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49	Keywords separated by ' - '	antigen presenting cells - dendritic cells - dry powder - polymeric nanoparticles - pulmonary delivery of vaccines	

50 Foot note
information

Nanocarriers Targeting Dendritic Cells for Pulmonary Vaccine Delivery

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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

ABBREVIATIONS

AMs	Alveolar macrophages	33
APCs	Antigen presenting cells	38
BAL	Bronchoalveolar lavage	39
CLRs	C-type lectin receptors	42
DCs	Dendritic cells	43
DPI	Dry powder inhalations	46
FD	Freeze-drying	48
HLA	Human leukocyte antigen	50
ILs	Interleukins	52
LN	Lymph node	53
MHC	Major histocompatibility complex	56
MN	Mannan	58
NPs	Nanoparticles	60
PCL	Poly-ε-caprolactone	62
PEG	Polyethylene glycol	63
PEI	Polyethyleneimine	66
PLA	Poly lactide or poly-L-lactic acid	68
PLGA	Poly lactic-co-glycolic-acid	70
PRRs	Pattern recognition receptors	72
PVA	Polyvinyl alcohol	73
SCF	Supercritical fluid	76
SD	Spray-drying	78
SFD	Spray-freeze drying	80
TLRs	Toll-like receptors	82
TMC	N-Trimethyl chitosan	83
VLPs	Virus-like particles	86

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

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

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

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

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

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

Table I List of Reported Cases by Disease According to World Health Statistics (WHS) 2011 t1.1

Disease	Reported Cases (WHS 2011) ^a	t1.2
Diphtheria	857	t1.3
Malaria	81,735,305 (1990–2009)	t1.4
Measles	222,318	t1.5
Mumps	546,684	t1.6
Tetanus	9,836	t1.7
Tuberculosis	5,797,317	t1.8
Pneumonia (Children <5 years)	~1,400,000 (18% of all child deaths in 2008) (120)	t1.9

^aData 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). 147 148

PULMONARY VACCINE DELIVERY 149

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 patent-
 ed 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 biopharma-
 ceuticals, 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). 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176

Anatomy of the Human Lung 177

The human lung, weighing about 1 kg, is divided by the
 pleural membranes into three lobes on the right and two 178 179

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

The submucosal glands and the ‘goblet cells’ (present on the bronchial surface) secrete mucus onto the bronchial surfaces. The submucosal glands also help in producing an electrolyte solution on which the mucus rests. The mucus covering the airways is transported towards the mouth with the coordinated movement of cilia present on top of the ciliated columnar cells. This mucus transported to the mouth is then swallowed. This process of mucus movement from the bronchial surfaces to the mouth for swallowing is mainly responsible for removing any foreign material that lands on the bronchial surfaces (25).

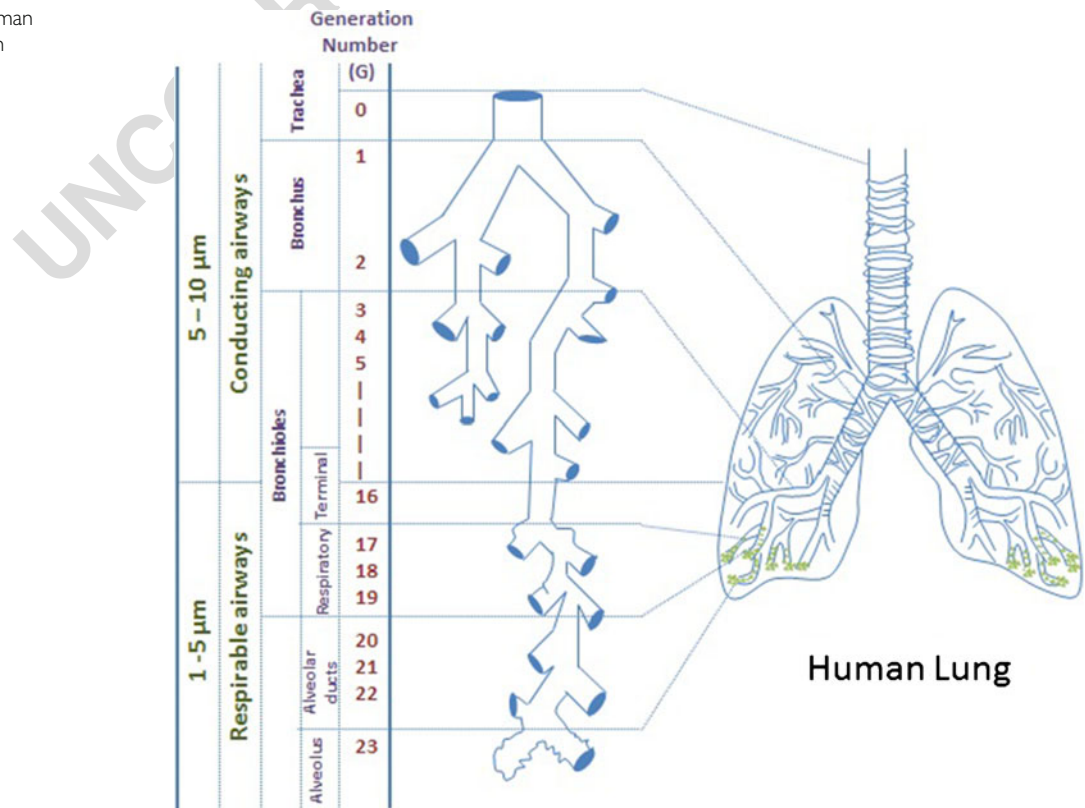
The alveoli and the pulmonary capillaries are separated by a barrier composing of endothelial cells, interstitial space, and pneumocytes (pulmonary epithelial cells). The pneumocytes

