



Novel antigen delivery systems

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Abstract

Vaccines represent the most relevant contribution of immunology to human health. However, despite the remarkable success achieved in the past years, many vaccines are still missing in order to fight important human pathologies and to prevent emerging and re-emerging diseases. For these pathogens the known strategies for

making vaccines have been unsuccessful and thus, new avenues should be investigated to overcome the failure of clinical trials and other important issues including safety concerns related to live vaccines or viral vectors, the weak immunogenicity of subunit vaccines and side effects associated with the use of adjuvants. A major hurdle of developing successful and effective vaccines is to design antigen delivery systems in such a way that optimizes antigen presentation and induces broad protective immune responses. Recent advances in vector delivery technologies, immunology, vaccinology and system biology, have led to a deeper understanding of the molecular and cellular mechanisms by which vaccines should stimulate both arms of the adaptive immune responses, offering new strategies of vaccinations. This review is an update of current strategies with respect to live attenuated and inactivated vaccines, DNA vaccines, viral vectors, lipid-based carrier systems such as liposomes and virosomes as well as polymeric nanoparticle vaccines and virus-like particles. In addition, this article will describe our work on a versatile and immunogenic delivery system which we have studied in the past decade and which is derived from a non-pathogenic prokaryotic organism: the "E2 scaffold" of the pyruvate dehydrogenase complex from *Geobacillus stearothermophilus*.

Key words: Vaccines; Antigen display; Delivery systems; E2 scaffold; Immune response

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Core tip: Several promising strategies of vaccination have been proposed over the past years to treat and/or prevent infectious and cancer diseases. These include live attenuated or inactivated viral vaccines, recombinant viral vectors, DNA vaccines, subunit vaccines, nanoparticle carriers, and lipid-based delivery systems such as liposomes and virosomes. Although some of these suffer from certain limitations (*e.g.*, safety concerns, weak immunogenicity, adverse side-effects associated with adjuvants), recent advances in vaccine technology have

provided further insights for guiding vaccine design. Here, we review the current status of antigen delivery systems with emphasis on a versatile and immunogenic vaccine delivery candidate: the "E2 scaffold".

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LIVE ATTENUATED AND INACTIVATED, RECOMBINANT SUBUNIT VACCINES

Currently, the majority of vaccines licensed for human uses include live-attenuated and inactivated or killed vaccines^[1]. They came from disease-causing viruses or bacteria manipulated *in vitro* to reduce or attenuate the pathogenicity, without altering the antigenic properties. Vaccines are manufactured using several different methods^[2]. They may contain live microorganisms attenuated by repeated passages in cell-culture or animal embryos; inactivated (viral) or killed (bacterial) microorganisms that have lost the ability to replicate by physical, chemical or radiation treatments; inactivated toxins and conjugated subunits^[3] (Table 1). Live attenuated vaccines currently available on the market include those against measles, mumps, rubella, varicella, influenza, rotavirus, and smallpox. Most of them are formulated as dry solids. Commercially available killed or inactivated vaccines, toxoids and subunit vaccines include several products, most of them being formulated in liquid dosage forms to treat other diseases, *e.g.*, rabies, meningitis, diphtheria, tetanus, poliomyelitis, *Haemophilus influenzae* type b, pertussis and hepatitis B. These vaccines are able of eliciting both humoral and cell-mediated immune responses^[4]; however, some safety, stability, and efficacy concerns must be considered when developing these vaccines. Live attenuated vaccine can eventually mutate into a more virulent form capable of causing diseases^[5], whereas inactivated or killed vaccines and protein subunit vaccines generally generate weak immune responses often requiring the use of adjuvants^[6]. Many live attenuated vaccines are capable of eliciting virus-specific T cell and B cell responses and long-term immunity by mimicking the natural infection, and therefore they usually do not require the use of adjuvants. However, for some viruses vaccines have been very difficult to develop, due to the absence of tissue culture systems that allow for efficient propagation and production in a scalable setting. They tend to be more difficult and expensive to store and to distribute, since viability must be maintained, often requiring formulation approaches for stabilization^[7]. On the other hand, killed/inactivated vaccines have a number of disadvantages. The major challenge is that since cells are never infected with the live microbe, these vaccines are generally not effective at eliciting a full adaptive immune response. They do not

give rise to pathogen-specific cytotoxic T cells, thus often requiring multiple booster shots and co-administration with adjuvants to increase antigenicity and to create long-term immunity, with subsequent local reactions at the vaccine site. However, for the absence of living pathogens these types of vaccines are usually safe compared to live attenuated vaccines.

Overall, these technologies have allowed to achieve the successes of vaccinology in the last century and to produce the vaccine formulations available on the market. However, many new vaccines are needed and for them new strategies have to be found^[8]. In this context, the development of novel delivery technologies aimed to design safer and more effective vaccines is a relevant topic.

