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| 48 | Abstract | | Pulmonary vaccine delivery has gained significant attention as an alternate route for vaccination without the use of needles. Immunization through the pulmonary route induces both mucosal and systemic immunity, and the delivery of antigens in a dry powder state can overcome some challenges such as cold-chain and availability of medical personnel compared to traditional liquid-based vaccines. Antigens formulated as nanoparticles (NPs) reach the respiratory airways of the lungs providing greater chance of uptake by relevant immune cells. In addition, effective targeting of antigens to the most 'professional' antigen presenting cells (APCs), the dendritic cells (DCs) yields an enhanced immune response and the use of an adjuvant further augments the generated immune response thus requiring less antigen/dosage to achieve vaccination. This review discusses the pulmonary delivery of vaccines, methods of preparing NPs for antigen delivery and targeting, the importance of targeting DCs and different techniques involved in formulating dry powders suitable for inhalation. |

50   Foot note
information

EXPERT REVIEW

Nanocarriers Targeting Dendritic Cells for Pulmonary Vaccine Delivery

Nitesh K. Kunda • Satyanarayana Somavarapu • Stephen B. Gordon • Gillian A. Hutcheon • Imran Y. Saleem

ABSTRACT Pulmonary vaccine delivery has gained significant attention as an alternate route for vaccination without the use of needles. Immunization through the pulmonary route induces both mucosal and systemic immunity, and the delivery of antigens in a dry powder state can overcome some challenges such as cold-chain and availability of medical personnel compared to traditional liquid-based vaccines. Antigens formulated as nanoparticles (NPs) reach the respiratory airways of the lungs providing greater chance of uptake by relevant immune cells. In addition, effective targeting of antigens to the most 'professional' antigen presenting cells (APCs), the dendritic cells (DCs) yields an enhanced immune response and the use of an adjuvant further augments the generated immune response thus requiring less antigen/dosage to achieve vaccination. This review discusses the pulmonary delivery of vaccines, methods of preparing NPs for antigen delivery and targeting, the importance of targeting DCs and different techniques involved in formulating dry powders suitable for inhalation.

KEY WORDS antigen presenting cells · dendritic cells · dry powder · polymeric nanoparticles · pulmonary delivery of vaccines

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ABBREVIATIONS

AMs	Alveolar macrophages
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
CLRs	C-type lectin receptors
DCs	Dendritic cells
DPI	Dry powder inhalations
FD	Freeze-drying
HLA	Human leukocyte antigen
ILs	Interleukins
LN	Lymph node
MHC	Major histocompatibility complex
MN	Mannan
NPs	Nanoparticles
PCL	Poly-ε-caprolactone
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PLA	Polylactide or poly-L-lactic acid
PLGA	Poly lactic-co-glycolic-acid
PRRs	Pattern recognition receptors
PVA	Polyvinyl alcohol
SCF	Supercritical fluid
SD	Spray-drying
SFD	Spray-freeze drying
TLRs	Toll-like receptors
TMC	N-Trimethyl chitosan
VLPs	Virus-like particles

INTRODUCTION

New therapeutic biopharmaceuticals have made it possible to treat and/or prevent many diseases which were untreatable a decade ago (1). The majority of these biopharmaceuticals are administered via parenteral routes because they are degraded by acid and proteases in the stomach or

have high first-pass metabolism and as such are not suitable for oral delivery. The formulation of biopharmaceuticals in non-invasive delivery systems in order to make them more acceptable to patients has gained significant attention but the pharmaceutical challenges are stability, integrity and effectiveness within the therapeutic dose (1,2). The leading non-invasive systems are buccal, nasal, pulmonary, sublingual and transdermal routes—this review will focus on the pulmonary route and on vaccine delivery in particular.

Pulmonary delivery of vaccines has gained major attention for achieving both mucosal and systemic immunity (3). An optimum formulation containing antigens in the dry state as nanoparticles (NPs) can result in greater stability and a better immune response compared to traditional liquid-based vaccines (3). NPs as colloidal carriers offer protection of biopharmaceuticals against degradation, and targeted delivery to specific sites of action. NPs can be developed with variable physico-chemical characteristics such as size, structure, morphology, surface texture and composition, and thus can be delivered either orally, parenterally or locally (4).

This review discusses the pulmonary delivery of vaccines, methods of preparing NPs, the importance of targeting dendritic cells (DCs) (antigen presenting cells-APCs) and different techniques involved in making dry powders suitable for inhalation. Progress in the delivery of biopharmaceuticals via buccal (5–7), nasal (8), sublingual (9) and transdermal (10) routes has previously been reported elsewhere and is beyond the scope of this review.

Since the term 'vaccination' was coined by Edward Jenner in 1796, it has been arguably the most important scientific advance in the battle against infectious disease (11). According to the World Health Organization (WHO), around 2.5 million children's lives are saved each year due to the availability of vaccines against a variety of antigens (12). However, in low and middle income countries (LMIC) a lack of infrastructure such as cold-chain and trained medical personnel essential for the administration of traditional liquid-based vaccine formulations, means that many eligible children and adults are not vaccinated (12). Table I below provides a list of reported cases by disease according to World Health Statistics (WHS) 2011 (13). Hence, there is a global need to develop effective and reliable vaccine strategies that are non-invasive, easily accessible and affordable (14). To address the issues with liquid-based vaccine formulations in LMIC, non-invasive routes of delivery, which do not have the requirements of cold-chain or trained personal are being investigated (3).

Of all the non-invasive routes of delivery, pulmonary delivery can overcome some of the current challenges of vaccination such as invasiveness, accessibility, and vaccine stability and integrity by delivering vaccines as dry powder inhalations (DPI) (14). In addition, the pulmonary route has

Table I List of Reported Cases by Disease According to World Health Statistics (WHS) 2011

Disease	Reported Cases (WHS 2011)[a]
Diptheria	857
Malaria	81,735,305 (1990–2009)
Measles	222,318
Mumps	546,684
Tetanus	9,836
Tuberculosis	5,797,317
Pneumonia (Children <5 years)	~1,400,000 (18% of all child deaths in 2008) (120)

[a] Data provided not necessarily for the year 2011, more details at http://www.who.int/whosis/whostat/2011/en/index.html

gained much attention as it is the main entry portal for pathogens (2,15).

PULMONARY VACCINE DELIVERY

Pulmonary delivery as a route of drug administration can be traced back 4000 years to India where people suffering from cough suppressed it by inhaling the leaves of *Atropa Belladonna* (16). Later in the 19th and 20th centuries, people suffering from asthma smoked cigarettes containing tobacco and stramonium powder to alleviate their symptoms (16). The first inhaling apparatus for dry powder delivery was patented in London in 1864 (17). Since then much progress has been made in developing devices such as nebulizers, metered dose inhalers and DPIs for delivery of therapeutics. With recent advancements in pulmonary delivery devices and recombinant protein technology the first peptide DPI formulation, Exubera (Nektar/Pfizer), was approved and released into the market in January 2006. This was soon withdrawn for several reasons including bulkiness of the device, complicated administration, contraindication in smokers and insufficient evidence with regulatory bodies regarding the patients preference of Exubera (inhaled dosage form) compared to other dosage forms (18). This led, however, to further research and development of DPI of biopharmaceuticals, and currently many investigations are being pursued by the pharmaceutical industry such as the AIR system (Alkermes/Eli Lilly), the Technosphere system (Mannkind) and Kos inhaled insulin (Kos Pharm/Abbott) for Type I/II diabetes, and Granulocyte-colony-stimulating factor (G-CSF) for Neutropenia (Amgen) (19). This has been followed by investigations into DPI of vaccines (20–24).

Anatomy of the Human Lung

The human lung, weighing about 1 kg, is divided by the pleural membranes into three lobes on the right and two

180 lobes on the left (25). Once inhaled, the air passes through
181 the nose and mouth, from the larynx to trachea and to the
182 series of around 16 generations of conductive bronchi and
183 bronchioles (25,26). From the 17th generation of bron-
184 chioles, alveoli begin to appear in the walls (respiratory
185 airways) and by the 20th generation of airways, the entire
186 walls are composed of alveoli, commonly referred to as
187 alveolar ducts. At the 23rd generation, the alveolar
188 ducts end in blind sacs, lined with alveoli, and are referred
189 to as alveolar sacs (Fig. 1) (25–27). It is estimated that
190 on an average a human lung consists of about 300 million
191 alveoli providing a surface area of exchange of 80–90 sq. m
192 (25,28).