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

Lung as a Delivery Site for Drugs

The lung is an excellent choice for the delivery of biopharmaceuticals for the treatment of both local and systemic disorders as it offers several advantages such as; large surface area (80 sq. m), dense vasculature, rapid absorption leading to an immediate onset of action, thin alveolar epithelium, less enzymatic activity than gut and a high capacity for solute exchange (29). With regards to the delivery of vaccines, a high density of APCs including alveolar macrophages (AMs), DCs and B cells represent an ideal target to induce a strong immune response resulting in both mucosal and systemic immunity (14). Recent research has confirmed that the induction of an immune response at one mucosal site elicits an immune response at distant mucosal sites by mucosal lymphocyte trafficking leading to both mucosal and systemic immunization (15,30). There is some evidence that mucosal immunization may also reduce the dosage required to achieve the desired immunity compared to liquid formulations administered via the parenteral route (3).

Fig. 1 Diagram of the human lung and particle deposition based on size.



232 **Pulmonary vs Parenteral Vaccine Delivery**

233 In development of novel anti-tuberculosis vaccines, Ballester
 234 M *et al.* demonstrated, that inhaled vaccine compared favorably to an intradermal route of delivery. In particular,
 235 vaccination with NP-Ag85B and immune-stimulatory oligo-
 236 nucleotide CpG as a Th1-promoting adjuvant via the pul-
 237 monary route modified the pulmonary immune response
 238 and provided significant protection following a *Mycobacterium*
 239 *tuberculosis* (*Mtb*) aerosol challenge (31).
 240

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

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

264 **Dendritic Cells**

265 Dendritic cells (DCs) were first identified in 1868 by Paul
 266 Langerhans in the basal layer of the epidermis (34). How-
 267 ever, it took more than a century to properly identify them
 268 as white blood cells related to macrophages and monocytes,
 269 and to understand their importance in the control of immu-
 270 nity (34,35). In 2011, the Nobel Prize in Physiology or
 271 Medicine was awarded to Ralph M. Steinman for his dis-
 272 covery of DCs and their role in adaptive immunity paving
 273 the way for more research in the field of immunity and
 274 vaccines (36). It has become evident over the years that
 275 DCs are APCs, true ‘professionals’ (37) with exceptional
 276 capability to internalize, process and present antigens
 277 through major histocompatibility complex (MHC) class I
 278 and II pathways. DCs induce a strong immune response
 279 by activating naïve T-cells which are produced in the bone
 280 marrow and have the capability to respond to novel patho-
 281 gens 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 macro-
 phages and the B-cells (40).
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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 im-
 mature 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), how-
 ever, 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).
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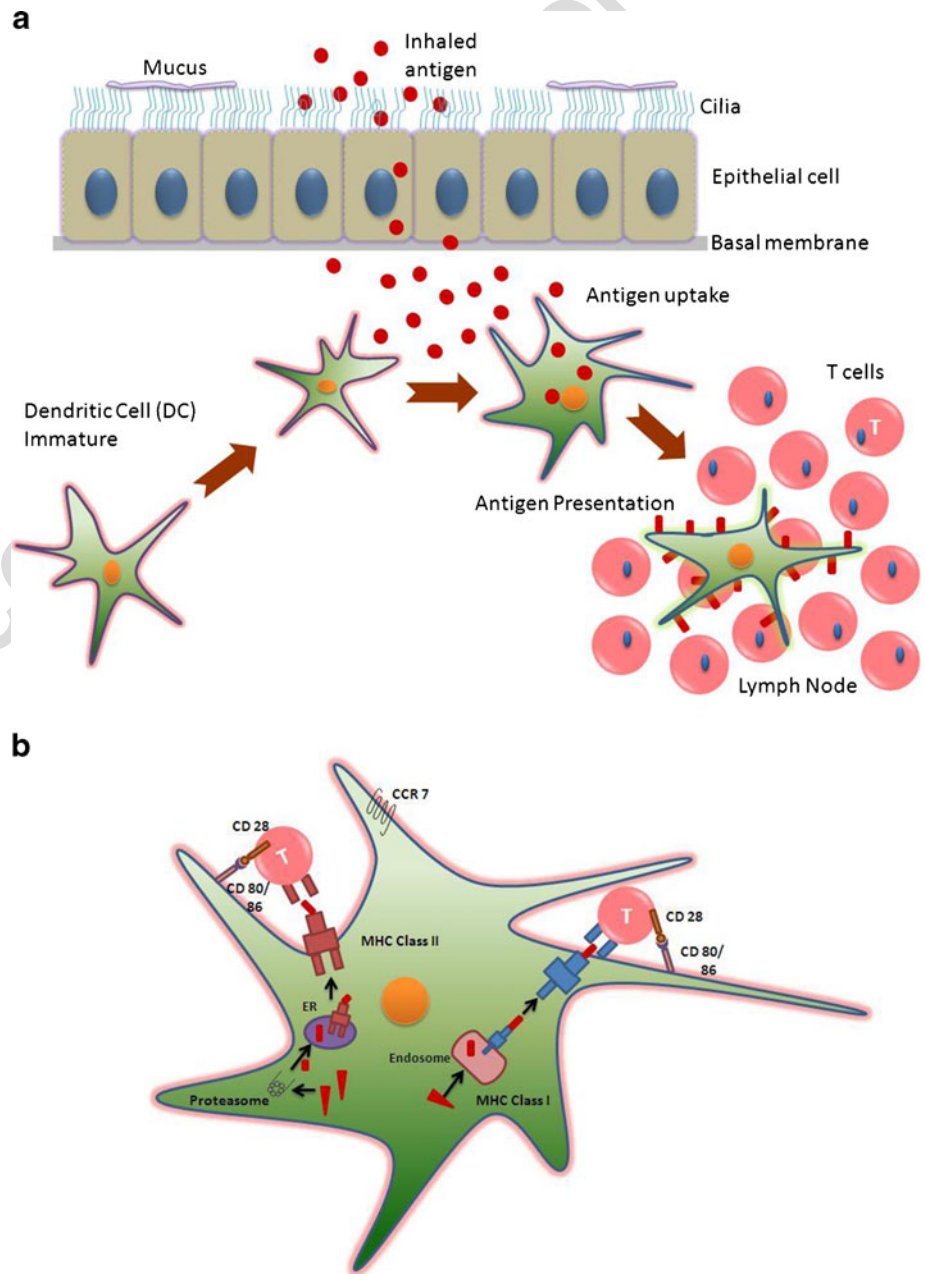
The human DCs are identified by over expression of
 human leukocyte antigen (HLA) DR (major histocompati-
 bility complex class II) with the absence of monocyte, lym-
 phocyte, natural killer cell and granulocyte lineage markers
 (43). In addition, the specific markers for identifying the
 mDCs include CD11c⁺, CD1a⁺, BDCA-1⁺, BDCA-3⁺,
 HLA-DR⁺ whereas for the pDCs they are CD11c⁻, HLA-
 DR⁺, BDCA-2⁺ and CD123⁺ (43,46,47).
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Inhaled antigens or antigen particulates are believed to
 encounter the wide spread DC network that lines the alve-
 olar epithelium and are subsequently taken up by cellular
 processes extending in to the alveolar lining fluid (33). Anti-
 gens 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
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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).