DNA VACCINES

DNA vaccines have emerged as a safer alternative to live and inactivated vaccines for treating human and animal infections, allergy, autoimmune disorders and cancer diseases^[9]. They exhibit several advantages over traditional strategies in terms of safety, stability, ease of manufacturing, and immunogenicity (Table 1). As DNA-based plasmid vaccines are non-live, non-replicating, non-spreading vaccines, there is a little or no risk of mutation or reversion to the virulent form as with viral vectors, therefore raising fewer safety concerns. They are easy to manufacture and to manipulate compared with live attenuated vaccines, and the DNA product is highly stable and easily stored, without requiring refrigeration procedures. DNA vaccines can activate innate immunity and both arms of the adaptive immune response without inducing anti-vector antibodies unlike viral vector particles, thus being theoretically suitable for repeated booster shots. Furthermore, recent innovations in plasmid host strain and vector engineering increased plasmid manufacturing quality and yield, transgene expression levels, transfection efficiency, for a safer and more effective gene platform compared to first generation vectors^[10,11]. Essentially, plasmid DNA vaccines consist of purified vectors that combine an eukaryotic region - which includes a strong enhancer/promoter for the expression of transgene coding for antigenic/therapeutic proteins or peptides in mammalian cells and the transcript termination/polyadenylation (poly A) sequence for mRNA transcript stabilization - with a prokaryotic region that provides selection and propagation in host bacteria. Although the exact mechanism by which DNA vaccines work still remains unclear, recent advances have provided a deeper understanding of the molecular and immunological mechanisms of action of these vectors^[12-14]. Generally, once the DNA plasmid is administered *via* intradermal, intravenous, intraperitoneal, subcutaneous, nasal or intramuscular route, the plasmid is internalized into the host cells (myocytes and antigen-presenting cells), it translocates to the cellular nucleus where the host cellular machinery initiates the transgene transcription followed by the cytoplasmic translation of the transgene into protein. Plasmid-encoded proteins may be processed in transfected

Table 1 Overview of the different vaccine formulations

Vaccine type	Description	Advantages	Disadvantages	Immunogenicity	Examples
Live attenuated vaccines	Living weakened microbes that generally show reduced pathogenicity	Induce a protective immune response by activating both B and T cell responses; induce long-term immunity; do not require adjuvants; unable to spread and cause infection	They can revert towards virulent forms or can be insufficiently attenuated for immunosuppressed individuals with risk of infection; difficult to produce in a scalable setting; heat-labile; quality and safety requirements	Humoral and cytotoxic immune responses	Smallpox; yellow fever; rabies; measles; mumps; rubella; typhoid; influenza; rotavirus; varicella
Killed/inactivated vaccines	Bacteria (killed vaccines) or viruses (inactivated vaccines) inactivated by chemical or physical treatments	Due to the absence of living pathogens they do not revert towards virulent forms and can be used in immunodeficient hosts; not heat-labile	Repeated booster shots and adjuvants (with subsequent local reactions at the vaccine site) are required to optimally trigger the adaptive immune system and generate long-term immunity; do not give rise to cytotoxic T cells; poor induction of mucosal immunity; difficult to produce in a scalable setting; quality and safety requirements	Humoral immunity	Diphtheria; tetanus; pertussis; haemophilus influenzae type b; poliomyelitis; rabies; meningitis; Japanese encephalitis; cholera; hepatitis A; hepatitis B
Toxoids vaccines	Purified exotoxins chemically inactivated into toxoids that retain the ability to induce toxin-neutralizing antibodies	Safe and stable. There is no possibility of reversion to pathogenicity or spread of live microbe to other animals	Poorly immunogenic; need adjuvants and large amounts or multiple doses to ensure efficient activation of the adaptive immune response and generation of long-last immunity; local reactions at vaccine site	B cell activation (T cell dependent)	Diphtheria, tetanus, and pertussis toxoids; acellular pertussis vaccines; anthrax secreted proteins
Subunit/polysaccharide vaccines	Antigenic components of pathogens: partly or fully purified protein antigens or capsular polysaccharides	Can be chemically linked to protein carrier	Variable degree of immunogenicity; need adjuvants (and often multiple doses); frequent local reactions at the injection site	T-dependent and/or T-independent immune responses	Hepatitis B and Haemophilus influenzae type b; influenza; meningococcus, pneumococcus, and Haemophilus influenzae type B polysaccharides
Plasmid DNA	Genetically engineered vectors expressing antigens of interest	Inability to revert to pathogenic forms; activation of innate and adaptive immune responses; highly stable; easy storage and transport; large-scale production; optimization of plasmids and transcript is possible	Not-useful for non-protein immunogens; lower immunogenicity in human compared to mice; low transfection efficiency	Activation of antigen-specific B cells, CD4+ and CD8+ T cells	Infectious haematopoietic necrosis virus; West Nile virus; melanoma; growth hormone releasing hormone
Vectored vaccines	Live recombinant viral and bacterial vectors expressing heterologous antigens	Ability to induce specific humoral and cellular immune responses; high transduction efficiency; highly effective in dividing and non-dividing cells; production of high levels of antigens inside target cells; sustained gene expression; vector itself can provide an adjuvant effect	High expense; toxic side effects; limits on transgene size; potential for insertional mutagenesis; anti-vector immunity; difficult to manufacture and store	B cell, CD4+ and cytotoxic CD8+ T cell activation	Adenovirus; adeno-associated virus; retrovirus; lentivirus; Herpes simplex virus; <i>Salmonella</i>
Nanoparticles	Nano-scale size materials made of polymers, proteins or lipids used as carrier systems (e.g., PLGA, liposomes, virosomes, Virus-like particles)	Ability to induce humoral and cellular immune responses; increased antigen uptake, processing and presentation; controlled/sustained release of vaccine target; depot effect; targeted delivery; adjuvanticity; high encapsulation; improved cargo bioavailability; transport efficiency; enhanced permeability; biodegradability and biocompatibility	Challenges in vaccine formulation, production, stabilization. Immunotoxicity can occur	B-cell, CD4+ and cytotoxic T-cell responses	Hepatitis A virus; influenza; human papilloma virus; hepatitis B virus; hepatitis E virus