193 The submucosal glands and the 'goblet cells' (present on
194 the bronchial surface) secrete mucus onto the bronchial sur-
195 faces. The submucosal glands also help in producing an elec-
196 trolyte solution on which the mucus rests. The mucus covering
197 the airways is transported towards the mouth with the coor-
198 dinated movement of cilia present on top of the ciliated
199 columnar cells. This mucus transported to the mouth is then
200 swallowed. This process of mucus movement from the bron-
201 chial surfaces to the mouth for swallowing is mainly responsi-
202 ble for removing any foreign material that lands on the
203 bronchial surfaces (25).

204 The alveoli and the pulmonary capillaries are separated by
205 a barrier composing of endothelial cells, interstitial space, and
206 pneumocytes (pulmonary epithelial cells). The pneumocytes

207 are divided into two types, type I and type II cells. Type I are
208 very flat and cover the alveolar surface whereas type II are
209 irregularly shaped containing lamellar bodies that are secreted
210 as surfactant, and they can further divide and produce type I
211 and type II cells (25).

Lung as a Delivery Site for Drugs

213 The lung is an excellent choice for the delivery of biophar-
214 maceuticals for the treatment of both local and systemic
215 disorders as it offers several advantages such as; large surface
216 area (80 sq. m), dense vasculature, rapid absorption leading
217 to an immediate onset of action, thin alveolar epithelium,
218 less enzymatic activity than gut and a high capacity for
219 solute exchange (29). With regards to the delivery of vac-
220 cines, a high density of APCs including alveolar macro-
221 phages (AMs), DCs and B cells represent an ideal target to
222 induce a strong immune response resulting in both mucosal
223 and systemic immunity (14). Recent research has con-
224 firmed that the induction of an immune response at one
225 mucosal site elicits an immune response at distant muco-
226 sal sites by mucosal lymphocyte trafficking leading to
227 both mucosal and systemic immunization (15,30). There
228 is some evidence that mucosal immunization may also
229 reduce the dosage required to achieve the desired immunity
230 compared to liquid formulations administered via the
231 parenteral route (3).

Fig. 1 Diagram of the human lung and particle deposition based on size.
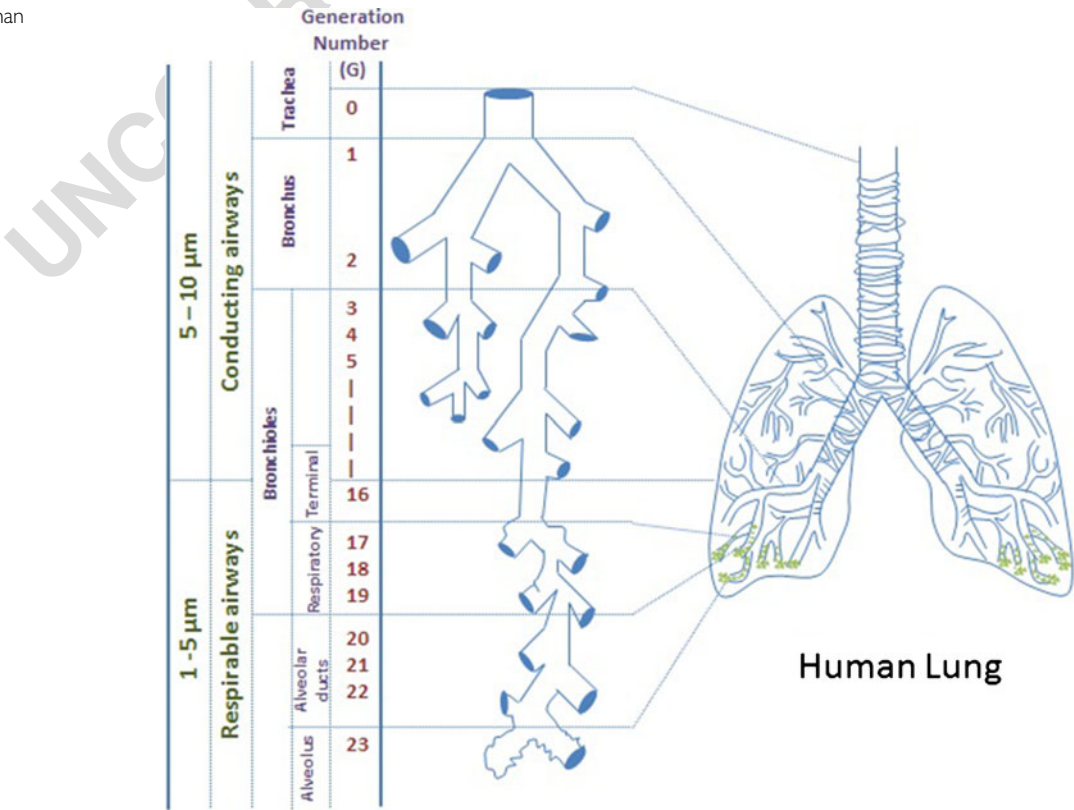
Pulmonary *vs* Parenteral Vaccine Delivery

In development of novel anti-tuberculosis vaccines, Ballester M *et al.* demonstrated, that inhaled vaccine compared favorably to an intradermal route of delivery. In particular, vaccination with NP-Ag85B and immune-stimulatory oligonucleotide CpG as a Th1-promoting adjuvant via the pulmonary route modified the pulmonary immune response and provided significant protection following a *Mycobacterium tuberculosis (Mtb)* aerosol challenge (31).

Muttil P *et al.* successfully prepared poly lactic-co-glycolic-acid (PLGA) NPs entrapping diphtheria CRM-197 antigen (CrmAg) with a size of 200 ± 50 nm by the emulsification solvent diffusion and double-emulsion methods. The NPs were then spray-dried with L-leucine and the resulting spray-dried powders of formalin-treated/untreated CrmAg nanoaggregates were delivered to the lungs of guinea pigs. This study evaluated the immune response elicited in guinea pigs following pulmonary and parenteral immunizations with the dry powders and the highest titer of serum IgG antibody was observed in guinea pigs immunized by the intramuscular route whereas high IgA titers were observed for dry powder formulations administered by the pulmonary route. This demonstrates that pulmonary immunization with dry powder vaccines leads to a high mucosal immune response in the respiratory tract and sufficient neutralizing antibodies in the systemic circulation to provide protection against diphtheria (32).

An ideal vaccine formulation for mass vaccination would induce the desired immunity upon administration of a single dose. Moreover, it is important to target APCs like DCs to illicit a strong and durable immune response with a single dose aimed at both systemic and mucosal immunity (33).

Dendritic Cells

Dendritic cells (DCs) were first identified in 1868 by Paul Langerhans in the basal layer of the epidermis (34). However, it took more than a century to properly identify them as white blood cells related to macrophages and monocytes, and to understand their importance in the control of immunity (34,35). In 2011, the Nobel Prize in Physiology or Medicine was awarded to Ralph M. Steinman for his discovery of DCs and their role in adaptive immunity paving the way for more research in the field of immunity and vaccines (36). It has become evident over the years that DCs are APCs, true 'professionals' (37) with exceptional capability to internalize, process and present antigens through major histocompatibility complex (MHC) class I and II pathways. DCs induce a strong immune response by activating naïve T-cells which are produced in the bone marrow and have the capability to respond to novel pathogens that have not been processed before (38,39). The role of DCs in initiating a primary immune response has now been shown to be greater than the role played by macrophages and the B-cells (40).

The lung is armed with an intricate network of DCs that can be found throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes (41,42). It is now apparent that there are at least five different subsets of DCs in the murine lung; resident DCs, plasmacytoid DCs, alveolar DCs, inflammatory DCs and interferon-producing killer DCs (41,42). The data for the subsets of DCs in the human lung is rare (43) owing to the need to obtain lung tissue, as they are not found in the bronchoalveolar lavage (BAL) fluid. However, studies on the human AMs are common as they are readily obtained from BAL (44). The AMs are primarily phagocytes with poor APC function and live in the air space, whereas immature DCs have high APC function but lower phagocytic function and live mainly in the interstitium (45). In the human lung, the mucosal surface in the conducting airways consists of ciliated epithelial cells, interspersed goblet cells, macrophages and DCs (46). The DC population in this region is mainly composed of myeloid DCs (mDCs), however, a fraction of plasmacytoid DCs (pDCs) can be found (46). These mDCs have a high capability for antigen uptake but less ability to stimulate the T cells (46). Moreover, the human DCs are generated from haematopoietic stem cells, mDCs from bone marrow–derived monocytic precursors and pDCs from lymphoid progenitors (34). The mDCs and pDCs are activated by a different set of pathogenic stimuli making them functionally distinct reflected by the different expression of cell surface receptors such as Toll-like receptors (TLRs) (34,46). The lung parenchyma consisting of lung interstitium, respiratory and terminal bronchioles, and alveoli is mainly composed of 80% macrophages with rest being DCs and T cells. The 'immature' resident DCs are highly capable of detecting, capturing and processing the encountered antigen (34,46).