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

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.



361 largely on them for cellular transport, whereas small particles
 362 (20–200 nm) and virus-like particles (VLPs) (30 nm) drained
 363 freely to the lymph nodes (LNs) and were present in LN-
 364 resident DCs and macrophages (54). However, this cannot be
 365 directly compared to pulmonary delivery as the DCs in the lung
 366 differ from those of the skin.

367 **Targeting Antigen to the DC**

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

374 Vaccines can be targeted to DCs in different ways (40,
 375 55–57). DCs contain pattern recognition receptors (PRRs)
 376 that aid in detecting the presence of a pathogen through
 377 interaction with pathogen-associated molecular patterns.
 378 More specifically, C-type lectin receptors (CLRs), a type of
 379 PRR, bind to sugar moieties (e.g., mannose, glucan) in a
 380 calcium-dependent manner present on the pathogen’s sur-
 381 face. This leads to antigen internalization through receptor
 382 mediated endocytosis resulting in antigen presentation to T-
 383 cells (58,59). Vaccines can also be targeted to DCs with anti-
 384 bodies having an affinity towards specific receptors present on
 385 their surface (e.g. anti-DEC205, anti-CD11c), internalization
 386 through phagocytosis and conjugation of danger signals that
 387 effectively bind to Toll-like receptors (TLRs) or cytokine
 388 receptors thereby inducing DC maturation (40,55). Table II
 389 lists some formulations that have been effectively targeted to
 390 DCs for an enhanced immune response. There are currently

no publications that establish targeting of pulmonary DCs 391
 through pulmonary delivery of dry powder vaccines. 392

Nanoparticles for Inhalation 393

Generally nanoparticles (NPs) are referred to as particles in the 394
 size range of 1–100 nm, however for drug delivery NPs larger 395
 than 100 nm are required for efficient drug loading, and have 396
 been in use for the last 40 years (60). NPs are used as drug 397
 carriers either by encapsulating, dissolving, surface adsorbing 398
 or chemically attaching the active substance (60). NPs have a 399
 large surface area-to-volume ratio and also an increased satu- 400
 ration solubility thus favoring application in the field of drug 401
 delivery. In delivery of NPs to the lung by inhalation, deposition 402
 takes place through impaction, sedimentation, interception or 403
 diffusion (Table III) depending on particle size, density, airflow, 404
 breathing rate, respiratory volume and the health of the indi- 405
 vidual (61,62). These are discussed in greater detail by Smyth 406
 HDC et al. (63) and definitions are summarized in Table III. 407

The deposition of particles in the lungs is evaluated using the 408
 aerodynamic particle size, which is defined as the diameter of a 409
 sphere (density-1 g/cm³) in air that has the same velocity as the 410
 particle in consideration (60). This is defined by the equation 411

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

where ρ is the mass density of the particle, ρ_a is the unit density 412
 (1 g/cm³) and d_g is the geometric diameter. 414