somatic cells *via* the TAP-dependent, endogenous pathway for the presentation on MHC class I molecules, whereas soluble/secreted plasmid product may simultaneously gain access to the major histocompatibility complex (MHC) class II exogenous pathway in phagocytic cells, for the activation of B cells, CD4+ and CD8+ T lymphocytes^[15]. Many reports emphasized on the ability of DNA vaccines to induce immune responses against a variety of infectious agents and cancers in preclinical animal models and more recently in clinical trials^[16,17]. Until now, four animal DNA products have been licensed for veterinary uses, demonstrating the well tolerated and safety profile of DNA vaccination. Although there are no US/FDA approved DNA vaccine for human uses, several DNA delivery strategies have been developed and improved in order to increase DNA vaccine performance, including the use of adjuvant plasmids expressing immunostimulatory molecules, such as costimulatory molecules, signaling proteins, cytokine, and chemokines^[18]. In addition, the use of mixed vaccines in prime-boost immunization strategies or in simultaneous delivery approaches resulted in an improved immunogenicity in several preclinical models against different pathogens such as HIV-1^[19]. Genetically engineered DNA can be administered by different methods following different routes, including physical approaches and viral and non-viral delivery systems^[20]. However, so far in human application the efficiency of DNA vaccination has not been so encouraging^[21].

GENE DELIVERY SYSTEMS: RECOMBINANT VIRAL AND BACTERIAL VECTORS

A huge amount of delivery systems based on recombinant viruses have emerged recently and have been widely employed as highly evolved natural vehicles for gene therapy and for vaccine purposes^[22]. Viral-based delivery systems consist of genetically engineered replication-defective viruses carrying a therapeutic gene expression cassette cloned into the viral backbone (Table 1). Viral vaccine vector systems, such as adenovirus (type 2 and 5), adeno-associated virus, retrovirus, lentivirus, poxvirus, alphavirus, herpes simplex virus (HSV), offer several potential advantages over traditional vaccines, even though each of them show some limitations and side effects^[23,24]. Viral vectors can produce high levels of antigens directly within the host cells; they can efficiently deliver antigens to specific subsets of immune cells [such as antigen-presenting cells (APCs)] and potentially act as adjuvant. They can be administered in different combination with other vaccines resulting in enhanced immune responses. However, some issues must be taken into consideration when using viral vectors for vaccination, including potential integration, transcriptional activation of oncogenes, pre-existing immunity against the viral vector, and limitations in transgenic capacity size. Several recombinant viral vectors, both RNA and DNA viruses, have been used and widely investigated as vaccines being able to express

the antigenic/therapeutic protein *in vivo* and to stimulate potent specific humoral and cellular immune responses^[25]. RNA viral vectors, such as retrovirus and lentivirus, allow long-term expression of the transgene, while DNA viral vectors allow expression in episomal form. Viral vectors based on adenovirus, adeno-associated virus, retrovirus, lentivirus and HSV represent those currently used in clinical trials, with adenovirus being the most commonly used, whereas others are under development^[26].

More recently, vaccine based on alphavirus vector has been considered a particular attractive option. All alphavirus vectors take advantage of extremely efficient RNA replication resulting in almost 200000 RNA copies from each RNA template^[27].

Although replication-deficient particles provide a high level of safety, there is still a marginal risk of the generation of replication-competent particles through non-homologous recombination. To minimize this risk, split helper vector systems with capsid and envelope genes expressed from separate vectors have been produced^[28]. Furthermore, the potential of alphavirus causing epidemics has raised additional concern. Regarding efficiency, recent alphavirus-based vaccines have been subjected to clinical trials. Disappointingly, no clinical benefit was found, indicating that these types of vaccines require further optimization.

In addition to viral vectors, recombinant bacterial carriers, derived from lactic acid bacteria, *Salmonella* and *L. monocytogenes* strains, have been used extensively as delivery systems being able to stimulate both systemic and mucosal immune responses^[29,30].

NANOPARTICLE DELIVERY SYSTEMS

Nanoparticle delivery systems offer several advantages over traditional vaccines. Due to their physicochemical characteristics - nanoparticle size, surface charge, biomaterials composition, hydrophobicity/hydrophilicity - and immunostimulatory properties, nanoparticles-based formulations have extensively been investigated as vaccine and drug delivery systems, adjuvants, nucleic acid delivery platforms, and nanocarriers for imaging approaches^[31-34]. Nanoparticle systems can be designed to optimally present antigens in their native conformations to the immune system in controlled, slow release formulations promoting their targeting to specific immune populations with attachment of targeting moiety. They can be engineered to improve antigenicity of the delivered antigens and thus acting as adjuvants. Moreover, by co-delivering antigen and adjuvant to the same antigen presenting cells, these nanocarriers can enhance immunogenicity of vaccines. The antigen multimeric display on the surface of some nanoparticle systems allows cross-linking of the B cell receptor, leading to an enhanced antibody response. Moreover, some of these nanoparticles can be designed for promoting the cytosolic delivery of antigens, enhancing cross-presentation *via* MHC-I pathway and thus leading to cytotoxic T-cell responses. In addition to increased antigen uptake, processing and presentation, nanocarriers also

offer the opportunity to encapsulate or entrap a variety of compounds, preventing their degradation, improving their solubility and half-life, providing site-specific targeting and a sustained release of compounds. Most of nanocarriers are biodegradable, biocompatible for different routes of administration (parenteral and non-parenteral administrations), exhibits low toxicity and stability, and they are able to induce strong humoral and cellular immune responses without anti-vector immunity^[35-37].

