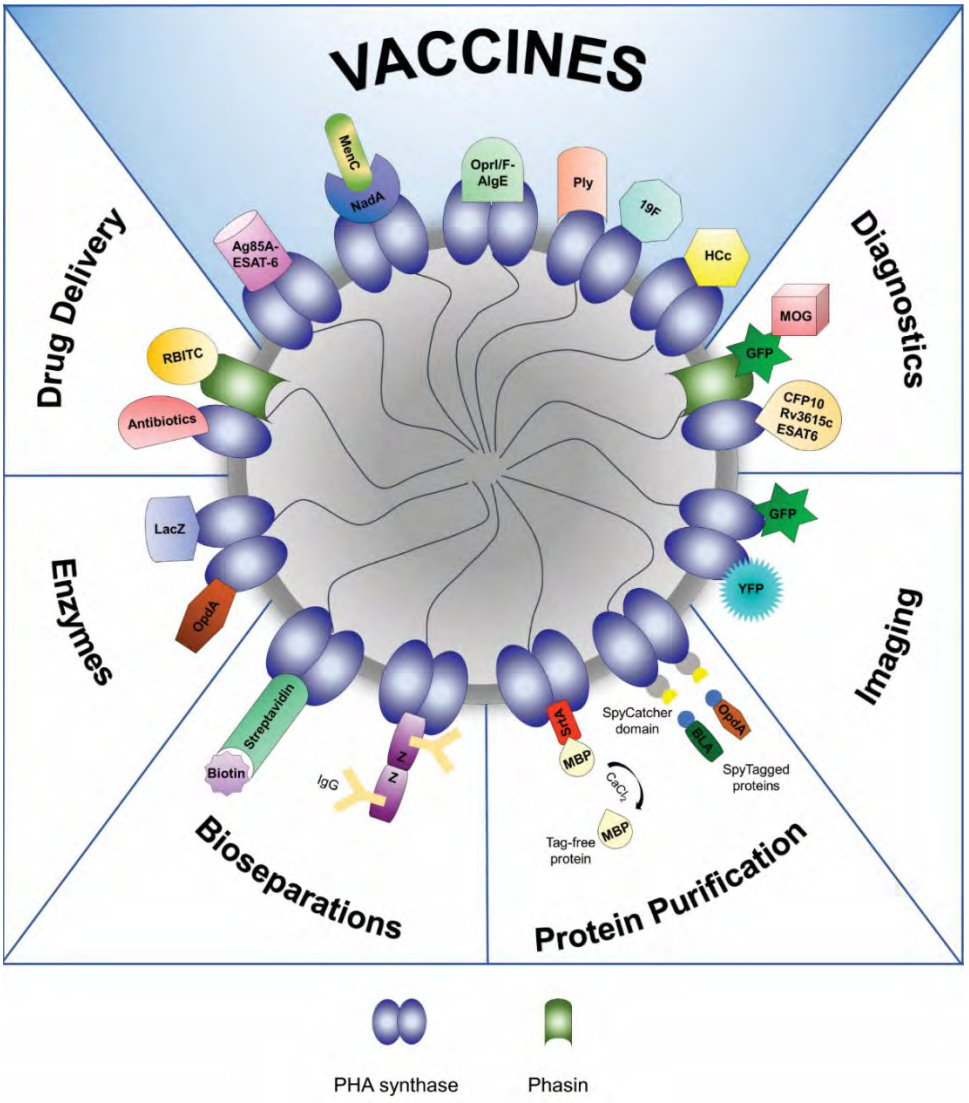

Figure 6. Medical and industrial applications of bioengineered PHA beads. PHA synthase, catalyses synthesis of PHA; Phasin, PHA specific structural protein hydrophobically interacting with PHA.