Particles greater than 10 μm (d_a) in size are commonly 415
 impacted in the throat or sedimented in the bronchial 416
 region whereas particles less than 1 μm (d_a) in size are 417
 exhaled and not likely to be deposited in the alveolar region. 418
 It is expected that particles in the size range of 1 to 5 μm (d_a) 419

t2.1 **Table II** Examples of Formulations Targeting Dendritic Cells (DCs)

t2.2	Formulation	Target	Model drug	Model	Ref
t2.3	Polyanhydride NPs with dimannose	Mannose receptor CD206	NA	<i>In vitro</i>	(58)
t2.4	MN-decorated PLGA NPs	Mannose receptor CD206	NA	<i>In vitro</i>	(121)
t2.5	PLGA NPs	DEC-205 receptor	Ovalbumin	Mice	(122)
t2.6	PLGA NPs	Humanized targeting antibody hDI (DC-SIGN)	FITC-TT/DQ Green BSA	<i>In vitro</i>	(123)
t2.7	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	<i>In vitro</i>	(56)
t2.8	Carbon magnetic NPs (CMNPs)	Endocytosis	Hen egg lysozyme (HEL)	Mice	(124)
t2.9	Polystyrene and PLGA microparticles	CD40, Fcγ, α(v)β3 and α(v)β5	NA	<i>In vitro</i>	(125)
t2.10	Acid degradable particles	DEC-205 receptor	Ovalbumin	Mice	(124)
t2.11	PAMAM dendrimer	Mannose receptor CD206	Ovalbumin	Mice	(126)
t2.12	Liposome (with tri-mannose) (L-Phosphatidylcholine + M3-DPPE)	Mannose receptor CD206	FITC-Ovalbumin	<i>In vitro</i>	(127)
t2.13	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

Nanocarriers Targeting Pulmonary Dendritic Cells

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 interactions
 426 (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 <i>in vivo</i> , 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)

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

466 In a study performed by Bivas-Benita M *et al.*, the potential of
 467 enhanced immunogenicity upon pulmonary delivery of DNA
 468 encapsulated in chitosan NPs was evaluated. Chitosan-DNA
 469 NPs were prepared by the complexation-coacervation method
 470 and the resultant DNA-loaded NPs had an average size of $376 \pm$
 471 59 nm ($n=5$), zeta-potential of 21 ± 4 mV ($n=5$) and a loading
 472 efficiency of 99%. Pulmonary administration of the chitosan-
 473 DNA NPs was shown to induce increased levels of IFN- γ
 474 secretion compared to pulmonary delivery of the plasmid in
 475 solution via the intramuscular immunization route. This indi-
 476 cates the plausibility of achieving pulmonary delivery of DNA
 477 vaccines with increased immunogenicity against tuberculosis
 478 compared to immunization through intramuscular route (71).

479 The polylactides PLA and PLGA are the most broadly
 480 investigated synthetic polymers in the field of drug delivery
 481 (66,67,72). These are rapidly hydrolyzed upon implantation
 482 into the body and are eventually removed by the citric acid
 483 cycle. The hydrolyzed products form at very slow rate and
 484 include lactic acid and glycolic acid which are biologically
 485 compatible and easily metabolized making them safe and
 486 non-toxic (66,73). However, the acidic degradation products
 487 can cause problems by eliciting inflammation and also a
 488 reduction in pH within the microparticles resulting in the
 489 hydrolysis of the biopharmaceuticals (74).

490 Muttli *et al.* prepared novel NP-aggregate formulations using
 491 poly(lactic-co-glycolic acid) (PLGA) and recombinant hepatitis
 492 B surface antigen (rHBsAg) and showed that the dry powder
 493 formulations elicited a high mucosal immune response after
 494 pulmonary immunization of guinea pigs without the need for
 495 adjuvants. They prepared three different formulations of dry
 496 powders by spray-drying with leucine, (1) rHBsAg encapsulated
 497 within PLGA/polyethylene glycol (PEG) NPs (antigen NPs,
 498 AgNSD), (2) a physical mixture of rHBsAg and blank PLGA/
 499 PEG NPs (antigen NP admixture (AgNASD)), and (3) rHBsAg
 500 encapsulated in PLGA/PEG NPs with free rHBsAg (antigen
 501 NPs plus free antigen). All the particles had mass median
 502 aerodynamic diameters (MMAD) of around $4.8 \mu\text{m}$ and a fine
 503 particle fraction (FPF) of 50%. After immunization the highest
 504 titre of serum IgG antibodies was observed in the control group
 505 immunized with alum adsorbed with rHBsAg (Alum Ag) (IM
 506 route) whereas the highest IgA titres were observed for animal
 507 groups immunized with powder formulations via the pulmo-
 508 nary route. It was also noteworthy guinea pigs immunized with
 509 AgNASD dry powder exhibited IgG titers above 1,000 mIU/
 510 ml in the serum (required 10 mIU/ml) suggesting the potential
 511 of administering novel dry powder formulations via the pulmo-
 512 nary route (75).