Nanoparticle delivery systems comprise a wide variety of nano-scale size materials (< 1 µm) including solid particulate delivery systems and emulsion delivery systems. Solid nanoparticles include synthetic or biodegradable polymers (nanospheres and nanocapsules) - such as poly(lactic-co-glycolic acid) (PLGA), chitosan, hydrogel capsules, poly(phosphazenes), polyanhydrides, poly(alkylcyanoacrylate) (PACA) and poly(methyl methacrylate) (PMMA) nanoparticles - solid lipid nanoparticles (SLNs), liposomal delivery systems, virosomes, immune stimulating complexes (ISCs), virus-like particles (VLPs), non-degradable nanoparticles, colloidal iron-based preparations and many others, while emulsions include heterogeneous liquid systems suitable for the entrapment of hydrophobic drugs, such as nanoemulsions and nanoliposomes (details in^[31-33,35]). Some formulations have proceeded to clinical trials and are commercially available, whereas many others are under preclinical development^[31].

POLYMERIC NANOPARTICLES

Polymer-based nanoparticle delivery systems (polymeric nanoparticles, polymeric micelles, dendrimers) have emerged as promising and innovative candidates to diagnose, monitor, treat, and prevent infectious, inflammatory and cancer diseases due to their excellent features - including biocompatibility and biodegradability, enhanced permeability, stability, low toxicity, improved cargo bioavailability, controlled/sustained release of vaccine targets, depot effect, high encapsulation and transport efficiency, targeted delivery^[38]. Polymeric nanoparticles (NPs) consist of polymeric colloidal nanoparticles prepared from biodegradable and biocompatible, natural or synthetic polymers, ranging in sizes from 10 nm to 1 µm. A wide variety of diagnostic and therapeutic compounds (such as hydrophilic and hydrophobic drugs, proteins, peptides, nucleic acids, biological macromolecules) can be entrapped or encapsulated within the polymeric matrix with good efficacy, protecting them from enzymatic degradation and thus improving their bioavailability, or adsorbed or chemically conjugated on their surface for antigen and targeted delivery. NPs can be made from many different polymer types including natural or synthetic polymers such as poly-D,L-lactide-co-glycolide (PLGA), polylactic acid (PLA), poly-ε-caprolactone (PCL), chitosan, gelatin, poly-alkyl-cyano-acrylates (PAC), gamma polyglutamic acid (γ-PGA), hyaluronan [or hyaluronic acid (HA)]^[34,35,39]. However, the most commonly studied polymers for parenteral and mucosal drug and antigen

delivery are biodegradable and biocompatible synthetic polymers - such as PLGA and PLA - since they provide biological compatibility with less toxicity^[40]. According to the structural organization, biodegradable nanoparticles are usually distinguished in nanospheres, where molecules are homogeneously dispersed, adsorbed or dissolved within the polymeric matrix, and nanocapsules, where a polymeric wall surrounds a vesicular core containing the agent of interest. Several methods have been developed to produce structurally stable optimized NPs, including encapsulation and adsorption of drugs, proteins, and nucleic acids^[39,40]. NPs can be prepared by polymerization of monomers following emulsion-based methods or by dispersion of polymers following nanoprecipitation (solvent displacement), salting out, or solvent evaporation methods^[39,40]. A huge amount of preclinical studies have emphasized the utility of PLGA/PLA-based nanoparticles as drug and antigen delivery systems. It has been reported that PLGA/PLA-based nanocarriers, carrying immunostimulatory molecules and/or vaccine antigens, confer antigenicity and immunogenicity to a large variety of antigens, being able to increase antigen-specific humoral and cellular immune responses^[40]. In addition, PLGA-based nanoparticles are able to specifically deliver vaccine compounds to antigen-presenting cells such as dendritic cells, enhancing cross-presentation and thus promoting CTL responses^[41]. PLGA nanoparticles are frequently used for encapsulating and successfully delivering a variety of anticancer drugs (reviewed in^[39]). Problems of stability, cytotoxicity and conservation may represent constraints that require further optimized formulations^[42].

LIPID-BASED ANTIGEN DELIVERY SYSTEMS: THE LIPOSOME FAMILY

Liposomal carrier systems

Liposomes and liposomal-based delivery systems represent a promising technology to deliver a variety of compounds to target sites. Various kinds of lipid vesicles belong to the liposome family, including LPD (liposomes-protamine-DNA complexes), polymerized targeted-liposomes, PEGylated liposomes, archaeosomes, ISCOMs (immune stimulating complex), virosomes, niosomes and many other, which are classified according to their structures, composition, and preparation^[43]. Essentially, they are spherical, uni- or multi-lamellar, nano or micro-sized vesicles composed of a phospholipid bilayer capable of encapsulating or incorporating bioactive molecules. Hydrophilic water-soluble compounds can be entrapped within the aqueous hollow cavity, whereas hydrophobic molecules can be intercalated into or attached on the phospholipid bilayer. Several methods of liposome preparation techniques including manufacturing process and process controls have been developed, although all the methods share a common general procedure^[43]. Liposome formulations with optimized properties - such as high stability, long blood circulation half-life (GM glycolipid

or PEG polymer-coated liposomes), enhanced target efficiency and activity (immunoliposomes), controllable and prolonged release properties, low toxicity, improved adjuvant and immunostimulatory properties - can be achieved by modulating the lipid membrane composition (neutral, anionic, and cationic lipid species), the liposome size, the net charge and the hydrophilicity of the liposomal surface, and/or by encapsulating additional adjuvants ("conventional" and second-generation liposomes, the stealth technology^[44-46]). Since liposomes were first described in 1960, these nanoparticulate carriers were investigated for various purposes - including industrial, pharmaceutical, clinical and therapeutic applications (from vaccination to cancer treatment, gene therapy with cationic liposomes, and diagnostic imaging), due to their adjuvant activity, immunostimulatory properties, safety, biodegradability, and tolerability, following intramuscular, subcutaneous, oral, or intravenous administrations^[44,46]. Many reports emphasized on the utility of liposomes as adjuvanted vaccine candidates and drug delivery systems, due to their ability to induce specific immune responses toward the encapsulated or surface-attached antigen, and to treat various diseases, including cancers, infectious, and auto-immunity (reviewed in^[46]). Currently, several liposomal formulations are commercially available and clinically approved^[44-46].