The human DCs are identified by over expression of human leukocyte antigen (HLA) DR (major histocompatibility complex class II) with the absence of monocyte, lymphocyte, natural killer cell and granulocyte lineage markers (43). In addition, the specific markers for identifying the mDCs include $CD11c^+$, $CD1a^+$, $BDCA-1^+$, $BDCA-3^+$, $HLA-DR^+$ whereas for the pDCs they are $CD11c^-$, $HLA-DR^+$, $BDCA-2^+$ and $CD123^+$ (43,46,47).

Inhaled antigens or antigen particulates are believed to encounter the wide spread DC network that lines the alveolar epithelium and are subsequently taken up by cellular processes extending in to the alveolar lining fluid (33). Antigens are then processed and fragments of antigenic peptides are presented on the surface through MHC class I and II pathways for recognition by the T-cell receptors present on T-cells (40). This process is often referred to as antigen

335 presentation and typically takes place in the regional lymph
336 node after chemokine dependent migration of the antigen
337 loaded DC. Also, APCs perceive danger signals from cells
338 and offer co-stimulatory signals (48) through co-stimulatory
339 molecules present on their surface for recognition by recep-
340 tors on recirculating T-cells to initiate an immune response
341 in the lymph node (40). Upon encountering the danger
342 signals, immature DCs change to a mature stage where they
343 present the antigen on their surface. This step is usually
344 concurrent with the migration of DCs from peripheral tissue
345 to the lymph node for T-cell activation (Fig. 2). It is believed
346 that soon after antigen presentation, the DCs undergo apo-
347 ptosis in the lymph nodes (40).

348 Antigen uptake by DCs occurs by macro-pinocytosis,
349 receptor-mediated endocytosis (macrophage mannose recep-
350 tor) and/or phagocytosis (49–52). Recent research by Foged *et*
351 *al.* has shown that both particle size and surface charge of the
352 material to be delivered plays an important role in determining
353 the uptake by human DCs derived from blood. Furthermore, it
354 was recognised that for optimal uptake by DCs the preferred
355 particle size was 0.5 μm (diameter). Uptake of large particles
356 (∼ 1 μm) was greatly enhanced when they displayed a positive
357 surface charge (53). In addition, a study conducted by Mano-
358 lova *et al.* revealed that upon intracutaneous injection of poly-
359 styrene beads of varying sizes the large particles (500–2000 nm)
360 associated with DCs from the site of injection and depended

Fig. 2 Antigen uptake and presentation by dendritic cells (DCs) in the airways. / **a** Upon exposure of an inhaled antigen the immature DCs migrate towards the site of attack. DCs at this stage express a wide variety of receptors (Fc, C-type lectin receptors etc.) and uptake the antigen. Simultaneously, some DCs upregulate the CC-chemokine receptor 7 (CCR7) and migrate towards the lymphatic vessels expressing CC-chemokine ligand 21 (CCL-21) where they are carried to the draining lymph node. After antigen uptake and activation, high amounts of peptide-loaded major histocompatibility complex (MHC) molecules and T-cell co-stimulatory receptors appear on the surface of DCs. The DCs then migrate to the lymph nodes and activate the antigen specific T-cells. / **b** After antigen uptake, the antigen is either processed through MHC class I (either through endogenous or exogenous pathway) or MHC class II (the antigen is degraded in endosomes and the obtained polypeptide is transported and loaded onto MHC II molecules) and DCs present it on their surface for specific T-cell activation. *ER – Endoplasmic reticulum.
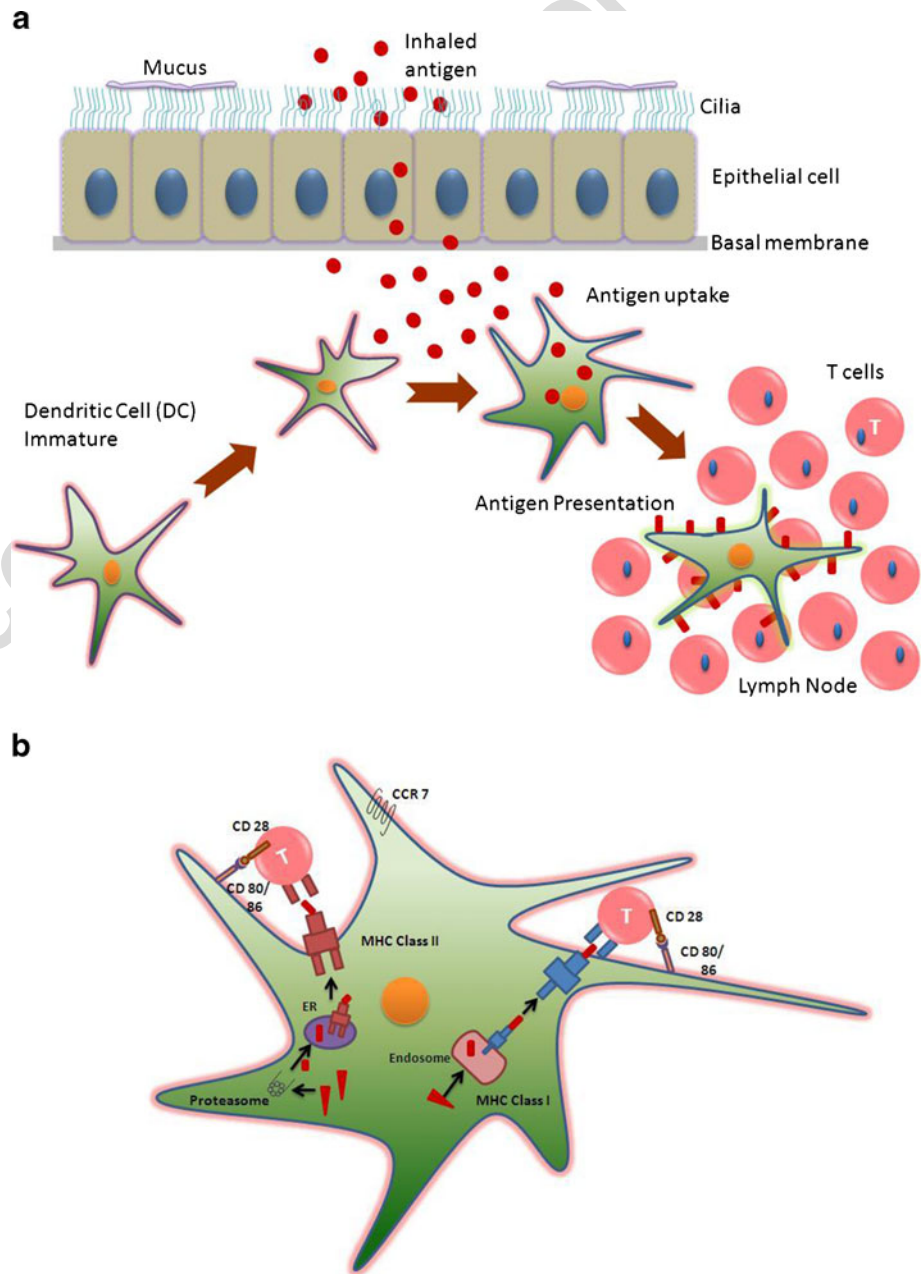
largely on them for cellular transport, whereas small particles (20–200 nm) and virus-like particles (VLPs) (30 nm) drained freely to the lymph nodes (LNs) and were present in LN-resident DCs and macrophages (54). However, this cannot be directly compared to pulmonary delivery as the DCs in the lung differ from those of the skin.