Since PHB had been approved by US Food and Drug Administration (FDA) for clinical studies, and the 3-hydroxybutyric acid component of PHB is a natural constituent of human blood, this type of PHA has been suggested as safe for use as antigen carrier in vaccine formulations.[130-131] There are a few bioengineered bacterial hosts that have been reported to produce PHB beads

displaying antigens. *Lactococcus lactis*, a Gram-positive and generally regarded as safe (GRAS) food-grade bacterium, was engineered to produce antigen-coated PHA beads. Although *L. lactis* is beneficial due to the absence of lipopolysaccharide (LPS) endotoxins that entail the need of intensive and costly purification processes,[132] the current low production yields prohibit its use as production host. *E. coli* has been the primary host for recombinant PHB bead production due to the availability of extensive genetic tools and high yield PHB production.[121] The drawback of containing endotoxins in *E. coli* can be overcome through innovative engineering of *E. coli* i.e. the use of ClearColi™ BL21(DE3) in which crucial enzymes involved in LPS synthesis pathways were knocked out.[133] Endotoxin-free *E. coli* was the preferred production host in recent studies describing development PHB bead-based particulate vaccines. In a recent study, the natural mcl-PHA producer, *Pseudomonas aeruginosa,* was bioengineered to produce mcl-PHA beads displaying *P. aeruginosa* antigens as particulate vaccines with the advantage of host-derived impurities expanding the antigen repertoirs but with the disadvantage of low PHA yields.[134] This is currently the only study describing the use of mcl-PHA beads as antigen carrier and further investigations are needed to confirm their applicability as particulate vaccines.

Particulate vaccines, such as PHA beads displaying protein or carbohydrate antigens, offer an exciting alternative to soluble subunit vaccines by inducing strong humoral and cell-mediated immune responses which are necessary to control and prevent specific infectious diseases.[135]

Importantly, PHB beads itself are non-immunogenic, i.e. show no carrier suppression, which makes them an ideal antigen carrier system for vaccine uses.[136] Their inherent adjuvant properties may not require addition of adjuvants for induction of strong immune responses.[21] Such adjuvant properties could be due to the small size (<1 μm) of PHB beads that mimic the size of pathogens. Several studies demonstrated the higher immunogenicity of antigen-coated PHA beads compared to their soluble antigen counterparts.[137] The antibody and cytokine profiles in animal trials showed induction of Th1, Th2 and Th17 type immunity. In addition, there were no reports of adverse effects in animal trials, demonstrating the safety of the PHB and mcl-PHA beads. The following paragraphs describe the design of antigen-coated PHA beads for vaccine development against selected infectious diseases.

***Mycobacterium tuberculosis*.** Tuberculosis (TB) is an infectious disease caused by pathogens of the *M. tuberculosis* complex.[138-140] This disease has led to approximately 2 million deaths and 10 million new cases each year globally.[140-141] Live, attenuated *M. bovis*, known as BCG, is the currently available vaccine used to protect against TB. However, it mainly prevents TB in children and cannot provide adequate protection in adults.[139-140] Inaccurate diagnosis is impairing TB disease management worldwide. [140] PHB beads were engineered to display mycobacterial antigens and to serve as vaccine against TB.[136, 142-143] Parlane *et al.*[142] have successfully bioengineered *E. coli* and *L. lactis* to produce spherical PHB beads displaying two immunogenic TB antigens, Ag85A and ESAT6. These PHB beads stimulated strong cellular immune responses and induced protective immunity against mycobacterial infection in mice.[142] Mycobacterial vaccine candidates, Ag85B-TB10.4 (designated H4) and Ag85B-TB10.4-Rv2660c (designated H28) have been considered in clinical trials and were recently attached to PHB beads which induced strong and specific immune responses.[25] The formulated PHB bead-based TB vaccines, i.e. after the