513 Recently a new class of biodegradable polymers, polyke-
 514 tals, have been developed and are largely being investigated
 515 for drug delivery purposes (76,77). This class of polymers

516 have non-acidic degradation products and pH-sensitive
 517 ketal linkages in their backbone. These polyketals offer
 518 several advantages for vaccine delivery such as exhibiting
 519 pH-dependent hydrolysis but yet are degradable in acidic
 520 phagolysosomes. Polyketal copolymers degrade into bio-
 521 compatible small molecules minimizing inflammation com-
 522 pared to PLGA. An aliphatic polyketal, poly(cyclohexane-1,4-
 523 diyl acetone dimethylene ketal) (PCADK) degrades into ace-
 524 tone and 1,4-cyclohexanedimethanol which are both biocom-
 525 patible, and has a hydrolysis half-life of 24 days at pH 4.5 (77).
 526 This was later modified to a co-polyketal termed PK3 synthe-
 527 sized from 1,4-cyclohexanedimethanol and 1,5-pentanediol
 528 with a hydrolysis half-life of 1.8 days at pH 4.5 (64) making
 529 it much suitable for vaccine delivery.

530 Heffernan MJ and Murthy N successfully prepared acid-
 531 sensitive polyketal NPs that released the loaded therapeutics in
 532 the acidic environments of tumors, inflammatory tissues and
 533 phagosomes. Polyketal NPs, 280–520 nm in diameter, were
 534 prepared by an oil-in-water (O/W) emulsion method using
 535 poly(1,4-phenyleneacetone dimethylene ketal) (PPADK), a
 536 new hydrophobic polymer that undergoes acid-catalysed hy-
 537 drolysis into low molecular weight hydrophilic compounds.
 538 (76). Heffernan *et al.* used polyketal PK3 to formulate a model
 539 vaccine that elicits CD8+ T cell responses. PK3 microparticles
 540 encapsulating ovalbumin (OVA), poly(inosinic acid)-poly(cyti-
 541 dylic acid) (poly(I:C)) - a TLR3 (Toll like receptor) agonist and a
 542 double-stranded RNA analog were prepared using single
 543 emulsion method. PK3-OVA-poly(I:C) microparticles (1–
 544 $3 \mu\text{m}$) at a dosage of $0.01 \mu\text{g}/\text{mL}$ were then supplied to murine
 545 splenic DCs and a higher percentage of IFN γ -producing
 546 CD8+ T cells, TNF- α and IL-2 production in CD8+ T cells
 547 were observed than with DCs treated with PK3-OVA par-
 548 ticles or soluble OVA/poly(I:C) implying polyketal PK3
 549 microparticles have potential for vaccine delivery (64).

550 Preparation of Polymer-Based Nanoparticles

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

558 **Emulsification/Solvent Evaporation and Nanoprecipitation.** E-
 559 mulsification/solvent evaporation, also referred to as solvent
 560 emulsion-evaporation, involves the emulsification of an or-
 561 ganic polymer solution into an aqueous phase followed by
 562 the evaporation of the organic solvent (78). The polymer
 563 with or without the drug is dissolved in a volatile organic
 564 solvent like acetone, ethyl acetate, chloroform or dichloro-
 565 methane etc. and is then transferred into stirring aqueous

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

572 Singh J *et al.* prepared diphtheria toxoid (DT) loaded
 573 poly-(ϵ -caprolactone) (PCL) NPs via a double emulsification
 574 solvent evaporation method (w/o/w) for investigating their
 575 potential as a mucosal vaccine delivery system. Briefly, DT
 576 was added to the internal aqueous phase containing 0.25 ml
 577 10%w/v polyvinyl alcohol (PVA). The solution was emulsi-
 578 fied with the organic phase comprising 100 mg of PCL in
 579 5 mL of dichloromethane (DCM), using a homogenizer at
 580 12,000 rpm for 2 min. The formulations were then stirred
 581 magnetically at ambient temperatures and pressure for 15–
 582 18 h to allow solvent evaporation and NP formation. The
 583 resultant NPs were approximately 267 ± 3 nm in size with a
 584 zeta-potential of -2.6 ± 1.2 mV. Also, the PCL NPs induced
 585 DT serum specific IgG antibody responses significantly
 586 higher than PLGA (79).

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

596 Lee JS *et al.* prepared poly(ethylene glycol)-poly(ϵ -capro-
 597 lactone) (MPEG-PCL) NPs via a nanoprecipitation method.
 598 Firstly, a predetermined concentration of MPEG-PCL block

599 copolymer was dissolved in 10 mL of organic solvent (ace-
 600 tone, acetonitrile or THF). This polymer solution was then
 601 added drop wise into deionized water (100 mL) under mag-
 602 netic stirring. The organic solvent was then evaporated under
 603 reduced pressure using a rotary evaporator, and the resultant
 604 NPs were isolated from the aqueous solution. Using different
 605 organic solvents and concentrations of polymer yielded NPs
 606 particles between ~ 50 to 150 nm (82).

607 **Emulsification and Solvent Displacement.** The emulsification
 608 and solvent displacement method is also known as emulsifica-
 609 tion solvent diffusion. This method involves the precipitation
 610 of the polymer from an organic solution and subsequent
 611 diffusion of the organic solvent into an aqueous phase (78).
 612 The solvent that aids in the formation of emulsion must be
 613 miscible with water. For example, the organic polymer solu-
 614 tion can be added to an aqueous phase, which often contains a
 615 stabilizer, under strong stirring. Upon the formation of the
 616 emulsion (O/W), a large quantity of water is added so as to
 617 dilute it favoring the diffusion of additional organic solvent
 618 from the dispersed droplets. This process leads to the precipi-
 619 tation of the polymer (81). An interfacial turbulence is created
 620 between the two phases as the solvent diffuses resulting in the
 621 formation of smaller particles and is believed that as the water-
 622 miscible solvent concentration increases the NPs tend to ac-
 623 quire a smaller size (80) (Fig. 3c).