Virosomes as vaccine and delivery system

In 1975, using preformed liposomes, Almeida *et al.*^[47] first generated lipid vesicles (named virosomes) containing the envelope proteins, Hemagglutinin and Neuraminidase, purified from influenza virus. Essentially, virosomes are lipid-based semi-synthetic complexes (approximately 150-200 nm in diameter) comprising of functional viral envelope glycoproteins protruding from the surface of a phospholipid bilayer membrane. These lipid vesicles closely mimic the native viral envelope but are devoid of the nucleocapsid including the viral genome of the parenteral virus they are derived from, thus they are not able to replicate. Functionally reconstituted glycoproteins retain the receptor binding property and the pH-dependent membrane fusion activities of the native viral proteins. These functional characteristics have been exploited in the design of vaccine adjuvant and carrier system to deliver molecules^[48-51]. After the first description of influenza virosomes, different envelope glycoproteins have been reconstituted to produce virosomes with full biological fusion activity, through detergent solubilization and detergent removal procedures^[48,51,52]. Several methods have been described to manufacture virosomes, including antigen loading, and DNA-binding to cationic-virosomes for gene delivery. Essentially, these procedures rely on the use of lipids (egg-derived, purified viral membrane lipids: first-generation virosomes or synthetic phospholipids: second-generation vaccines), envelope proteins (plant-expressed or purified from the inactivated parental virus), and heterologous compounds (details in^[51]). A variety of compounds,

including antigens, nucleic acids, drug molecules, cancer chemotherapeutic agents, tumor-associated antigen, antibody (targeted-virosomes), can be encapsulated within the aqueous lumen of virosomes, and adsorbed or cross-linked to their surface^[53]. Virosomes are qualified for administration *via* different routes (intramuscular, intradermal, intranasal, vaginal routes); they ensure a rapid uptake of the delivered molecule by immune cells (APCs and B cells), for MHC class I and class II presentation. Heterologous antigens exposed on the surface primarily evoke humoral immune responses, while the encapsulation approach give rise to CTL responses; thus, virosomes activate both arms of the adaptive immune response^[48]. In addition, due to the presence of the antigenic viral glycoproteins, virosomes can be used as vaccine adjuvant and carrier system to induce immune responses against the viral envelope and the unrelated antigen, being suitable for prophylactic and therapeutic immunizations^[46,54]. First-generation virosomes and virosomal adjuvanted formulations are currently applied in commercial vaccines (Hepatitis A vaccines: Epaxal and Epaxal junior; Influenza vaccines: Inflexal V and FluAd). Moreover, several promising virosome vaccine candidates (Malaria, HCV, breast cancer, HIV, Candida vaccines) are currently in preclinical and in clinical development^[51].

VIRUS-LIKE PARTICLE DELIVERY TECHNOLOGY

Virus like particles (VLPs), also called pseudovirions, are composed of one or more viral structural proteins (capsid and/or envelope proteins) that retain the ability to self-assemble into multimeric structures (or subviral particles) when expressed *in vitro* using recombinant protein expression systems - including plant, yeast, bacteria, viral vectors, insect cells (baculovirus technology), and mammalian cells^[55-57]. They form highly organized monomeric or oligomeric structures with a well-defined geometry (usually icosahedral or rod-like) and diameter ranging approximately from 20 to 120 nm, closely mimicking the native virus but unable to replicate since they lack the infectious viral genome. Thus, VLP-based vaccines offer a safer and more appealing alternative to live, attenuated and inactivated vaccination strategies. Intrinsic characteristics of VLP - such as the particulate nature and the size, the highly ordered and repetitive structure, the charge surface - coupled with immunogenic properties and adjuvanticity, make them particularly attractive as vaccine candidates, targeted drug carriers and antigen delivery systems for prophylactic and therapeutic applications: from vaccination against viral, bacterial, parasitic and fungal infections to gene therapy, immunotherapy against a variety of chronic diseases, including allergies, neurodegenerative and autoimmune disorders, cancers (VLPs targeting self-antigens)^[55,57]. Particulate delivery systems similar in size and geometry to pathogens, such as VLPs, are efficiently uptaken by professional antigen-presenting cells for both MHC class

I and II presentation; they efficiently reach lymphoid organs where they can directly interact with immune cells. Most importantly, the highly repetitive surface structures (PAMPs) can induce maturation of antigen-presenting cells (DCs, B cells) by triggering TLRs and cross-linking B cell receptors. These properties increase the ability of VLPs to stimulate strong B and T cell-mediated immune responses^[58]. Subviral particles, genetically engineered plant viruses, insect-derived virus-like particles, are suitable as presentation scaffold and adjuvant platform for multimeric display of foreign antigens in a correct, ordered and highly repetitive three-dimensional configuration, to optimally present B and T-cell epitopes and activate immune cells. Antigenic determinants (continuous or conformational immunological epitopes) can be incorporated into adequate permissive insertion sites at high density per particle by genetic fusion (chimeric VLPs) or by *in vitro* chemical conjugation (conjugated VLPs), without compromising the correct folding of VLPs, leading to optimized formulations^[59]. Currently, several VLP-based vaccine candidates for human diseases are under clinical development including those directed against Influenza A virus, Norwalk virus, Ebola and Marburg viruses, Hepatitis C virus, HIV and Malaria. To date, VLP-based vaccines for human papilloma virus (HPV), hepatitis B virus (HBV), and hepatitis E virus (HEV) have already been licensed and are commercially available worldwide^[59].