Targeting Antigen to the DC

Antigen can be targeted to DCs, for enhanced immune response, by making particles that bind to the specific receptors expressed on the DC surface (49–51). Effective targeting of vaccines to the DCs results in the possibility of a reduced vaccine dose, less side effects, improved efficacy and enhanced immune response (40).

Vaccines can be targeted to DCs in different ways (40, 55–57). DCs contain pattern recognition receptors (PRRs) that aid in detecting the presence of a pathogen through interaction with pathogen-associated molecular patterns. More specifically, C-type lectin receptors (CLRs), a type of PRR, bind to sugar moieties (e.g., mannose, glucan) in a calcium-dependent manner present on the pathogen's surface. This leads to antigen internalization through receptor mediated endocytosis resulting in antigen presentation to T-cells (58,59). Vaccines can also be targeted to DCs with antibodies having an affinity towards specific receptors present on their surface (e.g. anti-DEC205, anti-CD11c), internalization through phagocytosis and conjugation of danger signals that effectively bind to Toll-like receptors (TLRs) or cytokine receptors thereby inducing DC maturation (40,55). Table II lists some formulations that have been effectively targeted to DCs for an enhanced immune response. There are currently no publications that establish targeting of pulmonary DCs through pulmonary delivery of dry powder vaccines.

Nanoparticles for Inhalation

Generally nanoparticles (NPs) are referred to as particles in the size range of 1—100 nm, however for drug delivery NPs larger than 100 nm are required for efficient drug loading, and have been in use for the last 40 years (60). NPs are used as drug carriers either by encapsulating, dissolving, surface adsorbing or chemically attaching the active substance (60). NPs have a large surface area-to-volume ratio and also an increased saturation solubility thus favoring application in the field of drug delivery. In delivery of NPs to the lung by inhalation, deposition takes place through impaction, sedimentation, interception or diffusion (Table III) depending on particle size, density, airflow, breathing rate, respiratory volume and the health of the individual (61,62). These are discussed in greater detail by Smyth HDC et al. (63) and definitions are summarized in Table III.

The deposition of particles in the lungs is evaluated using the aerodynamic particle size, which is defined as the diameter of a sphere (density-1 g/cm^3) in air that has the same velocity as the particle in consideration (60). This is defined by the equation

$$d_a = d_g \sqrt{\rho/\rho_a}$$

where ρ is the mass density of the particle, ρ_a is the unit density (1 g/cm3) and d_g is the geometric diameter.

Particles greater than 10 μm (d_a) in size are commonly impacted in the throat or sedimented in the bronchial region whereas particles less than 1 μm (d_a) in size are exhaled and not likely to be deposited in the alveolar region. It is expected that particles in the size range of 1 to 5 μm (d_a)

Table II Examples of Formulations Targeting Dendritic Cells (DCs)

Formulation	Target	Model drug	Model	Ref
Polyanhydride NPs with dimannose	Mannose receptor CD206	NA	In vitro	(58)
MN-decorated PLGA NPs	Mannose receptor CD206	NA	In vitro	(121)
PLGA NPs	DEC-205 receptor	Ovalbumin	Mice	(122)
PLGA NPs	Humanized targeting antibody hD1 (DC-SIGN)	FITC-TT/DQ Green BSA	In vitro	(123)
PLGA NPs coated with streptavidin	gp120, ManLAM, Lex, aDC-SIGN 1, aDC-SIGN 2, aDC-SIGN 3	DQ-BSA, gp100$_{272-300}$ and FITC-TT	In vitro	(56)
Carbon magnetic NPs (CMNPs)	Endocytosis	Hen egg lysozyme (HEL)	Mice	(124)
Polystyrene and PLGA microparticles	CD40, Fcg, α(v)β3 and α(v)β5	NA	In vitro	(125)
Acid degradable particles	DEC-205 receptor	Ovalbumin	Mice	(124)
PAMAM dendrimer	Mannose receptor CD206	Ovalbumin	Mice	(126)
Liposome (with tri-mannose) (L-Phosphatidylcholine + M3-DPPE)	Mannose receptor CD206	FITC-Ovalbumin	In vitro	(127)
Niosomes (coated with polysaccharide o-palmitoyl MN)	Mannose receptor CD206	TT	Albino Rats	(128)

M3- DPPE trimannose-dipalmitoylphosphatidylethanolamine, ManLAM Mannosylated lipoarabinomannan, MN Mannan, Niosomes Sorbiton Span 60, cholesterol, stearylamine, PAMAM Polyamidoamine, PLGA poly lactic-co-glycolic-acid, TT Tetanus Toxoid, NA Not Applicable

t3.1	**Table III** Broad Descriptions of Impaction, Sedimentation, Interception and Diffusion	
t3.2	Impaction	The delivered particles, due to inertia, do not change their path and as the airflow changes with bifurcations they tend to get impacted on the airway surface. This is mostly experienced by large particles and is highly dependent on the aerodynamic properties of the particles.
t3.3	Sedimentation	The settling down of the delivered particles. This is generally observed in the bronchioles and alveoli.
t3.4	Interception	This occurs when particles, due to their shape and size, interact with the airway surface and is experienced when the particles are close to the airway wall.
t3.5	Diffusion	Is the transport of particles from a region of higher concentration to lower concentration, is observed for particles that are less than 0.5 μm in diameter and occurs in the regions where the airflow is low. This is highly dependent on the geometric diameter of the particles.

420 avoid deposition in the throat and reach the respirable airways
421 (Fig. 1) and the periphery of the lung (61). Particles less than
422 1 μm (referred to as NPs) are driven by diffusion and are most
423 likely to be exhaled, hence they are therefore often delivered
424 within microparticles. In addition, upon long term storage
425 NPs tend to aggregate due to high particle-particle interac-
426 tions (60). Microparticles prepared from NPs are typically
427 about 1–5 μm in size and usually also encompass inert phar-
428 maceutical excipients (sugars, amino acids etc.) that act as
429 carriers. The excipients dissolve upon encountering the respi-
430 ratory environment thereby releasing the NPs.

431 Different types of NPs have been explored for vaccine
432 delivery and antigenic peptides or proteins are either surface
433 adsorbed or encapsulated within the NPs. Table IV outlines
434 some types of NPs evaluated for vaccine delivery.

435 This review focuses on polymer-based NPs because they have
436 been extensively investigated as vaccine delivery systems due to
437 their enhanced uptake by phagocytic cells, thereby facilitating
438 antigen internalization and presentation in DCs. In addition,
439 both antigen and materials that augment the immune response
440 (adjuvants) can be encompassed together in nanocomposite
441 microparticles, resulting in their simultaneous delivery (64).

Polymer-based Nanoparticles

442

443 Wide varieties of polymers, both natural and synthetic, have
444 been exploited to form biodegradable NPs. In addition, some
445 of the polymers can act as adjuvants themselves (65). Natural
446 polymers that have been widely investigated for formulating
447 NPs include albumin, alginate, chitosan, collagen, cyclodex-
448 trin and gelatin; synthetic polymers include polyesters, poly-
449 lactides, polyacrylates, polylactones and polyanhydrides
450 (66,67). While natural polymers have a relatively short dura-
451 tion of drug release, synthetic polymers can be tailored to
452 release the drug over days to several weeks allowing the usage
453 of a single dose rather than multiple doses (65).