emulsification with adjuvant dimethyldioctadecylammonium bromide, showed a high degree of monodispersity and exhibited positively charged surfaces.[25] H4 and H28 displaying PHB beads showed advantageous immunological properties as they induced strong humoral (IgG1 and IgG2c) and cell-mediated (IFNγ and IL17A) immune responses as opposed to the respective soluble antigens.[25] In the context of TB, antigen-coated PHA beads were also designed to serve as immunodiagnostic reagents for development of a bovine TB diagnostic skin test.[144-145] Immunogenic TB antigens CFP10, ESAT6, Rv3615c, and Rv3020c are present in *M. tuberculosis* and the corresponding orthologue antigens are also present in other pathogenic mycobacterial species, such as *M. bovis*.[146-148] PHB beads displaying CFP10, ESAT6, Rv3615c, and Rv3020c were able to specifically distinguish TB infected from non-infected cattle when used in a skin test. The selected diagnostic antigens are not present in the majority of non-pathogenic environmental mycobacteria and the Bacille Calmette-Guérin vaccine strain[146-150], hence enable specific detection of TB even in a vaccinated background. Display of antigens on PHB beads strongly enhanced immunogenicity of respective antigens resulting in significantly lower antigen concentration required for the TB diagnostic test.[144-145]

***Streptococcus pneumoniae*.** Pneumonia according to UNICEF and WHO is "the forgotten killer of children". *S. pneumoniae* is one of the major causes of this disease worldwide. It is classified as a Gram-positive bacterium, behaving as a commensal in the human nasopharyngeal flora. Others common illnesses caused by *S. pneumoniae* infections are bacteraemia, sinus infections, meningitis and ear infections. In severe cases, the diseases can cause hearing loss, brain damage and death.[151] There are currently several pneumococcal vaccines licensed. These vaccines are based on the pneumococcal capsular polysaccharide (CPS) which has a high diversity resulting in more than 91 serotypes in total with 23 of these known to be virulent. The first pneumococcal

vaccines was a formulation of purified CPS from 23 serotypes (Pneumovax, PPV23), aiming for preventing severe infection caused in adults.[152] The second was the conjugate vaccines where the CPS is covalently linked to the protein carrier (Prevenar 7 and 13® (Wythe) and Synflorix 15® (GSK)).[153] These vaccines protect from 7–15 different serotypes, which are the most common cause of serious infections. The limitation of CPS-based vaccines is that they trigger an immune response serotype dependent and therefore not effective against new emerging serotypes.[154] Consequently, the development of vaccines that contain conserved antigens, providing a serotype-independent protective immunity is urgently needed.

Recently, recombinant PHB beads have been used to generate particulate vaccines against *S. pneumoniae*. In particular, pneumococcal surface protein A (PsaA) [155], a conserved protein found on the bacteria cell surface, which is vital for adhesion to host cells, was displayed on PHB beads (PsaA-PHB beads). Mice immunized with the PsaA-PHB beads were able to produce higher and specific IgG titers in comparison with soluble PsaA.[20] In a related study, PHB beads were coated with pneumolysin (Ply) which is a serotype-independent conserved protein found on the cytoplasm of *S. pneumoniae*.[156] Additionally, the CPS from serotype 19F was chemically conjugated to non-antigen coated PHB beads to assess the antigen carrier properties of the beads. The study showed that mice immunized with the antigen-coated PHB beads generated specific and higher IgG1 levels than mice immunized with soluble Ply or CPS conjugated to the tetanus toxoid (controls). The induced IgG antibodies recognized Ply in whole cell lysates of six different *S. pneumoniae* serotypes. In addition, mice vaccinated with Ply-coated PHB beads trigger the production of IFN-γ and IL17A. Overall these studies showed that PHB beads could effectively be coated with pneumococcal proteins and/or carbohydrates and be used as a particulate vaccine with enhanced immunological properties.[156]

