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48	Abstract	<p>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.</p>	
49	Keywords separated by ' - '	antigen presenting cells - dendritic cells - dry powder - polymeric nanoparticles - pulmonary delivery of vaccines	

50 Foot note  
information

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EXPERT REVIEW

4 **Nanocarriers Targeting Dendritic Cells for Pulmonary**  
5 **Vaccine Delivery**

7 Nitesh K. Kunda · Satyanarayana Somavarapu · Stephen B. Gordon · Gillian A. Hutcheon · Imran Y. Saleem  
8

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11 **ABSTRACT** Pulmonary vaccine delivery has gained significant  
12 attention as an alternate route for vaccination without the use of  
13 needles. Immunization through the pulmonary route induces  
14 both mucosal and systemic immunity, and the delivery of anti-  
15 gens in a dry powder state can overcome some challenges such  
16 as cold-chain and availability of medical personnel compared to  
17 traditional liquid-based vaccines. Antigens formulated as nano-  
18 particles (NPs) reach the respiratory airways of the lungs pro-  
19 viding greater chance of uptake by relevant immune cells. In  
20 addition, effective targeting of antigens to the most 'professional'  
21 antigen presenting cells (APCs), the dendritic cells (DCs) yields  
22 an enhanced immune response and the use of an adjuvant  
23 further augments the generated immune response thus requir-  
24 ing less antigen/dosage to achieve vaccination. This review  
25 discusses the pulmonary delivery of vaccines, methods of pre-  
26 paring NPs for antigen delivery and targeting, the importance of  
27 targeting DCs and different techniques involved in formulating  
28 dry powders suitable for inhalation.  
29

30 **KEY WORDS** antigen presenting cells · dendritic cells ·  
31 dry powder · polymeric nanoparticles · pulmonary delivery of  
32 vaccines

<b>ABBREVIATIONS</b>	33
AMs Alveolar macrophages	36
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 Polylactide 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

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**INTRODUCTION** 88

New therapeutic biopharmaceuticals have made it possible 89  
to treat and/or prevent many diseases which were untreat- 90  
able a decade ago (1). The majority of these biopharma- 91  
ceuticals are administered via parenteral routes because 92  
they are degraded by acid and proteases in the stomach or 93

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) <sup>a</sup>	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

<sup>a</sup>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  
 147 pathogens (2,15). 148

**PULMONARY VACCINE DELIVERY** 149

150 Pulmonary delivery as a route of drug administration can be  
 151 traced back 4000 years to India where people suffering from  
 152 cough suppressed it by inhaling the leaves of *Atropa Belladonna*  
 153 (16). Later in the 19th and 20th centuries, people suffering  
 154 from asthma smoked cigarettes containing tobacco and  
 155 stramonium powder to alleviate their symptoms (16). The  
 156 first inhaling apparatus for dry powder delivery was patent-  
 157 ed in London in 1864 (17). Since then much progress has  
 158 been made in developing devices such as nebulizers,  
 159 metered dose inhalers and DPIs for delivery of therapeutics.  
 160 With recent advancements in pulmonary delivery devices  
 161 and recombinant protein technology the first peptide DPI  
 162 formulation, Exubera (Nektar/Pfizer), was approved and  
 163 released into the market in January 2006. This was soon  
 164 withdrawn for several reasons including bulkiness of the  
 165 device, complicated administration, contraindication in  
 166 smokers and insufficient evidence with regulatory bodies  
 167 regarding the patients preference of Exubera (inhaled dosage  
 168 form) compared to other dosage forms (18). This led, however,  
 169 to further research and development of DPI of biopharma-  
 170 ceuticals, and currently many investigations are being pursued  
 171 by the pharmaceutical industry such as the AIR system  
 172 (Alkermes/Eli Lilly), the Technosphere system (Mannkind)  
 173 and Kos inhaled insulin (Kos Pharm/Abbott) for Type I/II  
 174 diabetes, and Granulocyte-colony-stimulating factor (G-CSF)  
 175 for Neutropenia (Amgen) (19). This has been followed by  
 176 investigations into DPI of vaccines (20–24). 176