454 Biodegradable polymers have gained significant attention
455 for the preparation of NPs for drug delivery and are often
456 favored as they offer several advantages such as controlled or
457 sustained drug release, biocompatibility with the surrounding
458 tissues and cells, low toxicity, are nonthrombogenic and are
459 more stable in the blood (66,68). Biodegradable polymer-based
460 NPs also offer an additional advantage for vaccine delivery
461 systems by acting as adjuvants and aiding in activating both
462 cellular and humoral immune responses (69). It has been

t4.1	**Table IV** Examples Of Nanoparticles Currently Being Evaluated For Vaccine Delivery				
t4.2	Nanoparticles	Description	Size	Vaccine	Ref
t4.3	Micelles (Peptide Cross-linked micelles-PCMs)	PCMs are composed of block copolymers and encapsulate immuno stimulatory DNA in the core and bind peptide antigens through disulphide linkages. In the presence of a high concentration of glutathione they deliver antigenic peptides and immuno stimulatory DNA to APCs	50 nm	HIV peptide vaccine	(129)
t4.4	Liposomes	Dimyristoyl phosphatyl-choline (DMPC):cholesterol(CH)-(7:3) liposomes were prepared by dehydration-rehydration followed by freezing-thawing method. The enzyme, GUS, was successfully encapsulated and showed encouraging activity following aerosolization	~ 6.4 μm (with 1:4 liposome:mannitol)	β-Gluc-uronidase – enzyme (GUS)	(130)
t4.5	Polymersomes	poly(g-benzyl-L-glutamate)-K (PBLG50-K) polymersomes were prepared by the solvent removal method and influenza hemagglutinin (HA) was surface adsorbed. When tested *in vivo*, polymersomes acted as an immune adjuvant and showed an improved immunogenicity.	250 nm	influenza hemagglutinin (HA) – subunit vaccine	(131)
t4.6	Polymer-based	Porous poly-L-lactic acid (PLA) and poly lactic-co-glycolic-acid (PLGA) NPs were prepared by a double-emulsion-solvent evaporation method encapsulating HBsAg and were tested for pulmonary delivery in rat spleen homogenates. The study demonstrated enhanced immune responses.	474–900 nm	hepatitis B surface antigen (HBsAg)	(24)

reported that upon phagocytosis by APCs, such as DCs, these NPs release the antigen intercellularly and elicit CD8+ and CD4+ T cell responses (70).

In a study performed by Bivas-Benita M *et al.*, the potential of enhanced immunogenicity upon pulmonary delivery of DNA encapsulated in chitosan NPs was evaluated. Chitosan-DNA NPs were prepared by the complexation-coacervation method and the resultant DNA-loaded NPs had an average size of 376± 59 nm (*n*=5), zeta-potential of 21±4 mV (*n*=5) and a loading efficiency of 99%. Pulmonary administration of the chitosan-DNA NPs was shown to induce increased levels of IFN-γ secretion compared to pulmonary delivery of the plasmid in solution via the intramuscular immunization route. This indicates the plausibility of achieving pulmonary delivery of DNA vaccines with increased immunogenicity against tuberculosis compared to immunization through intramuscular route (71).

The polylactides PLA and PLGA are the most broadly investigated synthetic polymers in the field of drug delivery (66,67,72). These are rapidly hydrolyzed upon implantation into the body and are eventually removed by the citric acid cycle. The hydrolyzed products form at very slow rate and include lactic acid and glycolic acid which are biologically compatible and easily metabolized making them safe and non-toxic (66,73). However, the acidic degradation products can cause problems by eliciting inflammation and also a reduction in pH within the microparticles resulting in the hydrolysis of the biopharmaceuticals (74).

Muttil *et al.* prepared novel NP-aggregate formulations using poly(lactic-co-glycolic acid) (PLGA) and recombinant hepatitis B surface antigen (rHBsAg) and showed that the dry powder formulations elicited a high mucosal immune response after pulmonary immunization of guinea pigs without the need for adjuvants. They prepared three different formulations of dry powders by spray-drying with leucine, (1) rHBsAg encapsulated within PLGA/polyethylene glycol (PEG) NPs (antigen NPs, AgNSD), (2) a physical mixture of rHBsAg and blank PLGA/ PEG NPs (antigen NP admixture (AgNASD), and (3) rHBsAg encapsulated in PLGA/PEG NPs with free rHBsAg (antigen NPs plus free antigen). All the particles had mass median aerodynamic diameters (MMAD) of around 4.8 μm and a fine particle fraction (FPF) of 50%. After immunization the highest titre of serum IgG antibodies was observed in the control group immunized with alum adsorbed with rHBsAg (Alum Ag) (IM route) whereas the highest IgA titres were observed for animal groups immunized with powder formulations via the pulmonary route. It was also noteworthy guinea pigs immunized with AgNASD dry powder exhibited IgG titers above 1,000 mIU/ ml in the serum (required 10 mIU/ml) suggesting the potential of administering novel dry powder formulations via the pulmonary route (75).

Recently a new class of biodegradable polymers, polyketals, have been developed and are largely being investigated for drug delivery purposes (76,77). This class of polymers have non-acidic degradation products and pH-sensitive ketal linkages in their backbone. These polyketals offer several advantages for vaccine delivery such as exhibiting pH-dependent hydrolysis but yet are degradable in acidic phagolysosomes. Polyketal copolymers degrade into biocompatible small molecules minimizing inflammation compared to PLGA. An aliphatic polyketal, poly(cyclohexane-1,4-diyl acetone dimethylene ketal) (PCADK) degrades into acetone and 1,4-cyclohexanedimethanol which are both biocompatible, and has a hydrolysis half-life of 24 days at pH 4.5 (77). This was later modified to a co-polyketal termed PK3 synthesized from 1,4-cyclohexanedimethanol and 1,5-pentanediol with a hydrolysis half-life of 1.8 days at pH 4.5 (64) making it much suitable for vaccine delivery.

Heffernan MJ and Murthy N successfully prepared acid-sensitive polyketal NPs that released the loaded therapeutics in the acidic environments of tumors, inflammatory tissues and phagosomes. Polyketal NPs, 280–520 nm in diameter, were prepared by an oil-in-water (O/W) emulsion method using poly(1,4-phenyleneacetone dimethylene ketal) (PPADK), a new hydrophobic polymer that undergoes acid-catalysed hydrolysis into low molecular weight hydrophilic compounds. (76). Heffernan *et al.* used polyketal PK3 to formulate a model vaccine that elicits CD8+ T cell responses. PK3 microparticles encapsulating ovalbumin (OVA), poly(inosinic acid)–poly(cytidylic acid) (poly(I:C)) - a TLR3 (Toll like receptor) agonist and a double-stranded RNA analog were prepared using single emulsion method. PK3-OVA-poly(I:C) microparticles (1–3 μm) at a dosage of 0.01 μg/mL were then supplied to murine splenic DCs and a higher percentage of IFNγ-producing CD8+ T cells, TNF-α and IL-2 production in CD8+ T cells were observed than with DCs treated with PK3-OVA particles or soluble OVA/poly(I:C) implying polyketal PK3 microparticles have potential for vaccine delivery (64).

Preparation of Polymer-Based Nanoparticles

Different methods have been employed to synthesize polymer-based NPs depending on the subsequent application and type of drug. Polymer-based NPs can either encapsulate or surface adsorb the drug (68,78). Here we review some of the most widely used methods to prepare polymer-based NPs. However, a more detailed review and analysis of these methods can be found at Reis P *et al.* (78) and Avnesh K *et al.* (68).

Emulsification/Solvent Evaporation and Nanoprecipitation. Emulsification/solvent evaporation, also referred to as solvent emulsion–evaporation, involves the emulsification of an organic polymer solution into an aqueous phase followed by the evaporation of the organic solvent (78). The polymer with or without the drug is dissolved in a volatile organic solvent like acetone, ethyl acetate, chloroform or dichloromethane etc. and is then transferred into stirring aqueous

phase with or without the presence of an emulsifier or stabilizer. This emulsion is then sonicated to evaporate the organic solvent and form NPs (68) (Fig. 3a). The size of the resultant particles can be controlled by varying the type, viscosity and amount of organic and aqueous phases, stir rate and temperature (78).

Singh J *et al.* prepared diphtheria toxoid (DT) loaded poly-(ε-caprolactone) (PCL) NPs via a double emulsification solvent evaporation method (w/o/w) for investigating their potential as a mucosal vaccine delivery system. Briefly, DT was added to the internal aqueous phase containing 0.25 ml 10%w/v polyvinyl alcohol (PVA). The solution was emulsified with the organic phase comprising 100 mg of PCL in 5 mL of dichloromethane (DCM), using a homogenizer at 12,000 rpm for 2 min. The formulations were then stirred magnetically at ambient temperatures and pressure for 15–18 h to allow solvent evaporation and NP formation. The resultant NPs were approximately 267 ± 3 nm in size with a zeta-potential of -2.6 ± 1.2 mV. Also, the PCL NPs induced DT serum specific IgG antibody responses significantly higher than PLGA (79).

The nanoprecipitation method is a single step method which is usually employed for entrapping hydrophobic drug moieties. In this method, the drug and the polymer are dissolved in a water-miscible solvent, such as acetone, acetonitrile or methanol (80). This organic phase is then added drop-wise to an aqueous phase with or without an emulsifier/stabilizer under magnetic stirring (68). NPs are formed due to rapid solvent diffusion and the solvent is finally removed from the emulsion under reduced pressure (81) (Fig. 3b).