Neisseria meningitidis: Meningitis is a significant cause of disease and mortality in children from developing and developed countries. *N. meningitidis* is an encapsulated Gram-negative bacterium responsible for most of meningitis/meningoencephalitis cases reported worldwide. The disease also leaves around 20% of survivors permanently disabled.[157] There are thirteen different meningococcal serogroups identified based on the CPS chemical structure. Six of them, serogroups A, B, C, X, Y and W, are responsible for almost all meningococcal diseases worldwide.[158-159] There are a few licensed vaccines (Menomune®, and Menactra® (Sanofi Pasteur), Menveo® and 4CMenB Bexsero® (Novartis), Nimenrix® (GSK) and Trumenba® (Pfizer, 2014)) against the various serotypes except for X.[159] The CPS-based vaccines are efficient to prevent infections from the serogroups, except B, which CPS resembles the human oligosaccharides made of sialic acid. To overcome this limitation, serotype-independent subunit vaccines included conserved protein such as *Neisseria* adhesin A (NadA), factor H binding protein (fHbp), *Neisseria* heparin binding antigen (NHBA), Genome-derived antigen (GNA) 2091 and GNA 1030.[13, 160-163] These protein vaccines triggered serogroup independent protective immunity in humans. Despite the contributions of these vaccines to decrease the incidence of meningococcal disease, these bacteria remain one of the leading global causes of meningitis.[159, 164] There is a need for vaccines that offer better and broader protective immunity but that are also more affordable for developing countries where the disease is most prevalent.

To develop a particulate vaccine against *N. meningitidis* infections, vaccine candidate antigen NadA was translationally fused to PhaC to form PHB beads displaying NadA in endotoxin-free *E. coli*.[21] Mice vaccinated with NadA-PhaC beads produced higher bactericidal IgG antibodies than soluble NadA. The NadA-PhaC beads, plain PHB beads (no antigen displayed), soluble NadA and Diphtheria toxoid, were further conjugated with the CPS from *N. meningitidis* serogroup C aiming

to generate a multivalent particulate vaccine. Immunoassays using commercial anti-CPS (MenC) monoclonal antibodies confirmed the successful conjugation of CPS to the carrier proteins. An immunological comparison between all vaccine prototypes after mice immunization showed that vaccination with NadA-coated PHB particles containing the CPS produced a strong, specific Th1/Th2 immune response aligned with bactericidal antibodies associated with protective immunity.[21] Overall, the study showed that multivalent antigen- coated PHB beads could be designed and could serve as particulate vaccine inducing protective immunity against *N. meningitidis*.[21]

***Pseudomonas aeruginosa*:** *P. aeruginosa* is an ubiquitous Gram-negative bacterium and an opportunistic human pathogen that causes life threatening nosocomial infections.[165] It contains the third largest regulatory network described in bacteria, comprising of 690 genes and 1,020 regulatory interactions between their products.[166] This contributes to its ability to adapt to a range of environments including the host during infection.[167] Healthy individuals are not affected by *P. aeruginosa* but it can cause serious infections in immunocompromised individuals, such as hospitalized patients in the intensive care unit (ICU), on mechanical ventilators, individuals with severe burns and wounds as well as individuals suffering from cystic fibrosis (CF).[15, 168] Treatment of *P. aeruginosa* infections remain challenging due to their intrinsic multidrug resistance and ability to acquire further resistance.[169-170] Also, *P. aeruginosa* can produce a biofilm that creates a barrier against the immune response and antibiotic treatment while enhancing resistance through emerging super-resistant cells, which are responsible for the biofilm re-development after antibiotic treatment.[171] Hence, once *P. aeruginosa* established infections, it is difficult to eradicate despite intensive antibiotic therapy.[172] The rise of multidrug-resistant *P. aeruginosa* justifies alternative strategies to effectively combat *P. aeruginosa* infections. Vaccination may be the most