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

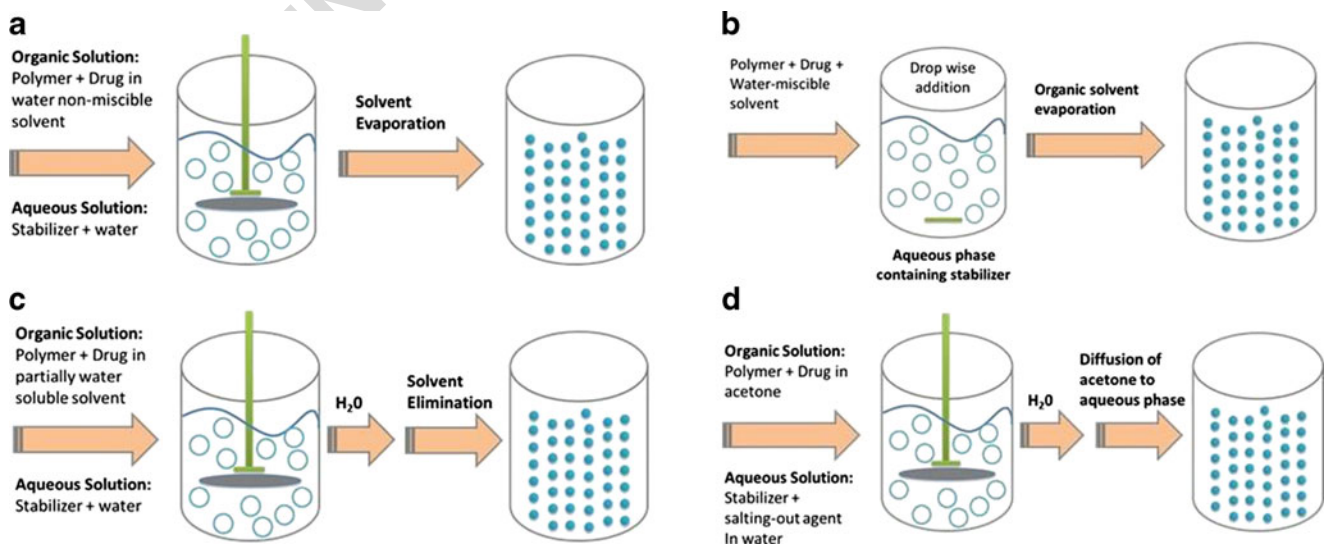


Fig. 3 Schematic representation of **a** emulsification/solvent evaporation technique, **b** emulsification and solvent displacement technique, **c** salting-out technique and **d** nanoprecipitation technique.

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 ($MgCl_2 \cdot 6H_2O$) 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).
 660

661 **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

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

It is important to note that the chemical structure of the
 drug/vaccine, the polymer and the conditions of drug loading
 influence the amount of drug/vaccine bound to the NPs and
 the type of interactions that occur between them (81). In addition,
 the encapsulation or adsorption of a drug/vaccine depends
 on the disease to be treated or prevented, route of administration,
 manufacturing feasibility and economic challenges.

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

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

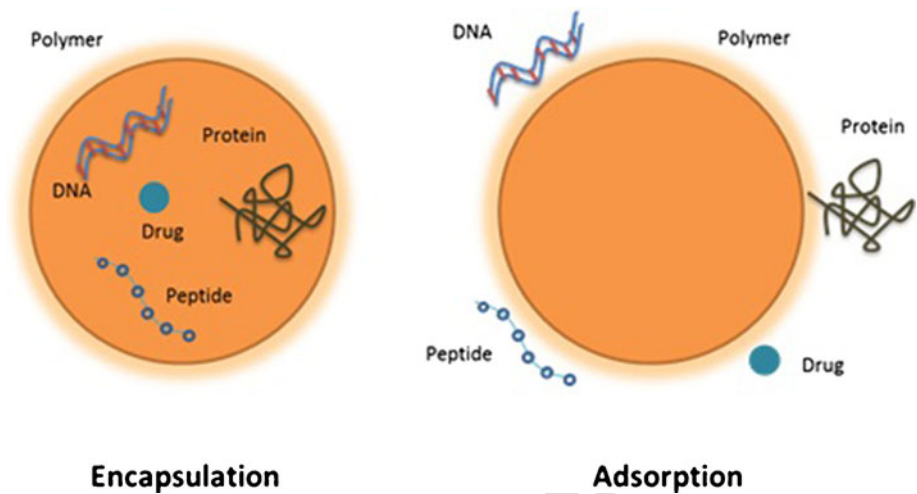
Adjuvants

Modern day vaccines contain pure recombinant or synthetic
 antigens that are less immunogenic than live or killed whole
 organism vaccines. Thus, in order to obtain a strong immune
 response upon administration of antigen and provide long term
 protection against the infection, adjuvants are included within
 the formulation (92). Adjuvants are substances used in combination
 with an antigen to produce a stronger and more robust immune
 response than the antigen alone (93). Adjuvants also provide a depot for the

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

t5.2 Method	Advantages	Disadvantages
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.