Lee JS *et al.* prepared poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) NPs via a nanoprecipitation method. Firstly, a predetermined concentration of MPEG-PCL block copolymer was dissolved in 10 mL of organic solvent (acetone, acetonitrile or THF). This polymer solution was then added drop wise into deionized water (100 mL) under magnetic stirring. The organic solvent was then evaporated under reduced pressure using a rotary evaporator, and the resultant NPs were isolated from the aqueous solution. Using different organic solvents and concentrations of polymer yielded NPs particles between ~50 to 150 nm (82).

Emulsification and Solvent Displacement. The emulsification and solvent displacement method is also known as emulsification solvent diffusion. This method involves the precipitation of the polymer from an organic solution and subsequent diffusion of the organic solvent into an aqueous phase (78). The solvent that aids in the formation of emulsion must be miscible with water. For example, the organic polymer solution can be added to an aqueous phase, which often contains a stabilizer, under strong stirring. Upon the formation of the emulsion (O/W), a large quantity of water is added so as to dilute it favoring the diffusion of additional organic solvent from the dispersed droplets. This process leads to the precipitation of the polymer (81). An interfacial turbulence is created between the two phases as the solvent diffuses resulting in the formation of smaller particles and is believed that as the water-miscible solvent concentration increases the NPs tend to acquire a smaller size (80) (Fig. 3c).

Ranjan AP *et al.* have recently prepared biodegradable NPs containing indocynanine green (ICG) using chitosan modified poly(L-lactide-co-epsilon-caprolactone) (PLCL): poloxamer (Pluronic F68) blended polymer by an emulsification solvent diffusion technique. PVA and chitosan were used as stabilizers in the process of making the NPs. The average particle size of the resultant NPs was between $146\pm$
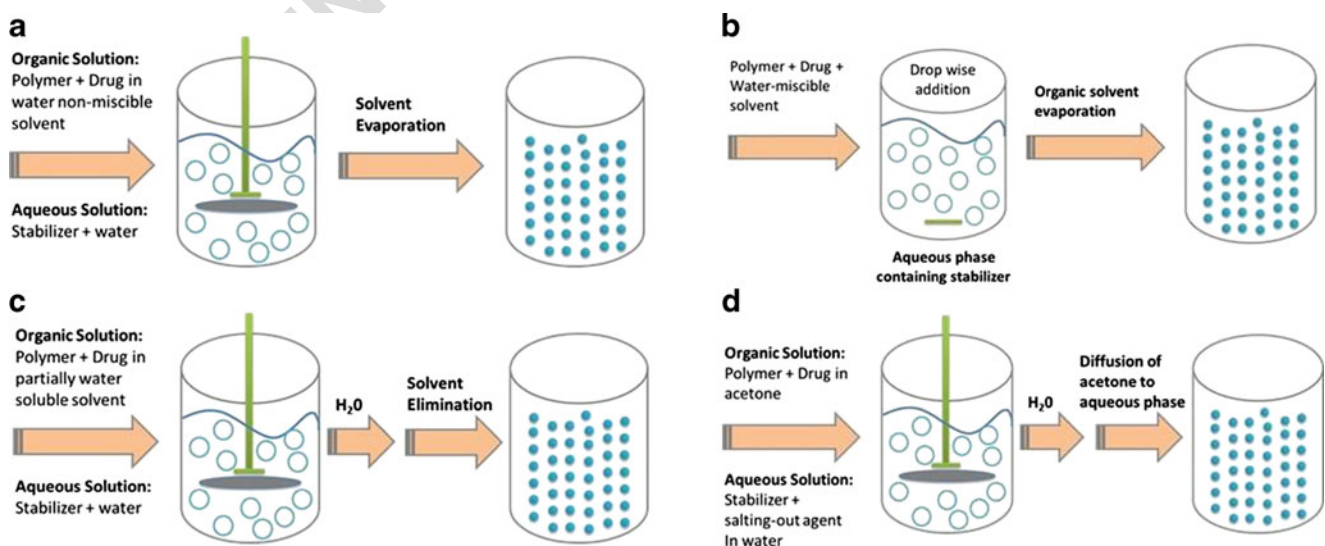

Fig. 3 Schematic representation of **a** emulsification/solvent evaporation technique, **b** emulsification and solvent displacement technique, **c** salting-out technique and **d** nanoprecipitation technique.

631 3.7 to 260±4.5 nm and the zeta potential progressively
632 increased from −41.6 to +25.3 mV with increasing amounts
633 of chitosan (83).

634 *Salting Out.* The salting out method is based on the separation
635 of a water-miscible organic phase from an aqueous solution by
636 adding salting out agents (78,80,84). Briefly, the polymer is
637 dissolved in a water-miscible organic solvent such as acetone
638 or tetrahydrofuran (THF) which is then added under strong
639 stirring to an aqueous solution containing salting out agents
640 (for example magnesium chloride, calcium chloride) and an
641 emulsifier or stabilizer to form an O/W emulsion (80,81,85).
642 This O/W emulsion is diluted by adding a large volume of
643 water under mild stirring thus reducing the salt concentration/
644 ionic strength and favouring the movement of the water-
645 miscible organic solvent into the aqueous phase. This process
646 leads to the formation of nanospheres and as a final step the
647 NPs formed are freed from the salting out agents either by
648 centrifugation or cross-flow filtration (80) (Fig.3d).

649 Konnan YN *et al.* prepared sub-200 nm NPs using a
650 salting out method. Typically, a solution of PLGA and
651 PLA in THF was added under mechanical stirring to an
652 aqueous phase containing PVA and magnesium chloride
653 hexahydrate (MgCl$_2$.6H$_2$O) as a salting out agent forming
654 an O/W emulsion. To this, a large volume of water was
655 added favoring migration of the water-miscible organic
656 solvent into the aqueous phase forming NPs which were
657 later purified by cross flow filtration (86).

658 Table V lists some of the advantages and disadvantages
659 of nanoparticle preparation methods (77).

Encapsulation or Adsorption

662 A high loading capacity is one of the most desired qualities of
663 NP-based vaccines. The main advantage of having a high
664 loading capacity is that the amount of polymer required to
665 carry the drug/vaccine is reduced (81) hence minimizing any
666 toxic effects from the polymer. Drugs/vaccines can be loaded
667 into or onto NPs using two approaches (Fig. 4) (87). The first is
668 encapsulation where the drug/vaccine is incorporated into the

669 NP at the time of preparation; the second is adsorption where
670 the drug/vaccine is either chemically or physically adsorbed
671 onto the NP after preparation.

672 It is important to note that the chemical structure of the
673 drug/vaccine, the polymer and the conditions of drug loading
674 influence the amount of drug/vaccine bound to the NPs and
675 the type of interactions that occur between them (81). In addi-
676 tion, the encapsulation or adsorption of a drug/vaccine depends
677 on the disease to be treated or prevented, route of administra-
678 tion, manufacturing feasibility and economic challenges.

679 Bivas-Benita M *et al.* prepared PLGA–polyethyleneimine
680 (PEI) NPs by an interfacial deposition (88) method. The
681 resultant NPs were loaded with Mycobacterium tuberculosis
682 (Mtb) Antigen 85B (Ag85B) by adding the NP suspension to
683 25 μg/mL DNA plasmid solution. The characterization stud-
684 ies revealed that the particle size increased from 235 to
685 275 nm when resuspended in water and 271 nm in saline with
686 the mean zeta potential increase from +38.8 mV to +40.6 mV
687 respectively. The NPs greatly stimulated human DCs resulting
688 in the secretion of IL-12 and TNF-α at comparable levels to
689 that observed after stimulation using lipopolysaccharide
690 (LPS) (89).

691 Biodegradable polymer-based NPs have been widely ex-
692 plored and appear to be well tolerated when administered into
693 the body. These NPs have gained significant attention and are
694 being accepted as effective delivery systems with the develop-
695 ment of NP based vaccines (90,91). In addition, the NP based
696 vaccines need to be formulated appropriately, as dry powders
697 and at low cost to help achieve effective mass vaccination.

