Dear Author

Here are the proofs of your article.

- This article has a short turn-around time. We need to receive your corrections **within 48 hours**. If we do not receive your corrections within 48 hours, we will send you a reminder. Succeeding reminders will be sent every 24 hours until we receive your corrections.
- You can submit your corrections **online,** via **e-mail** or by **fax**.
- For **online** submission please insert your corrections in the online correction form. Always indicate the line number to which the correction refers.
- You can also insert your corrections in the proof PDF and **email** the annotated PDF.
- For **fax** submission, please ensure that your corrections are clearly legible. Use a fine black pen and write the correction in the margin, not too close to the edge of the page.
- Remember to note the **journal title**, **article number**, and **your name** when sending your response via e-mail or fax.
- **Check** the metadata sheet to make sure that the header information, especially author names and the corresponding affiliations are correctly shown.
- **Check** the questions that may have arisen during copy editing and insert your answers/ corrections.
- **Check** that the text is complete and that all figures, tables and their legends are included. Also check the accuracy of special characters, equations, and electronic supplementary material if applicable. If necessary refer to the *Edited manuscript*.
- The publication of inaccurate data such as dosages and units can have serious consequences. Please take particular care that all such details are correct.
- Please **do not** make changes that involve only matters of style. We have generally introduced forms that follow the journal's style. Substantial changes in content, e.g., new results, corrected values, title and authorship are not allowed without the approval of the responsible editor. In such a case, please contact the Editorial Office and return his/her consent together with the proof.
- Your article will be published **Online First** approximately three working days after receipt of your corrected proofs. This is the **official first publication** citable with the DOI. **Further changes are, therefore, not possible.**
- The **printed version** will follow in a forthcoming issue.

Please note

After online publication, subscribers (personal/institutional) to this journal will have access to the complete article via the DOI using the URL:

`http://dx.doi.org/10.1007/s11095-012-0891-5`

If you would like to know when your article has been published online, take advantage of our free alert service. For registration and further information, go to: http://www.springerlink.com.

Due to the electronic nature of the procedure, the manuscript and the original figures will only be returned to you on special request. When you return your corrections, please inform us, if you would like to have these documents returned.

Metadata of the article that will be visualized in OnlineFirst

1	Article Title	**Nanocarriers Targeting Dendritic Cells for Pulmonary Vaccine Delivery**	
2	Article Sub- Title		
3	Article Copyright - Year	**Springer Science+Business Media New York 2012 (This will be the copyright line in the final PDF)**	
4	Journal Name	Pharmaceutical Research	
5	Corresponding Author	Family Name	**Saleem**
6		Particle	
7		Given Name	**Imran Y.**
8		Suffix	
9		Organization	Liverpool John Moores University
10		Division	Formulation and Drug Delivery Research, School of Pharmacy and Biomolecular Sciences
11		Address	James Parson Building, Byrom Street, Liverpool L3 3AF, UK
12		e-mail	I.Saleem@ljmu.ac.uk
13	Author	Family Name	**Kunda**
14		Particle	
15		Given Name	**Nitesh K.**
16		Suffix	
17		Organization	Liverpool John Moores University
18		Division	Formulation and Drug Delivery Research, School of Pharmacy and Biomolecular Sciences
19		Address	James Parson Building, Byrom Street, Liverpool L3 3AF, UK
20		e-mail	
21	Author	Family Name	**Somavarapu**
22		Particle	
23		Given Name	**Satyanarayana**
24		Suffix	
25		Organization	University College London
26		Division	Department of Pharmaceutics, School of Pharmacy

27		Address	London, UK
28		e-mail	
29	Author	Family Name	**Gordon**
30		Particle	
31		Given Name	**Stephen B.**
32		Suffix	
33		Organization	Liverpool School of Tropical Medicine
34		Division	Respiratory Infection Group
35		Address	Liverpool, UK
36		e-mail	
37	Author	Family Name	**Hutcheon**
38		Particle	
39		Given Name	**Gillian A.**
40		Suffix	
41		Organization	Liverpool John Moores University
42		Division	Formulation and Drug Delivery Research, School of Pharmacy and Biomolecular Sciences
43		Address	James Parson Building, Byrom Street, Liverpool L3 3AF, UK
44		e-mail	
45	Schedule	Received	11 June 2012
46		Revised	
47		Accepted	18 September 2012