708 antigen favoring a slow release, reduce the dose of antigen
 709 required to generate a strong immune response, modulate
 710 the immune response, aid in targeting the APCs, and pro-
 711 vide danger signals helping the immune system respond to
 712 the antigen (92–94). The selection of an adjuvant depends
 713 on the antigen, delivery system, route of administration and
 714 possible side-effects. However, an ideal adjuvant should
 715 have a long shelf life and be safe, stable, biodegradable,
 716 economical and should not induce an immune response
 717 against themselves (92).

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

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

736 Wee JLK *et al.* used a sheep animal model to evaluate the
 737 delivery of ISCOMATRIX adjuvanted influenza vaccine via
 738 its mucosal site of infection for improved vaccine effectiveness.
 739 Upon pulmonary immunization with low antigen doses
 740 (0.04 µg) of adjuvanted influenza equivalent serum antibody
 741 levels were induced when compared to an almost 375-fold
 742 higher dose (15 µg) unadjuvanted influenza delivered subcu-
 743 taneously suggesting the successful use of this combination for
 744 improved protection (101).

DRY POWDER PREPARATION TECHNIQUES

745

746 The use of liquid suspensions of NPs are often accompanied by
 747 several disadvantages such as particle aggregation and sedi-
 748 mentation leading to physico-chemical instability, reduced or
 749 loss of biological activity of the drug, contamination, and
 750 hydrolysis leading to degradation of the polymer (102). To
 751 overcome these problems, preparations can be stored and
 752 transported in a dry form (102). In addition, for vaccines, the
 753 delivery of a dry powder by inhalation has the potential benefits
 754 of a) increased stability during transport and administration, b)
 755 increased safety by eliminating contamination risks and c)
 756 improved cost-effectiveness (103). The most commonly used
 757 methods for transforming liquid preparations into dry powders
 758 are freeze-drying, spray-drying, spray-freeze-drying and the
 759 use of super critical fluid technologies. Each of these methods
 760 has advantages and disadvantages and are selected depending
 761 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)	t6.3
	Potassium aluminium sulphate	t6.4
	Aluminium phosphate	t6.5
Oil emulsions	MF59	t6.6
Particulate adjuvants	Virosomes	t6.7
	ISCOMS (Immuno stimulating complexes)	t6.8
Microbial derivatives	Monophosphoryl lipid A-MPL ^(TM)	t6.9
Plant derivatives	QS-21 (Saponin)	t6.10
	ADVAX	t6.11
Miscellaneous	AS04 (liposome formulation containing MPLA & QS-21), polymeric adjuvants, CpG oligodeoxynucleotides, vitamins	t6.12

762 distribution, improved bioavailability, enhanced stability, im- 811
 763 proved dispersibility and controlled release (104,105). 812

764 **Freeze-Drying** 813

765 Freeze-drying, also known as lyophilisation, is commonly used 814
 766 in industry to ensure long term stability and preservation of the 815
 767 original properties of various biological products such as viruses, 816
 768 vaccines, proteins, peptides and their carriers; NPs and lip- 817
 769 osomes (102,106). This process comprises of removing water 818
 770 from a frozen sample by sublimation and desorption under 819
 771 vacuum (106) and can be divided into three steps: freezing 820
 772 (solidification), primary drying (ice sublimation) and secondary 821
 773 drying (desorption of unfrozen water) (102). However, this 822
 774 process is relatively slow, very expensive and generates various 823
 775 stresses on the biological product during both the freezing and 824
 776 drying steps (106). Protectants in the form of excipients are 825
 777 usually added to stabilize the products, avoid aggregation and 826
 778 to ensure acceptable tonicity and reconstitution (106,107). Sug- 827
 779 ars such as glucose, sucrose, trehalose, mannitol, lactose, dextran
 780 or maltose with or without surfactants such as poly(vinyl) alcohol
 781 or poloxamer 188 are often employed as protectants to stabilize
 782 the product and prevent coalescence (107,108). The concentra-
 783 tion and the NP/sugar mass ratio also play an important role in
 784 determining the stability and long term storage of the final
 785 product (102). Anhorn MG *et al.* evaluated the effect of different
 786 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).

795 **Spray-Drying**

796 Spray-drying is a one-step preparation of dry powders. It is a 829
 797 process that converts liquid feed (solution, suspension or col- 830
 798 loidal dispersion) into dry particles (109). The process can be 831
 799 divided into four parts (110): atomization (1), spray-air contact 832
 800 (2), drying (3) and separation (4). The liquid feed is atomized 833
 801 (1) to break the liquid into droplets and this spray form comes 834
 802 into contact with a hot gas (2), causing rapid evaporation of 835
 803 the droplets to form dry particles (3). The dry particles are 836
 804 then separated from the hot gas with the help of a cyclone (4) 837
 805 (105). Compared to particles obtained from micronization 838
 806 using milling, spray-dried particles are more spherical and 839
 807 have a homogenous size-distribution resulting in a higher 840
 808 respirable fraction which is advantageous for pulmonary del- 841
 809 ivery (105). In addition, spray-drying has the advantage of 842
 810 being; simple, easily scalable, cost-effective, suitable for heat-

sensitive products and enables high drug loading (110). An 811
 economically acceptable yield can now be achieved with the 812
 fourth and newest generation of laboratory-scale spray dryer 813
 developed by Büchi, the Nano Spray Dryer B-90. This nano 814
 spray dryer can generate particles of size ranging from 300 nm 815
 to 5 µm for milligram sample quantities at high yields (up to 816
 90%) (111). However, there is a chance of degradation of 817
 macromolecules during the process due to high shear stress 818
 in the nozzle and thermal stress while drying (105). Fourie PB 819
et al. (21) describes the challenges such as thermal stress, 820
 osmotic stress, and scalability involved with spray-drying of 821
 vaccines. Fourie PB *et al.* formulated a dry powder TB vaccine 822
 for delivery to the lung by preparing *Mycobacterium bovis* Bacillus 823
 Calmette–Guérin (BCG) spray-dried particles which, when 824
 administered into *M. tuberculosis* infected guinea-pigs, resulted 825
 in enhanced immunogenicity levels compared to an equal dose 826
 injected subcutaneously into control animals (21). 827