Adjuvants

699 Modern day vaccines contain pure recombinant or synthetic
700 antigens that are less immunogenic than live or killed whole
701 organism vaccines. Thus, in order to obtain a strong im-
702 mune response upon administration of antigen and provide
703 long term protection against the infection, adjuvants are
704 included within the formulation (92). Adjuvants are substan-
705 ces used in combination with an antigen to produce a
706 stronger and more robust immune response than the anti-
707 gen alone (93). Adjuvants also provide a depot for the

t5.1 **Table V** Advantages and Disadvantages of Nanoparticle Preparation Methods

	Method	Advantages	Disadvantages
t5.2			
t5.3	Emulsification/Solvent Evaporation	Hydrophilic and hydrophobic drugs can be encapsulated	Agglomeration of nanodroplets during evaporation
t5.4	Emulsification and Solvent Displacement	Control over the size of nanoparticles	Possibility of water-soluble drug leaking into the external aqueous phase, Large amounts of water to be removed
t5.5	Salting Out	High loading efficiency, Easy scale-up	Removal of electrolytes, Incompatibility of salting-out agents with drugs
t5.6	Nanoprecipitation	Simple, fast and reproducible, Easy scale-up, Low surfactant concentrations required	Less polymer in the organic phase

Fig. 4 The molecule of interest (DNA/Drug/Peptide/Protein) is either encapsulated (*Left*) within or surface adsorbed (*Right*) onto the polymer-based nanoparticle.
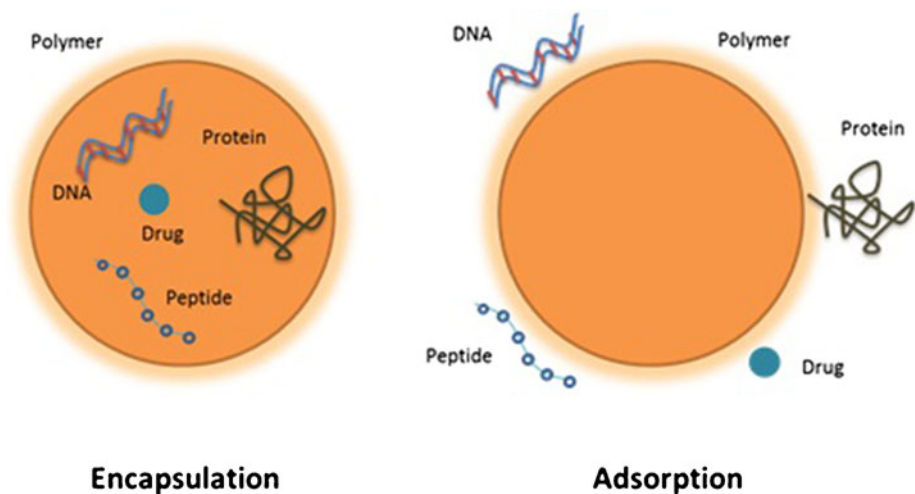
antigen favoring a slow release, reduce the dose of antigen required to generate a strong immune response, modulate the immune response, aid in targeting the APCs, and provide danger signals helping the immune system respond to the antigen (92–94). The selection of an adjuvant depends on the antigen, delivery system, route of administration and possible side-effects. However, an ideal adjuvant should have a long shelf life and be safe, stable, biodegradable, economical and should not induce an immune response against themselves (92).

Despite massive efforts over nearly 90 years into the research and development of adjuvants, the list of adjuvants that are clinically approved is short. The prime reason being their safety coupled with limited data on the predictability of safety using available animal models (95). The serious adverse events in the recent clinical trials of Merck's (96) and Novartis's (NCT00369031) (97) HIV vaccines using adenovirus- and toxin-based adjuvanted delivery systems has moved the research into further investigations in developing nutritive adjuvanted delivery systems (Vitamins A, C, D, E, flavonoids and plant oils). These may prove safer in clinical trials (98,99). Table VI lists adjuvants in development or licensed for human use.

Alum salts have a well-established safety record, are the most widely used human adjuvants and are used as standards to assess other adjuvants (92,93,95,100). Despite their wide use their mechanism is poorly understood and thus rarely induce human responses (92).

Wee JLK *et al.* used a sheep animal model to evaluate the delivery of ISCOMATRIX adjuvanted influenza vaccine via its mucosal site of infection for improved vaccine effectiveness. Upon pulmonary immunization with low antigen doses (0.04 μg) of adjuvanted influenza equivalent serum antibody levels were induced when compared to an almost 375-fold higher dose (15 μg) unadjuvanted influenza delivered subcutaneously suggesting the successful use of this combination for improved protection (101).

DRY POWDER PREPARATION TECHNIQUES

The use of liquid suspensions of NPs are often accompanied by several disadvantages such as particle aggregation and sedimentation leading to physico-chemical instability, reduced or loss of biological activity of the drug, contamination, and hydrolysis leading to degradation of the polymer (102). To overcome these problems, preparations can be stored and transported in a dry form (102). In addition, for vaccines, the delivery of a dry powder by inhalation has the potential benefits of a) increased stability during transport and administration, b) increased safety by eliminating contamination risks and c) improved cost-effectiveness (103). The most commonly used methods for transforming liquid preparations into dry powders are freeze-drying, spray-drying, spray-freeze-drying and the use of super critical fluid technologies. Each of these methods has advantages and disadvantages and are selected depending on the desired attributes such as narrow particle size

Table VI List of Adjuvants in Either Development, Testing or for Human Use

Category	Examples
Mineral Salts	Aluminium hydroxide (Alum)
	Potassium aluminium sulphate
	Aluminium phosphate
Oil emulsions	MF59
Particulate adjuvants	Virosomes
	ISCOMS (Immuno stimulating complexes)
Microbial derivatives	Monophosphoryl lipid A-MPL[TM]
Plant derivatives	QS-21 (Saponin)
	ADVAX
Miscellaneous	AS04 (liposome formulation containing MPLA & QS-21), polymeric adjuvants, CpG oligodeoxynucleotides, vitamins

distribution, improved bioavailability, enhanced stability, improved dispersibility and controlled release (104,105).

Freeze-Drying

Freeze-drying, also known as lyophilisation, is commonly used in industry to ensure long term stability and preservation of the original properties of various biological products such as viruses, vaccines, proteins, peptides and their carriers; NPs and liposomes (102,106). This process comprises of removing water from a frozen sample by sublimation and desorption under vacuum (106) and can be divided into three steps: freezing (solidification), primary drying (ice sublimation) and secondary drying (desorption of unfrozen water) (102). However, this process is relatively slow, very expensive and generates various stresses on the biological product during both the freezing and drying steps (106). Protectants in the form of excipients are usually added to stabilize the products, avoid aggregation and to ensure acceptable tonicity and reconstitution (106,107). Sugars such as glucose, sucrose, trehalose, mannitol, lactose, dextran or maltose with or without surfactants such as poly(vinyl) alcohol or poloxamer 188 are often employed as protectants to stabilize the product and prevent coalescence (107,108). The concentration and the NP/sugar mass ratio also play an important role in determining the stability and long term storage of the final product (102). Anhorn MG *et al.* evaluated the effect of different concentrations of sucrose, mannitol and trehalose as cryoprotectants on the physico-chemical characteristics of resulting NPs by analyzing the appearance, particle-size and polydispersity index (107). Long term stability studies indicated that the absence of cryoprotectants led to particle growth whereas their presence reduced aggregation. Particles freeze-dried with sucrose and trehalose at 2% and 3%w/v had more controlled particle size and these sugars appeared to be superior to mannitol at similar concentrations (107).

Spray-Drying

Spray-drying is a one-step preparation of dry powders. It is a process that converts liquid feed (solution, suspension or colloidal dispersion) into dry particles (109). The process can be divided into four parts (110): atomization (1), spray-air contact (2), drying (3) and separation (4). The liquid feed is atomized *(1)* to break the liquid into droplets and this spray form comes into contact with a hot gas *(2)*, causing rapid evaporation of the droplets to form dry particles *(3)*. The dry particles are then separated from the hot gas with the help of a cyclone *(4)* (105). Compared to particles obtained from micronization using milling, spray-dried particles are more spherical and have a homogenous size-distribution resulting in a higher respirable fraction which is advantageous for pulmonary delivery (105). In addition, spray-drying has the advantage of being; simple, easily scalable, cost-effective, suitable for heat-

sensitive products and enables high drug loading (110). An economically acceptable yield can now be achieved with the fourth and newest generation of laboratory-scale spray dryer developed by Büchi, the Nano Spray Dryer B-90. This nano spray dryer can generate particles of size ranging from 300 nm to 5 µm for milligram sample quantities at high yields (up to 90%) (111). However, there is a chance of degradation of macromolecules during the process due to high shear stress in the nozzle and thermal stress while drying (105). Fourie PB *et al.* (21) describes the challenges such as thermal stress, osmotic stress, and scalability involved with spray-drying of vaccines. Fourie PB *et al.* formulated a dry powder TB vaccine for delivery to the lung by preparing *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) spray-dried particles which, when administered into *M. tuberculosis* infected guinea-pigs, resulted in enhanced immunogenicity levels compared to an equal dose injected subcutaneously into control animals (21).