The current HPV vaccines are based on virus-like particles (VLPs). The first HPV vaccine to be licensed was Gardasil (Merck and Co., Inc.) - approved by the FDA in 2006 - a quadrivalent (HPV types 6, 11, 16 and 18) VLP-based vaccine made of the recombinant HPV major capsid protein L1 produced in *S. cerevisiae*. In 2009 the FDA approved Cervarix, a bivalent (HPV types 16 and 18) vaccine commercialized by GlaxoSmithKline (GSK). Both the HPV VLP vaccines have shown to have a sustained prophylactic efficacy in clinical trials against infection and genital disease, generating a long-lasting antibody response^[60]. VLP vaccines combine many of the advantages of the whole-virus vaccines and recombinant subunit vaccines. In addition, compared to individual proteins or peptides, they closely mimic the organization and conformation of authentic native viruses, leading to a more efficacious activation of the adaptive immune system. They can elicit a protective response without requiring multiple booster shots, thus significantly reducing the vaccine costs. VLPs do not need attenuation or inactivation - as the live attenuated and killed/inactivated vaccines - avoiding all the possible side effects of inactivation treatments on the epitope modifications. Moreover, but lacking the viral genome VLPs potentially yield safer vaccine candidates compared to whole-virus vaccines. However, some technical challenges need to be considered for VLP production^[56], essentially related to the limitations of the size of the expressed antigens and the choice of the expression systems. VLPs are normally expressed in bacteria, and therefore VLP assembly and stability, solubility, yield, endotoxin-free production, and composition may be potentially affected by all the

concerns related to the prokaryotic expression machinery. Baculovirus/insect cell systems allow high expression levels. However, co-production of enveloped baculovirus contaminants may significantly impact the vaccine efficiency, and even though VLPs expressed in mammalian cells undergo complex post-translational modifications, this system shows high production costs, low controllability and productivities. Currently, researchers are actively investigating methods to produce cheaper optimized VLP-based vaccines with increased half-life.

“E2 SCAFFOLD” AS A VERSATILE VACCINE DELIVERY SYSTEM

The E2 protein scaffold represents a versatile antigen delivery system (E2DISP) where antigenic determinants can be exposed on the surface of an icosahedral dodecahedral nanoparticle^[61,62]. The scaffold is composed of the E2 acetyltransferase protein derived from the pyruvate dehydrogenase (PDH) multienzyme complex of *Geobacillus stearothermophilus*. The PDH complex belongs to the family of 2-oxo acid dehydrogenase multienzyme complexes that catalyse the irreversible oxidative decarboxylation of 2-oxo acids. They comprise multiple copies of three different enzymes, and in the case of PDH of *Geobacillus stearothermophilus*, two of these enzymes, E1 and E3, assemble over the surface of a large structural scaffold formed by the multi-domain core enzyme, E2, a specific dihydrolipoyl acetyltransferase. The E2 polypeptide chain is composed of three independently folded domains separated by flexible linker regions: a lipoyl domain (LD) of 9.5 kDa, a peripheral (E1 and/or E3) subunit-binding domain (PSBD) of 5.3 kDa and a catalytic acetyltransferase core domain (CD) of 28 kDa (Figure 1A). The E2 CD forms trimers that assemble to generate a pentagonal dodecahedral protein scaffold resembling a virus-like particle (VLP) with icosahedral symmetry, composed of 60 identical E2 subunits (60-mer), that is 24 nm in diameter, with a molecular weight of 1.5 MDa, with an outer and inner domains of 240 Å and 50 Å, respectively^[63] (Figure 1A). In the field of antigen display, the acetyltransferase core domain (CD) of the E2 protein is of great potential utility (E2DISP) (Figure 1B). Two engineered plasmids, pET-HE2DISP and pET-E2DISP, allow to insert exogenous oligonucleotides coding for the antigen of interest at the 5' end of gene encoding the E2 CD, and thus to display foreign peptides/proteins as N-terminal fusions to CD (Figure 1B). Due to the stability and ability of this thermophilic protein to assembly *in vitro*^[64], it is possible to display 60 copies of heterologous polypeptides on the surface of the E2 macromolecular scaffold, still capable of self-assembly to the 60-mer. This property is particularly suitable for vaccine design. There is no limitation to the size of peptide displayed, given the ability of the E2 CD to naturally present 60 lipoyl domains plus 60 copies of the E1 (150 kDa) or E3 (100 kDa) enzymes. Domingo *et al.*^[61] demonstrated that a green fluorescent