Spray-Freeze Drying 828

Spray-freeze drying (SFD) is a drying process that usually 829
 involves atomization, rapid freezing and lyophilisation (112). 830
 A solution containing the drug is sprayed into a vessel that 831
 contains a cryogenic liquid such as nitrogen, oxygen or argon. 832
 As the boiling temperatures of these cryogenic liquids are very 833
 low they cause the droplets to freeze instantly. The resulting 834
 droplets are then collected and lyophilized to obtain porous dry 835
 powder particles suitable for respiration (105). The advantage 836
 of SFD is the ability to produce particles with adjustable sizes 837
 (112) and as it is conducted at sub-ambient temperature, ther- 838
 molabile polymers and highly potent biopharmaceuticals can 839
 be formulated into dry powder products (105). However, the 840
 major disadvantage of this technique is the stresses associated 841
 with freezing and drying, which may cause irreversible damage 842
 to proteins (113). This is displayed as structural denaturation, 843
 aggregation and loss of biological activity upon rehydration 844
 (105). In addition, loss of stability due to unfolding and aggre- 845
 gation remains a major challenge (113) and also the method has 846
 low process efficacy, is time consuming, and expensive (114). 847

Amorij J-P *et al.* showed that an influenza subunit vaccine 848
 powder prepared by SFD using oligosaccharide inulin as a 849
 stabilizer and delivered via the pulmonary route to BALB/c 850
 mice induced systemic humoral (IgG), cell-mediated (IL-4, 851
 IFN-γ) and mucosal immune responses (IgA, IgG). Whereas 852
 vaccination with a liquid subunit vaccine via either pulmonary 853
 or intramuscular route induced only systemic humoral (IgG) 854
 immune responses suggesting that powder vaccine formula- 855
 tions could be beneficial for immunization (23). 856

Supercritical Fluid Technology 857

Supercritical fluids (SCF) are compressed gases or liquids above 858
 their critical temperatures (T_c) and pressures (P_c), and possess 859

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 (CO₂), water, propane, acetone, nitrous oxide (N₂O),
 864 trifluoromethane, chlorodifluoromethane, diethyl ether, water,
 865 or CO₂ with ethanol (114). However, because of its accessible
 866 critical point at 31°C and 74 bar, its low cost and non-toxicity,
 867 CO₂ is the most widely used solvent in SCF. In addition, its
 868 low critical temperature makes supercritical (SC) CO₂ suitable
 869 for handling heat-labile solutes at conditions close to room
 870 temperature. Therefore, SC CO₂ 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

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

t7.1 **Table VII** Recent studies on dry powder particle-based vaccine delivery

t7.2	Disease	Antigen	Carrier/Stabilizer	Dry Powder Preparation	Size (μm)	Model	Ref
t7.3	Bacterial Infections	Bacteriophages	Trehalose, Leucine	SD	2.5–2.8	NA	(132)
t7.4	Diphtheria	Diphtheria Toxoid	Chitosan	SCF	3–4	GP	(119)
t7.5	Diphtheria	Diphtheria CRM-197 antigen	L-leucine	SD	~ 5	GP	(32)
t7.6	Hepatitis B	Recombinant hepatitis B surface antigen (rHBsAg)	Leucine	SD	4.8	GP	(75)
t7.7	Influenza	Influenza monovalent	Inulin	SD, SFD	2.6 (SD), 10.5 SFD)	M	(133)
t7.8	Influenza	Influenza subunit	Inulin	SFD	~ 10	M	(23)
t7.9	Tuberculosis	Ad35-vectored tuberculosis (TB) AERAS-402	Mannitol-cyclodextrin-trehalose-dextran, MCTD	SD	3.2–3.5	NA	(134)
t7.10	Tuberculosis	Bacille Calmette-Guerin (BCG)	Leucine	SD	2–3	GP	(135)
t7.11	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

929 local APCs that present antigen in a way to induce both
 930 mucosal and systemic immune response. Recent progress in
 931 targeting vaccines specifically to DCs for an enhanced im-
 932 mune response with low doses has paved way for developing
 933 new vaccine technology. Polymer-based NPs offer the ad-
 934 vantage of biodegradability, avoiding antigen degradation if
 935 encapsulated and through chemical attachments can target
 936 DCs. However, more research is needed to understand the
 937 fate of NPs after inhalation, their interaction with the biolog-
 938 ical cells and their toxicity (nanotoxicity). The method of
 939 formulation of NP based vaccines into dry powders is of equal
 940 importance as it provides the opportunity to maintain the
 941 stability and integrity of the antigen, ease of transport and
 942 administration. The right combination of polymer chemistry,
 943 polymer-based NPs, immunology, dry powder technology,
 944 delivery device and animal models will lead to the discovery
 945 of next generation of vaccine delivery systems.

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