**Anatomy of the Human Lung** 177

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

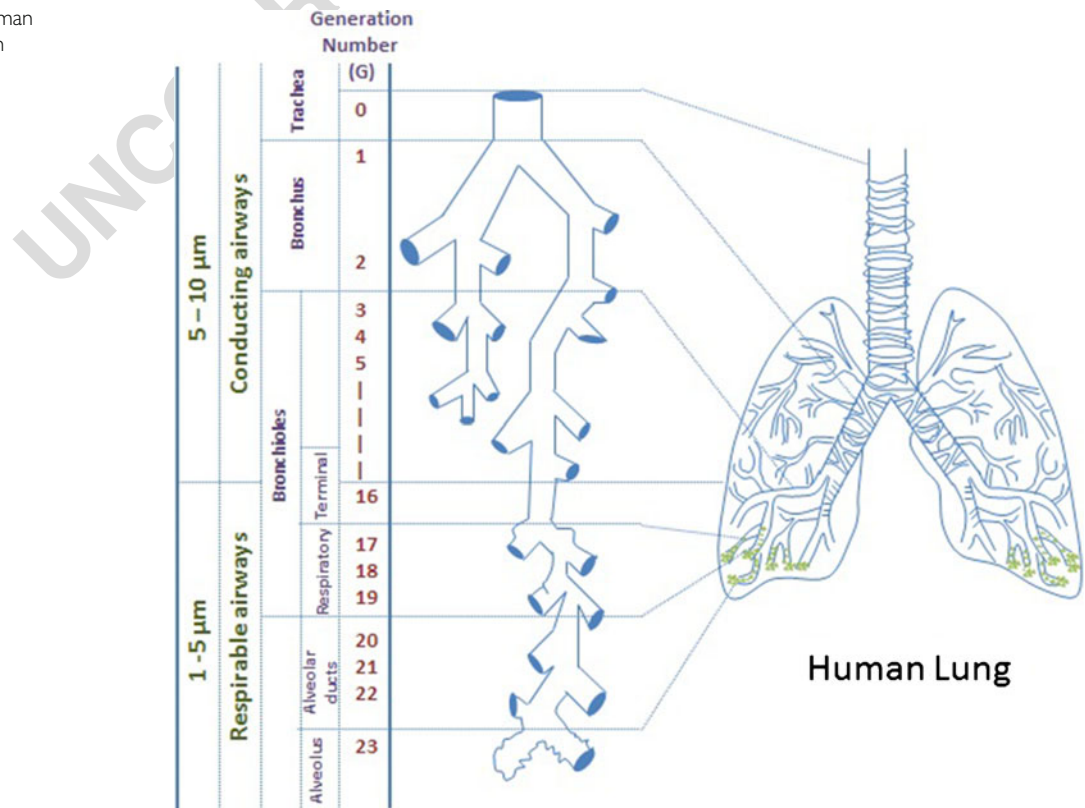
The alveoli and the pulmonary capillaries are separated by a barrier composing of endothelial cells, interstitial space, and pneumocytes (pulmonary epithelial cells). The pneumocytes