48 Abstract

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

49 Keywords separated by ' - '

antigen presenting cells - dendritic cells - dry powder - polymeric nanoparticles - pulmonary delivery of vaccines

50 Foot note information

Pharm Res
DOI 10.1007/s11095-012-0891-5

EXPERT REVIEW

Nanocarriers Targeting Dendritic Cells for Pulmonary Vaccine Delivery

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

Received: 11 June 2012 / Accepted: 18 September 2012

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

N. K. Kunda • G. A. Hutcheon • I. Y. Saleem (✉)
Formulation and Drug Delivery Research, School of Pharmacy and Biomolecular Sciences Liverpool John Moores University
James Parson Building, Byrom Street
Liverpool L3 3AF, UK
e-mail: I.Saleem@ljmu.ac.uk

S. Somavarapu
Department of Pharmaceutics, School of Pharmacy
University College London
London, UK

S. B. Gordon
Respiratory Infection Group, Liverpool School of Tropical Medicine
Liverpool, UK

ABBREVIATIONS

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

INTRODUCTION

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

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

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

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

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

Of all the non-invasive routes of delivery, pulmonary delivery can overcome some of the current challenges of vaccination such as invasiveness, accessibility, and vaccine stability and integrity by delivering vaccines as dry powder inhalations (DPI) (14). In addition, the pulmonary route has gained much attention as it is the main entry portal for pathogens (2,15).

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

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

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

PULMONARY VACCINE DELIVERY

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

Anatomy of the Human Lung

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

JrnlID 11095_ArtID 891_Proof# 1 - 21/09/2012

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).

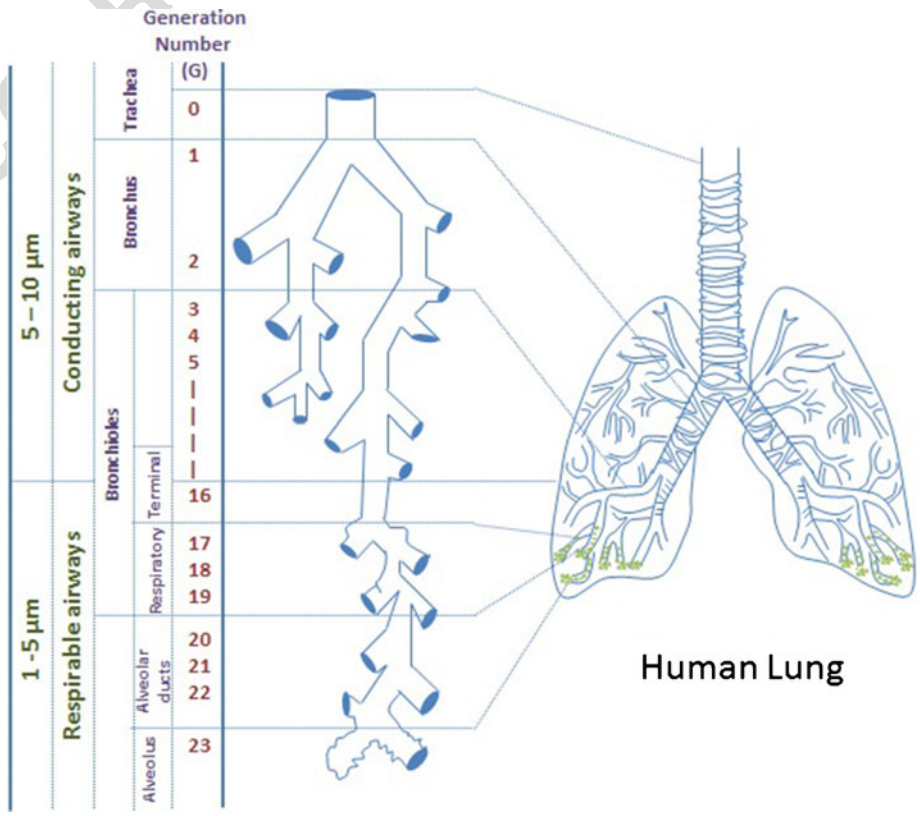

Fig. 1 Diagram of the human lung and particle deposition based on size.