Spray-Freeze Drying

Spray-freeze drying (SFD) is a drying process that usually involves atomization, rapid freezing and lyophilisation (112). A solution containing the drug is sprayed into a vessel that contains a cryogenic liquid such as nitrogen, oxygen or argon. As the boiling temperatures of these cryogenic liquids are very low they cause the droplets to freeze instantly. The resulting droplets are then collected and lyophilized to obtain porous dry powder particles suitable for respiration (105). The advantage of SFD is the ability to produce particles with adjustable sizes (112) and as it is conducted at sub-ambient temperature, thermolabile polymers and highly potent biopharmaceuticals can be formulated into dry powder products (105). However, the major disadvantage of this technique is the stresses associated with freezing and drying, which may cause irreversible damage to proteins (113). This is displayed as structural denaturation, aggregation and loss of biological activity upon rehydration (105). In addition, loss of stability due to unfolding and aggregation remains a major challenge (113) and also the method has low process efficacy, is time consuming, and expensive (114).

Amorij J-P *et al.* showed that an influenza subunit vaccine powder prepared by SFD using oligosaccharide inulin as a stabilizer and delivered via the pulmonary route to BALB/c mice induced systemic humoral (IgG), cell-mediated (Il-4, IFN-γ) and mucosal immune responses (IgA, IgG). Whereas vaccination with a liquid subunit vaccine via either pulmonary or intramuscular route induced only systemic humoral (IgG) immune responses suggesting that powder vaccine formulations could be beneficial for immunization (23).

Supercritical Fluid Technology

Supercritical fluids (SCF) are compressed gases or liquids above their critical temperatures (Tc) and pressures (Pc), and possess

860 several advantages of both gases and liquids (105). The density
861 and thus solvating power can be controlled by varying the
862 temperature and pressure. SCF can be prepared using carbon
863 dioxide (CO_2), water, propane, acetone, nitrous oxide (N_2O),
864 trifluoromethane, chlorodifluoromethane, diethyl ether, water,
865 or CO_2 with ethanol (114). However, because of its accessible
866 critical point at 31°C and 74 bar, its low cost and non-toxicity,
867 CO_2 is the most widely used solvent in SCF. In addition, its
868 low critical temperature makes supercritical (SC) CO_2 suitable
869 for handling heat-labile solutes at conditions close to room
870 temperature. Therefore, SC CO_2 has potential as an alterna-
871 tive to conventional organic solvents for use in solvent-based
872 processes for forming solid dosage forms (105).

873 There are two major principles for particle precipitation
874 with supercritical fluids. One employs SCF as a solvent and
875 the other as an antisolvent (115). In the first, the drug is
876 dissolved in the SCF followed by sudden decompression, after
877 which the solution is passed through an orifice and rapidly
878 expanded at low pressure. Rapid Expansion of a Supercritical
879 Solution (RESS) employs this principle (114). In the second
880 process, the solute is insoluble in SCF and hence utilizes SCF
881 as an antisolvent. A solute is dissolved in an organic solvent
882 and then precipitated by the SCF (antisolvent). Precipitation
883 occurs when the SCF is absorbed by the organic solvent
884 followed by expansion of the liquid phase and a decrease in
885 the solvation power leading to particle formation. The Gas
886 Anti-Solvent (GAS), Aerosol Solvent Extraction System
887 (ASES), Supercritical Fluid Antisolvent (SAS), Precipitation
888 with Compressed Antisolvent (PCA), Solution Enhanced Dis-
889 persion by Supercritical Fluids (SEDS), and supercritical fluid
890 extraction of emulsion (SFEE) are the processes that employ
891 this second principle (114). Using these techniques particles
892 can be formed in a well-ordered fashion to achieve the desired
893 morphology and any negative effects on the macromolecules
894 can be minimized (105,113). Thorough discussions of these
895 techniques including their advantages and disadvantages have

896 been recently published by Al-fagih I *et al.* (114) and elsewhere
897 (105,113,115–118).

898 The fine powders produced via SCF precipitation are often
899 less charged than those produced mechanically allowing them
900 to flow more freely and thus to be more easily dispersed from a
901 DPI. In addition, SCF processes allow the production of inhal-
902 able particles that are more uniform in terms of crystallinity,
903 morphology, particle-size distribution and shape than those
904 produced via jet milling. In spite of its potential, SCF is still
905 classified as an emerging technology that is still to be exploited
906 in DPI products; with concerns being raised over the potential
907 denaturing effects of the solvents/antisolvents used in this pro-
908 cess (105). Amidi M *et al.* prepared diphtheria toxoid (DT)
909 containing microparticles using a supercritical fluid (SCF)
910 spraying process and obtained dry powder microparticles with
911 a median volume diameter between 2 and 3 μm. Pulmonary
912 immunization of guinea pigs with DT-TMC (N-Trimethyl
913 chitosan) microparticles resulted in a strong immunological
914 response as reflected by the induction of IgM, IgG, IgG1 and
915 IgG2 antibodies comparable to or significantly higher than
916 those achieved after subcutaneous (SC) administration of
917 alum-adsorbed DT demonstrating an effective new delivery
918 system for pulmonary administered DT antigen (119).

919 Table VII highlights some recent studies that have
920 employed various dry powder preparation techniques and
921 the subsequent evaluation for vaccine delivery.

CONCLUSION

923 Pulmonary administration has gained significant attention
924 in the recent years as a potential non-invasive route for
925 vaccines, and has also shown great promise as an effective
926 means of vaccination. Much of the success is due to the
927 lung's large surface area (80 sq. m), and rich blood supply
928 leading to rapid absorption coupled with an abundance of

Table VII Recent studies on dry powder particle-based vaccine delivery

Disease	Antigen	Carrier/Stabilizer	Dry Powder Preparation	Size (μm)	Model	Ref
Bacterial Infections	Bacteriophages	Trehalose, Leucine	SD	2.5–2.8	NA	(132)
Diptheria	Diptheria Toxoid	Chitosan	SCF	3–4	GP	(119)
Diptheria	Diphtheria CRM-197 antigen	L-leucine	SD	~ 5	GP	(32)
Hepatitis B	Recombinant hepatitis B surface antigen (rHBsAg)	Leucine	SD	4.8	GP	(75)
Influenza	Influenza monovalent	Inulin	SD, SFD	2.6 (SD), 10.5 SFD)	M	(133)
Influenza	Influenza subunit	Inulin	SFD	~ 10	M	(23)
Tuberculosis	Ad35-vectored tuberculosis (TB) AERAS-402	Mannitol-cyclodextrin-trehalose-dextran, MCTD	SD	3.2–3.5	NA	(134)
Tuberculosis	Bacille Calmette-Guerin (BCG)	Leucine	SD	2–3	GP	(135)
Tuberculosis	Recombinant antigen 85B (rAg85B)	NA	SD	2.8	GP	(136)

SD Spray drying, *SFD* Spray-freeze drying, *SCF* Supercritical Fluid; *M* Mice, *GP* Guinea Pigs; *NA* Not Available

local APCs that present antigen in a way to induce both mucosal and systemic immune response. Recent progress in targeting vaccines specifically to DCs for an enhanced immune response with low doses has paved way for developing new vaccine technology. Polymer-based NPs offer the advantage of biodegradabiltiy, avoiding antigen degradation if encapsulated and through chemical attachments can target DCs. However, more research is needed to understand the fate of NPs after inhalation, their interaction with the biological cells and their toxicity (nanotoxicity). The method of formulation of NP based vaccines into dry powders is of equal importance as it provides the opportunity to maintain the stability and integrity of the antigen, ease of transport and administration. The right combination of polymer chemistry, polymer-based NPs, immunology, dry powder technology, delivery device and animal models will lead to the discovery of next generation of vaccine delivery systems.

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