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).  
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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). 282  
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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). 285  
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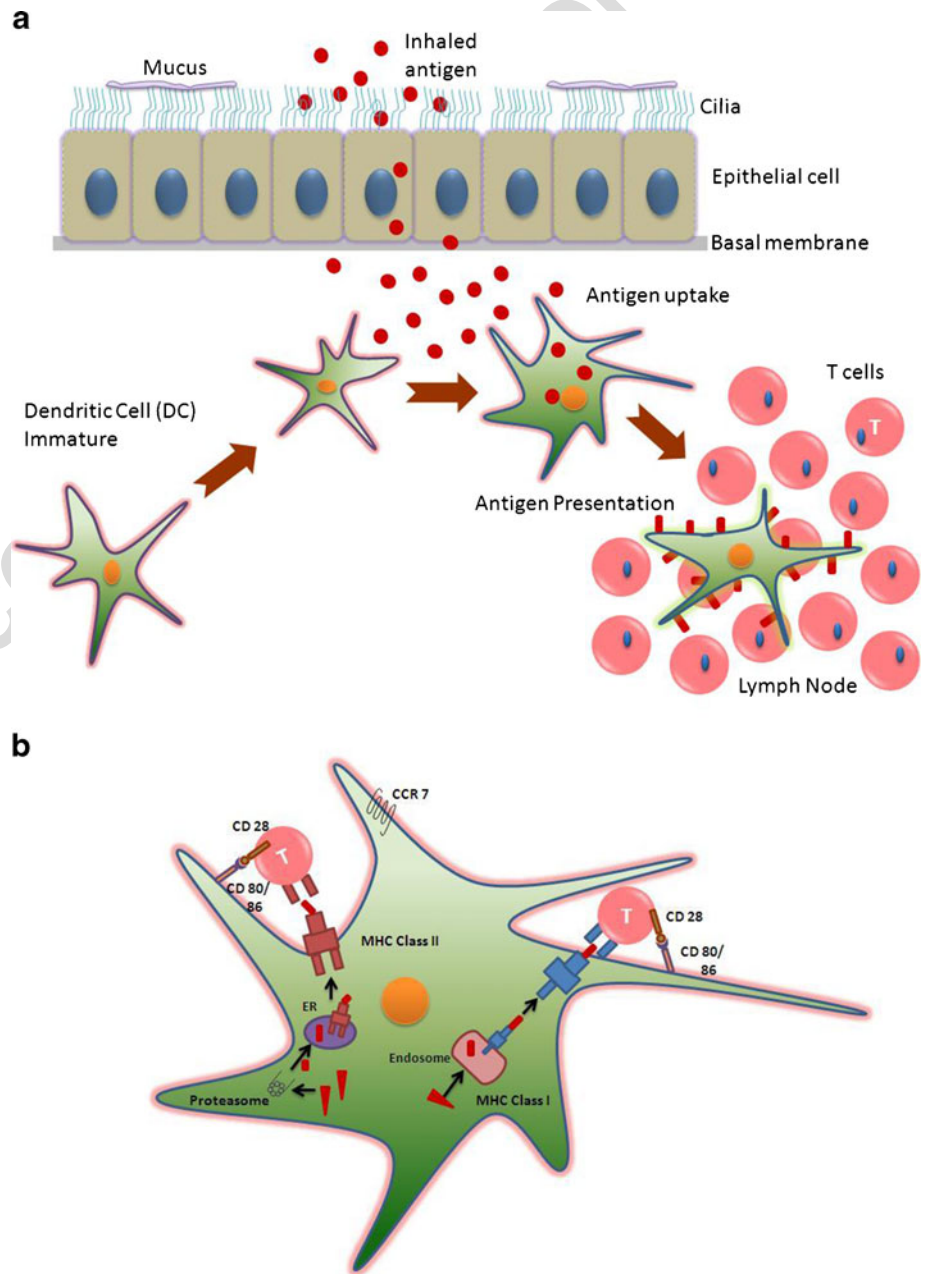
The human DCs are identified by over expression of  
 human leukocyte antigen (HLA) DR (major histocompati-  
 bility complex class II) with the absence of monocyte, lym-  
 phocyte, natural killer cell and granulocyte lineage markers  
 (43). In addition, the specific markers for identifying the  
 mDCs include CD11c<sup>+</sup>, CD1a<sup>+</sup>, BDCA-1<sup>+</sup>, BDCA-3<sup>+</sup>,  
 HLA-DR<sup>+</sup> whereas for the pDCs they are CD11c<sup>-</sup>, HLA-  
 DR<sup>+</sup>, BDCA-2<sup>+</sup> and CD123<sup>+</sup> (43,46,47). 319  
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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 327  
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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).

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 al.*  
 351 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  $\mu\text{m}$  (diameter). Uptake of large particles  
 356 ( $\sim 1 \mu\text{m}$ ) 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.



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  
 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 satu-  
 ration 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 indi-  
 vidual (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<sup>3</sup>) 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  $\rho$  is the mass density of the particle,  $\rho_a$  is the unit density  
 (1 g/cm<sup>3</sup>) and  $d_g$  is the geometric diameter.

Particles greater than 10  $\mu\text{m}$  ( $d_a$ ) in size are commonly  
 impacted in the throat or sedimented in the bronchial  
 region whereas particles less than 1  $\mu\text{m}$  ( $d_a$ ) 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  $\mu\text{m}$  ( $d_a$ )

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 <sub>272-300</sub> 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, Fc $\gamma$ , $\alpha$ (v) $\beta$ 3 and $\alpha$ (v) $\beta$ 5	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**

**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 $\mu\text{m}$ 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  $\mu\text{m}$  (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  $\mu\text{m}$  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

**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 $\mu\text{m}$ (with 1:4 liposome:mannitol)	$\beta$ -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  $21 \pm 4$  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- $\gamma$   
 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 $\gamma$ -producing  
 546 CD8+ T cells, TNF- $\alpha$  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  $267 \pm 3$  nm in size with a  
 584 zeta-potential of  $-2.6 \pm 1.2$  mV. Also, the PCL NPs induced  
 585 DT serum specific IgG antibody responses significantly  
 586 higher than PLGA (79).