Table 2 Preclinical studies based on E2 formulations

E2 construct	Description	Route	Immune response	Ref.
Gag(p17)-E2	HIV-1 Gag p17 matrix protein	sc	Mice immunized with Gag(p17)-E2 mounted a strong and sustained Ab response; the isotype of induced Abs was biased toward IgG1; CD8+ T cells primed with E2 particles were able to exert lytic activity and to produce IFN- γ	[65]
BS1-E2	Mimotope 1 from HIV-1 bridging sheet domain (BS)	IM ¹ /sc ¹	The E2-BS1 fusion peptide showed good antigenic results; a moderate neutralizing antibody response was found against two HIV-1 clade B and one clade C primary isolates	[67]
Env(V3)-E2	HIV-1 SF162 Env V3 loop peptide 291-336 from gp120 (HXB2 numbering)	Env-E2: IM ¹ ; pDNA ² : ID ¹	Env(V3)-E2 induced potent binding Ab and T-cell responses in mice, as well as autologous NAbs in rabbits, when co-immunized with pDNA; co-immunization with pDNA and E2 multimers generated potent immune responses after only two immunizations	[19]
Env(MPER)-E2	HIV-1 SF162 Env MPER peptide 649-689 from gp41 (HXB2 numbering)	Env-E2: IM ¹ ; pDNA ^{2,3} : ID ¹	MPER (membrane proximal external region) displayed on E2 focused Ab responses toward conserved region of HIV-1 Envelope when co-administered with pDNA lacking hypervariable loop regions	[66]
(1-11)-E2	Peptide 1-11 of beta-amyloid	sc	(1-11)E2 vaccine induced fast-rising, robust and persistent Ab responses to beta-amyloid; the Ab response was characterized by a marked prevalence of IgG1 over the IgG2a isotype	[68,69]

¹Routes of administration for rabbit immunizations; ²pDNA: codon-optimized HIV-1 SF162 plasmid DNA encoding gp160 full-length; ³Lacking hypervariable regions. sc: Subcutaneous; IM: Intramuscular; ID: Intradermal administration; Env: Envelope; gp: Glycoprotein; Ab: Antibody; NAbs: Neutralizing antibodies.

protein (EGFP) displayed on the E2 surface folded into its active form. We and others have successfully expressed and refolded several HIV-1 antigens and protein domains^[19,65-67]. In addition, peptides 1-11 and 2-6 of beta-amyloid were displayed as N terminal fusions of the E2 core domain^[68,69]. N-terminal fusion proteins are displayed without constraint on the surface of the E2 60-mer particles. Efficient expression was achieved in *Escherichia coli* (*E. coli*) cells. If soluble, proteins are purified as a large soluble aggregate, according to previously described methodologies^[64] with a yield of pure E2 particles of about 15 mg/L of cell culture. Insoluble aggregates can be purified from inclusion bodies (IBs)^[70]. It was shown that solubility and stability of HIV-1 Env-E2 fusion proteins substantially increased when they were refolded in the presence of the E2 wild type (E2wt) core protein, with no precipitation^[19,66]. In details, pure HIV-1 Env-E2 IBs can be solubilized in presence of 6 M GuHCl (guanidine hydrochloride) and then refolded in the presence of E2 wild-type core protein (E2 monomers without the N-terminal HIV-1 fusion) in step-down dialysis by slow removal of the denaturant in the presence of oxidizing agents and low molecular weight additives, as schematically shown in Figure 1C. HIV-1 Env(V3)-E2 construct was refolded with equimolar amounts of E2wt, requiring a 1:1 ratio of Env-E2 fusion protein: E2wt to remain fully soluble^[19]. Solubilized particles typically have more than 50 EU/mL of *E. coli*-derived endotoxin (lipopolysaccharide, LPS) as a result of expression in this system. Endotoxin levels can be reduced to less than 0.05 EU/mL using standard biochemical techniques^[71]. The resulting vaccines are non-replicative multimeric particles formed by exogenous antigens inserted on the surface of E2 60-mer scaffold protein that is able to confer high immunogenicity to the displayed determinants.

We previously described that epitopes displayed on the surface of E2 scaffold are able to elicit both B and T cell responses, demonstrating that E2 particles can reach both MHC class I and class II compartments for the processing and presentation of the displayed epitopes^[72-74], and we have investigated this system in various preclinical studies demonstrating the immunogenicity of E2-based vaccine formulations (resumed in Table 2). In particular, using this system, we demonstrated that mice immunized with the HIV-1 Gag (p17) protein displayed as an N-terminal fusion to the E2 CD [Gag (p17)-E2] mounted a strong and sustained humoral immune response. High titers of specific-antibodies were induced even in the absence of any adjuvants, and priming of transgenic mice with Gag(p17)-E2 particles induced antigen-specific cytotoxic CD8+ T cells able to produce IFN- γ ^[65]. Moreover, a moderate neutralizing antibody response was found in rabbits immunized with an E2 scaffold displaying a peptide mimotope of the HIV-1 gp120 bridging sheet^[67].

Furthermore, E2 multimeric scaffolds displaying HIV-1 neutralizing antigens, such as the HIV-1 Envelope (Env) V3 loop from gp120 glycoprotein, was able to elicit potent binding antibodies and T-cell responses in mice, as well as autologous neutralizing antibodies in rabbits, when co-immunized with an HIV Env glycoprotein (gp160) expression plasmid DNA^[19]. Interestingly, co-immunization of plasmid DNA vaccine with E2 multimeric scaffolds appeared to be more effective in eliciting rapid, specific, and sustained autologous neutralizing antibody responses as well as antigen-specific CD8+ T cells producing IFN- γ , compared to standard DNA-prime/protein-boost regimen. On this line, the E2 scaffold displaying the membrane proximal external region (MPER) from HIV-1 Env gp41 glycoprotein - N-terminally fused to E2 core domain - was able to focus humoral immune responses