cost-effective choice and can overcome the problem associated with multi-drug resistance of *P. aeruginosa*. Despite several decades of vaccine research and development, no vaccine against *P. aeruginosa* has reached the market, yet. There are several challenges in vaccine development against this pathogen such as *P. aeruginosa* has several virulence factors and multiple mechanisms to cause infections and conserved antigens are difficult to identify due to the high genotypic variability of the pathogen. [173-174] Adjuvants and antigen delivery systems are being explored by several research teams in order to improve vaccine efficiency against *P. aeruginosa*. Recently, a novel particulate vaccine was developed by bioengineering *P. aeruginosa* hijacking its inherent mcl-PHA production system to produce mcl-PHA beads displaying its own antigens: the outer membrane lipoprotein I (OprI), outer membrane protein (OMP) F (OprF), and OMP AlgE (OprI/F-AlgE). OprI and OprF are highly conserved, serotype independent and well-tolerated antigens, and OMP AlgE (alginate pore) is an alternative target that is suggested to be immunogenic and overproduced in the mucoid alginate overproducing variant found in the lung of CF patients.[134] Mouse trials without the addition of adjuvants showed robust induction of Th1-type immunity characterized by antigen-specific production of IFN-γ and IgG2c. The plain PHA beads without the OprI/F-AlgE antigen also produced antigen-specific antibody responses at similar levels to OprI/F-AlgE-PHA beads indicating an immune response to co-purified host cell proteins (HCPs). Also, mice immunized with the OprI/F-AlgE-PHA beads produced significantly higher and specific IgG titers in comparison to the soluble fusion antigens. Opsonophagocytic killing activity mediated by serum antibodies in PHA bead vaccinated groups was significantly greater than in groups vaccinated with soluble antigens. Development of antigen-coated mcl-PHA beads as particulate *P. aeruginosa* vaccine requires further assessment of protective immunity by challenging vaccinated animals with the pathogen.

Hepatitis C Virus (HCV). HCV is a small enveloped positive sense RNA virus. Exposure to HCV has given a 75 to 85% chance of developing chronic infection. The virus then causes hepatitis (inflammation of the liver) resulting in a variety of liver problems ranging from slight cirrhosis to liver cancer. As a result, HCV is noted as one of the primary reason for liver transplants in the USA.[175] There are seven defined genotypes of HCV known as GT1 to 7, which can differ by more than 50% in the genome sequence. These genotypes can again be divided into subtypes such as GT1a and GT1b.[176] There is currently no vaccine available for HCV, and as a result, treatment relies heavily on antiviral drugs.[175] The PHB bead technology was utilized in an attempt to develop a particulate vaccine against HCV.[177-178] This concept was first shown to be possible in 2011 when *L. lactis* was engineered to produce PHB beads coated with the HCV core (HCc) antigen. Mice immunized with the HCc-coated PHB beads showed a diverse cytokine response and high IgG1 antibody response, confirming that the particles were useful as particulate vaccines against the HCV infections.[177] Later, another more in-depth study was conducted using HCV core antigen-coated PHB particles produced in endotoxin-free *E. coli*.[178] Mice were immunized with the antigen-coated particles mixed with recombinant HCV proteins E1, E2 and NS3. After three injections, potent antibody response against E1 and E2 were observed together with functional T-cell immune responses. Mice vaccinated with HCc-coated PHB beads were protected against infection by a recombinant vaccinia virus expressing HCc, demonstrating efficacy of the PHB beads to serve as potential HCV vaccine.

Table 4 summarizes the currently developed PHA bead-based particulate vaccines and their efficacy in preclinical studies. The development of vaccine candidate against very different

pathogens (bacteria, viruses) suggests that the PHA beads offer an extensive design space and might provide a new particulate vaccine platform technology.