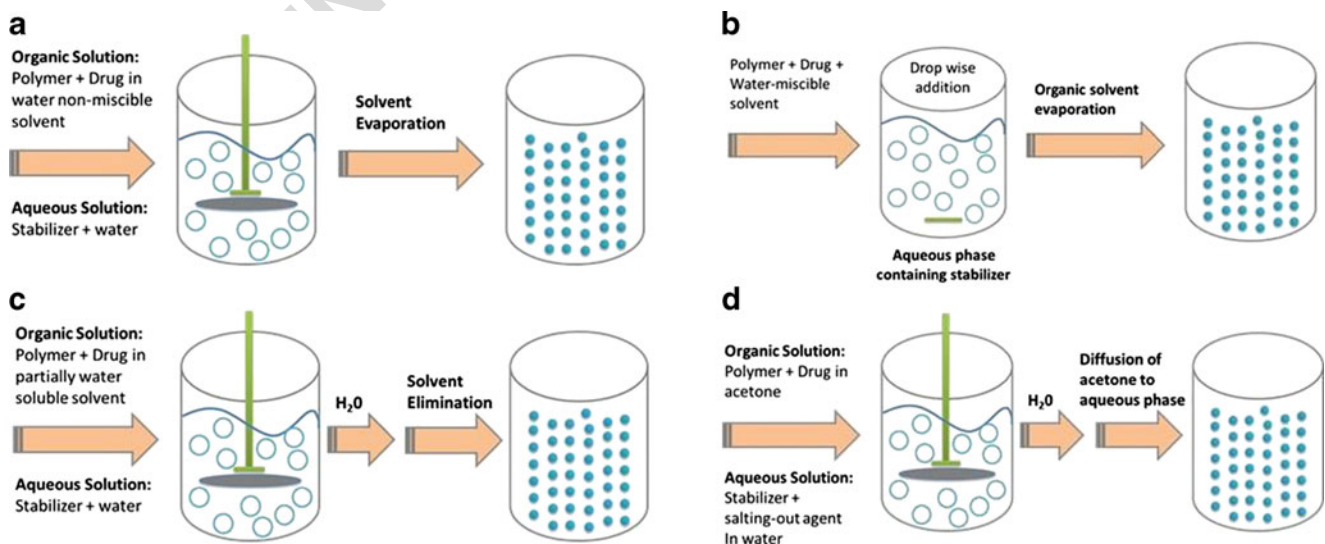
587 The nanoprecipitation method is a single step method  
 588 which is usually employed for entrapping hydrophobic drug  
 589 moieties. In this method, the drug and the polymer are dis-  
 590 solved in a water-miscible solvent, such as acetone, acetonitrile  
 591 or methanol (80). This organic phase 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  $260 \pm 4.5$  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- $\alpha$  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 develop-  
 ment 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**

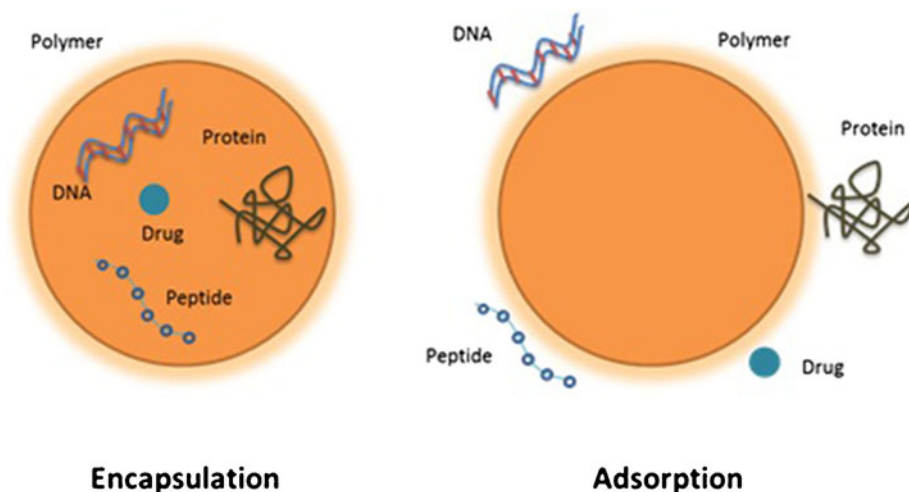
Modern day vaccines contain pure recombinant or synthetic  
 antigens that are less immunogenic than live or killed whole  
 organism vaccines. Thus, in order to obtain a strong im-  
 mune response upon administration of antigen and provide  
 long term protection against the infection, adjuvants are  
 included within the formulation (92). Adjuvants are substan-  
 ces used in combination with an antigen to produce a  
 stronger and more robust immune response than the anti-  
 gen 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 <sup>(TM)</sup>	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 de- 841  
 809 livery (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<sub>c</sub>) and pressures (P<sub>c</sub>), 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 (CO<sub>2</sub>), water, propane, acetone, nitrous oxide (N<sub>2</sub>O),  
 864 trifluoromethane, chlorodifluoromethane, diethyl ether, water,  
 865 or CO<sub>2</sub> 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<sub>2</sub> is the most widely used solvent in SCF. In addition, its  
 868 low critical temperature makes supercritical (SC) CO<sub>2</sub> suitable  
 869 for handling heat-labile solutes at conditions close to room  
 870 temperature. Therefore, SC CO<sub>2</sub> 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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