These properties make the E2DISP system an attractive option for vaccine delivery. Theoretically, there is no limitation to the size of peptide displayed on the E2 surface, given the potential of the E2 core domain to naturally accommodate 187 amino acid residues in the form of the two folded protein domains (LIP and PSBD domains) and two flexible linkers (Figure 1A). Displaying full-length protein as antigen may be a convenient option compared to peptide to provide optimal epitope diversity for antibody production and T cell induction. In this context, the E2DISP delivery may be particularly favorable to other types of antigen display systems - such as the Hepatitis B surface antigen vector that has a limit of approximately 36 amino acids^[76] or the chimeric human papilloma virus-simian/human immunodeficiency virus virus-like particle vaccine that can only accept approximately 60 amino acids of foreign

antigen^[77]. Repetitive presentation of an epitope in highly organized structures - as with E2 nanoparticle - can increase the ability of particulate delivery systems to stimulate stronger immune responses by triggering and cross-linking specific B cell antigen receptors. Within this context, the E2 nanoparticle may be particularly useful as repetitive antigen delivery system due to its potential to display up to 60 copies of an antigen of interest per particle. Moreover, the E2DISP delivery may function as presentation scaffold for multiple displays of antigens, all on the same E2 particle, in their native form to properly activate both the humoral and cellular branches of the immune response. The ability of E2-based vaccines to generate both CD8+ T cell responses and antibodies may represent an advantage over protein subunit vaccines, which primarily evoke humoral responses, and recombinant viral vectors being more effective at generating cellular immune responses.

Bacterial expression is the most common expression system employed for the expression and purification of heterologous recombinant proteins - as for the production of the E2 nanoparticles. However, proteins expressed in a prokaryotic-based system are not correctly modified - in terms of protein phosphorylation and glycosylation - and might precipitate in the form of inclusion bodies, thus affecting the protein folding. Moreover, as result of expression in *E. coli* cells, recombinant proteins are generally contaminated with the lipopolysaccharide (LPS) component of the outer cell membrane. Such a toxic component triggers secretion of pro-inflammatory cytokines, and it often requires extensive and expensive removal during protein purification, thus affecting the final yield. It was shown that solubility and stability of recombinant E2 scaffolds that precipitate into the insoluble fraction could increase when they are refolded *in vitro* from denaturing conditions in presence of the E2wt core protein. In addition, treatment by phase separation with Triton X-114 detergent leads to an endotoxin reduction of less than 0.05 EU/mL. However, alternative organisms and expression systems could be more useful for the expression and production of E2 nanoparticles in order to circumvent all the problems related to the *E. coli* expression machinery.

We previously explored the potential of the E2 antigen display system as an HIV-1 vaccine candidate. It was shown that E2-based multimeric vaccines displaying the V3 loop or the MPER region from the HIV-1 Envelope are able to focus and to direct antibody responses to conserved neutralization determinants. However, the V3 epitope displayed on the surface of E2 scaffold is not effective in generating broadly neutralizing antibodies (NAbs), and we can only generate low levels of neutralizing antibodies that are MPER-specific^[19,66]. Clearly, this current E2-based immunogen requires further optimization for advancement. A major goal of HIV-1 vaccine development is to find strategies for inducing high levels of broad-spectrum neutralizing antibodies. We hypothesize that the E2-mediated immune responses can likely be further enhanced using molecular

modeling to determine the appropriate regions of the E2 protein to serve as insertion sites for key neutralization determinants in order to improve presentation and thus immunogenicity of HIV-1 regions in this system.

Overall, the potential of this system is that it exhibits stability and no toxicity, it is able to induce sustained humoral and cellular antigen-specific immune responses without anti-vector immunity, and thus low-cost, non-replicating, non-integrating, non-pathogenic E2 vaccines could be designed and combined with other approaches to advance the field of vaccinology.

CONCLUSION

Vaccines play a pivotal role in host protection against infectious diseases and have significantly reduced mortality worldwide. However most of vaccine candidates have failed to completely protect individuals from emerging and re-emerging diseases/agents, with many diseases, such HIV/AIDS, tuberculosis, and malaria, being not yet preventable by vaccination. Hence, the development of new vaccine formulations is of fundamental priority. Several strategies have been developed over the years in order to achieve this goal, and the recent advances in the field of vaccine technology may provide valuable insights for the rational design of next-generation vaccine delivery systems. Historically, vaccinology has relied on the use of live attenuated, killed/inactivated, toxoid and subunit vaccines with most of them currently available on the market. Many live attenuated vaccines are able to stimulate humoral as well as cell-mediated immune responses, by mimicking the natural infection. However, some concerns still remain to be addressed when using attenuated/inactivated vectors as vaccines, including safety, instability and weak immunogenicity. Alternative strategies have been developed to provide safer and more effective vaccines. Recombinant DNA technology could be a useful approach, mainly due to the ability of DNA vaccines to elicit different types of immune response, providing many advantages over traditional vaccines in terms of safety, stability, costs of production, and ease of manufacturing. However, until now DNA vaccines have not been successful in non-human primates and humans. Recombinant viral vectors represent an attractive tool to deliver antigen and to stimulate stronger immune responses than DNA vaccines, with the majority of current clinical trials for gene therapy using viral vectors; however, biosafety and pre-existing immunity concerns must be taken into account when using viral vectors as vaccine. Nanoparticle-based delivery systems have arisen as promising vaccine candidates over traditional vaccines, mainly due their ability to elicit robust immune responses without toxicity and anti-vector immunity, even though these formulations suffer of problems of stability and conservation. Given this scenario, we have been studying in the past decade a delivery system based on a protein scaffold formed by a 60-mer assembled over the domain of the E2 component of the PDH complex from *Geobacillus stearothermophilus*. The E2 scaffold represents a versatile vaccine delivery candidate, being able to trigger both arms

of the adaptive immune response, combining good safety and stability with strong immunogenicity.

In conclusion, in this review we have described the advancement obtained in the recent past on the topic of antigen delivery systems for new vaccine formulations. Studies aimed to compare in controlled assay conditions should be performed in a near future in order to identify the most promising vaccination strategies.

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