Table 4. PHA beads vaccine examples and immune responses

Antigens on PHA beads (production host)	**Pathogen**	**Mode of immune response in mice**	**Protective Immunity in mice**	**Ref**
Ag85A–ESAT-6/ (*E.coli* and *Lactococcus lactis*)	*Mycobacterium tuberculosis*	Th1, Th17	IL17A, IFNγ and TNFα	[142]
Ag85B-TB10.4 (designated H4)/ (ClearColi)	*Mycobacterium tuberculosis*	Th1, Th17, Th2	IL17A, IFNγ and TNFα	[25]
Ag85B-TB10.4-Rv2660c (designated H28)/ (ClearColi)	*Mycobacterium tuberculosis*	Th1, Th17, Th2	IL17A, IFNγ and TNFα	[25]
OprI, OprF, OprI/F-AlgE *Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	Th1, Th2	IL17A, IFNγ, Opsonophagocytic antibodies	[134]

PAO1ΔCΔ8ΔF mutant				
PsaA, Ply, Serotype 19F CPS/ (ClearColi)	*Streptococcus pneumoniae*	Th2	IL17A, IFNγ, Opsonophagocytic antibodies against CPS	20, 156
Serogroup C CPS, NadA, fHbp/ (ClearColi)	*Neisseria meningitidis*	Th1, Th17, Th2	Bactericidal antibodies	21
HCc *Lactococcus lactis*	Hepatitis C virus	Th1, Th17	IL17A, IFNγ	177
Co.120 (Co), E1.340, and NS3/ (ClearColi/ *Lactococcus lactis*)	Hepatitis C virus	Th1	IL17A, IFNγ	178

It is currently unknown how the antigen-coated PHA beads induce an immune response. However, the elicited immune response, i.e. the induction of both a Th1 and Th2 immune responses, suggests uptake by phagocytosis and antigen-processing leading cross-presentation of epitopes on MHC class I and MHC class II molecules (**Figure 7**). As most antigen-coated PHA beads were smaller 200 nm, they might be transported to the lymph nodes and directly stimulate B cells to differentiate into plasma and memory B cells, i.e. induce a specific antibody response.

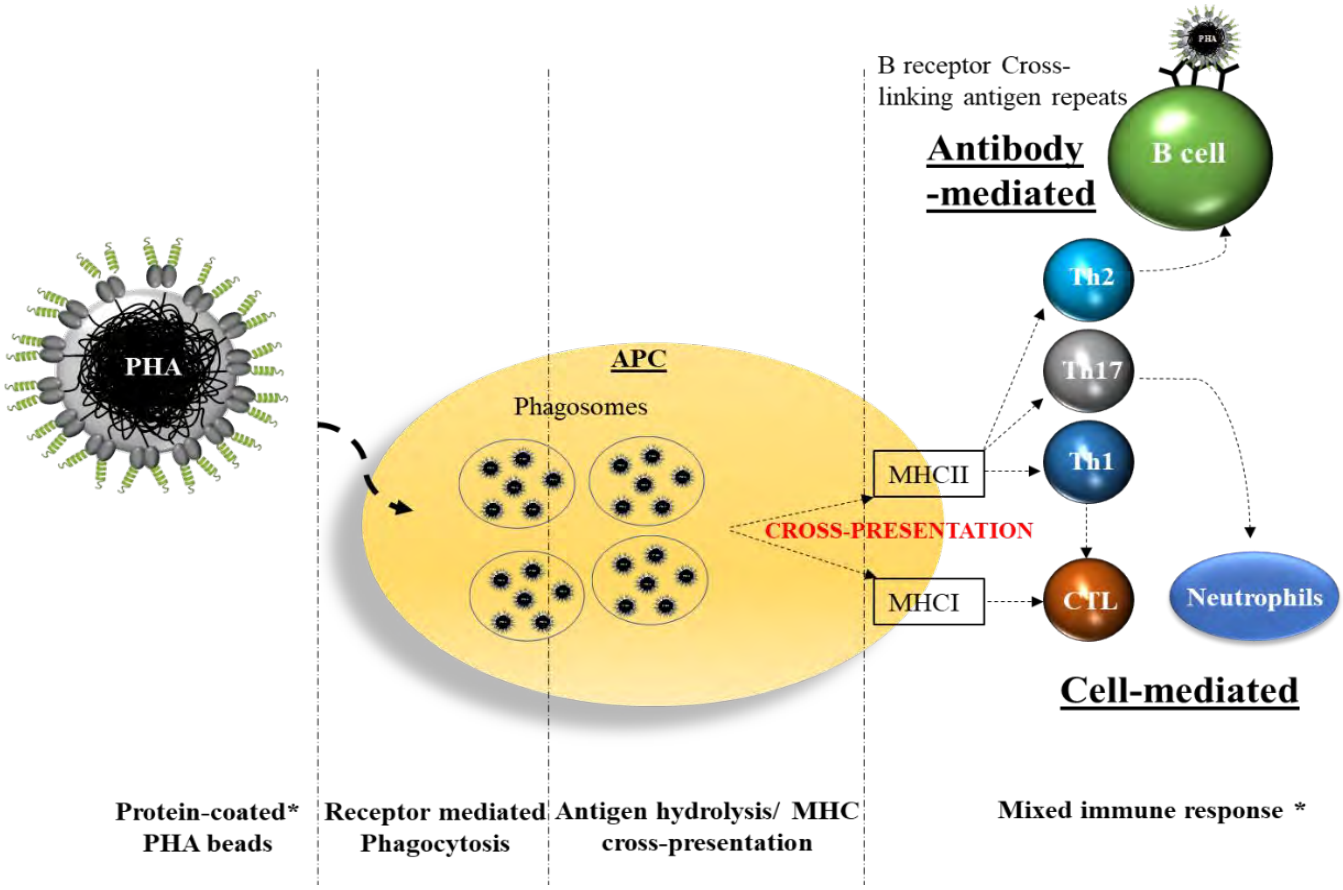

Figure 7. Proposed mode of action of antigen-coated PHA beads. *Examples are shown in **Table 4**.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The development of safe subunit vaccines had a substantial impact on prevention of infectious diseases worldwide.[1] However, these vaccines have been facing limitations such as low immunogenicity, the need for addition of adjuvants, and strain-specific immune responses. The high cost of vaccine production also limits vaccine availability in low-resource settings. A current strategy to overcome subunit vaccine limitation is the formulation of defined antigens into safe and immunogenic particulate vaccines. Particles based on synthetic or natural polyesters were

successfully engineered and processed to serve as antigen carrier systems. Significant efforts have been made to reduce production costs to make vaccines affordable, especially for developing countries.

Incorporation of antigens from pathogens such as e.g. *Salmonella typhi* and Hepatitis B virus into PLGA and PLA nano- and micro-particles either through encapsulation, adsorption or conjugation resulted in particulate vaccines, which induced strong and protective humoral and cellular immune responses in preclinical studies (**Table 2**). However, the inclusion of organic solvents during antigen loading, the use of high temperatures during the organic solvent removal, plus incompatibility between some excipients are detrimental to some antigens. Also, a few examples showed that degradation of PLGA particles lowered the pH dramatically impacting antigen integrity, which is often crucial for an effective immune response. From an industrial point of view, production of these particles can be quite challenging from batch to batch, in particular, to maintain consistent physicochemical properties during the multiple manufacturing steps. The additional costly manufacture of soluble antigen for incorporation into PLA/PLGA particles add a further layer of complexity to the development of particulate vaccines based on synthetic polyester.

Recent advances in the design and manufacturing of biologically assembled antigen-coated PHA beads using integrated synthetic biology and bioprocess engineering enabled one-step production of the carrier PHA beads and selected antigens in engineered bacteria. This one-step approach together with high yields over biomass enabled cost-effective manufacture of particulate vaccines (**Table 5**). In contrast to the PLA/PLGA particle approach, antigens and PHA beads self-assemble in inside the bacterial cell providing a gently environment to retain integrity of antigens (**Table 5**). The self-assembly process also mediates dense and oriented display of antigens at the surface of PHA beads, i.e. enabling enhanced presentation to immune cells for strong induction of

immune responses. A further advantage over PLA/PLGA particles is that the PHB degradation product, 3-hydroxybutyric acid, has a greater *pKa*, i.e. is less acidic and hence less toxic to antigen processing immune cells.

Table 5. Summary of advantages and disadvantages of chemically and biologically synthesised polyesters as antigen carrier systems

	Chemically synthesized polyester (e.g., PLA and PLGA particles)	Biologically synthesized polyester (e.g., PHA particles)
Advantages	• Controllable physicochemical properties (e.g., size, charge, etc.) achieved by tuning particle synthesis parameters, e.g., composition and molecular weight of the precursors, energy shearing, etc.	• Sustainable and scalable manufacturing processes utilizing endotoxin-free microbial cell factories and high-cell-density fermentation technology. • One-step approach, high yields over biomass, cost-effective manufacture particulate vaccine.

	• Controllable antigen release kinetics by controlling the particle hydrophobicity, size, and surface properties.	• Versatile incorporation of single or multiple antigen proteins and/or targeting peptides into the particle surface through genetic engineering.
	• Biodegradable and biocompatible.	• Rapid production of particulate vaccines through simultaneous particle formation and antigen attachment in recombinant cells. • Retained integrity of antigens
		• Biodegradable, biocompatible, • Not toxic 3-hydroxybutyric acid metabolization.

Disadvantages	• Acidic degradation product of PLGA may pose adverse effects on antigen integrity.	• Difficult to control the particle size in the host cells.
	• Tedious and challenging processes to remove toxic organic solvents and/or chemical surfactants without negatively affecting the antigen functionalities.	• Host cell proteins produced along with the particles represent potential impurities requiring additional purification steps.
	• Involve multiple steps to produce particulate vaccines, hence, difficult to control reproducibility and homogeneity of the particles in a large scale.	• The bead production yield and the fusion protein production displayed on the bead surface are largely influenced by the properties of target fusion

		protein (numbers of repeated sequences, charges, etc) and are therefore difficult to be predicted.

Abbreviation: PLA = poly (lactic acid). PLGA = poly (lactic-co-glycolic acid). PHA = polyhydroxyalkanoate.

When comparing PHAs as antigen carrier to the more established virus-like particles (self-assembling viral capsid proteins, reviewed elsewhere)[17] it becomes obvious that PHA particles represent unique particulate vaccine features of self-assembled shell-core (protein-PHA) structures exhibiting enhanced stability, improved design space towards presentation of large and multiple antigens combined with cost-effective manufacture at scale. The intrinsic properties of PHB beads, including biodegradability, biocompatibility, stability and versatile incorporation of antigens through genetic and chemical engineering, make PHA particles a flexible and effective carrier system for vaccine delivery. Extensive preclinical studies demonstrated the performance of antigen-coated PHB beads to induce protective humoral and cellular immune responses (**Table 4**).

Despite the advantageous immunological properties of PHB beads and the promising preclinical results, there is still a need to further explore the design space on how to incorporate single and multiple antigens for enhanced presentation to the immune system or to investigate how

physicochemical properties such as the Zeta potential and the particle size contribute to immunological properties. Therefore, further studies are required to elucidate the influence of these parameters on immune responses including the mode of action, i.e. how do PHB beads induce immune responses. Incorporation of DNA and immunomodulatory molecules, rather than antigenic proteins, peptide combinations or carbohydrates that have been displayed on PHB beads, may expand the antigenic repertoire and enhance the performance of PHB beads as particulate vaccines.

Considering the impressive examples outlined in this Review, it should be clear that bioengineered antigen-coated PHB beads offer a very promising alternative to subunit vaccines, representing next-generation synthetic particulate vaccines based on an unprecedented design space, unique physicochemical and immunological properties.

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The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

B.H.A.R. is co-founder and shareholder of PolyBatics Ltd that commercializes veterinary TB diagnostic products related to the PHA bead technology.

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