Pulmonary *vs* Parenteral Vaccine Delivery

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

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

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

Dendritic Cells

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

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

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

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

JrnlID 11095_ArtID 891_Proof# 1 - 21/09/2012

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

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

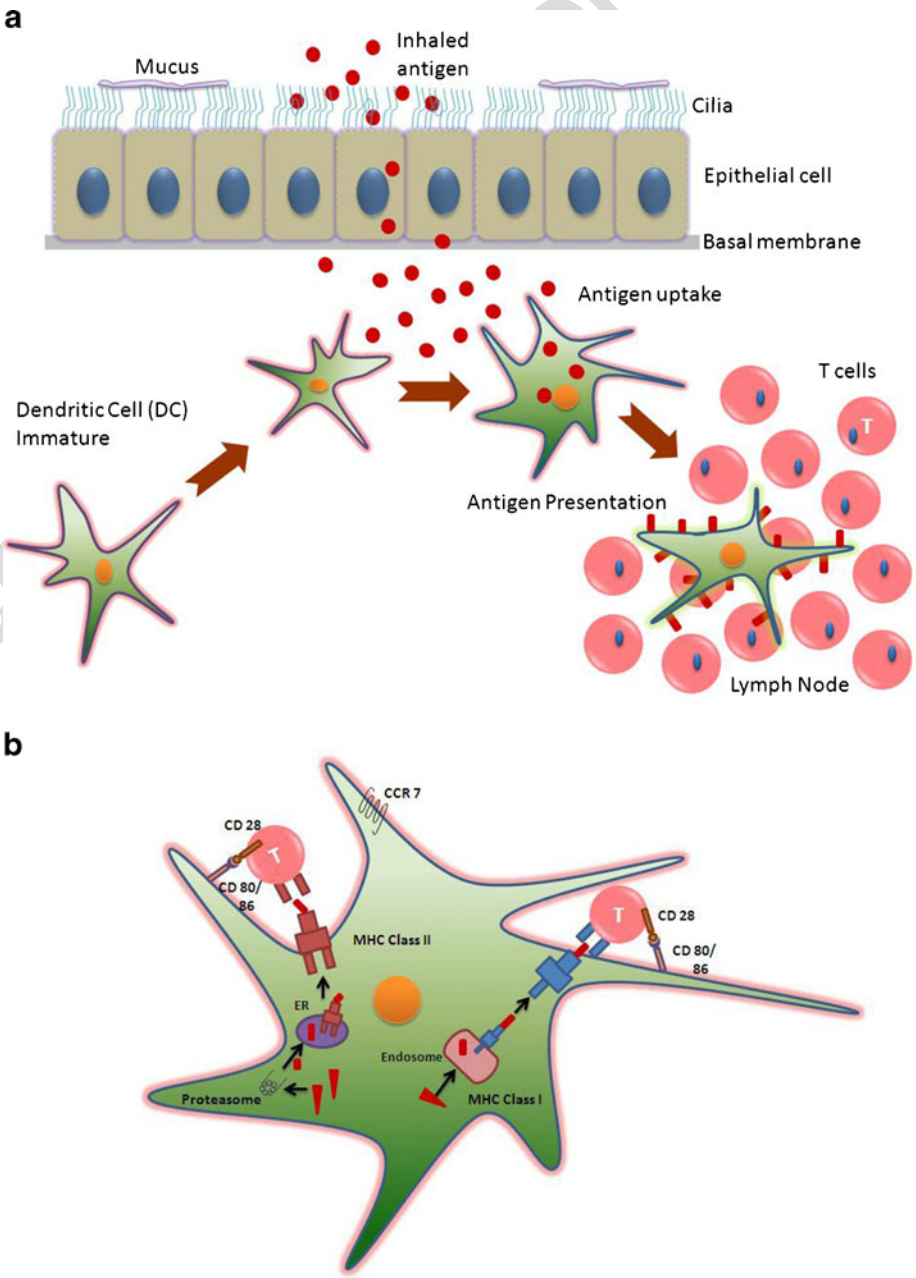

Fig. 2 Antigen uptake and presentation by dendritic cells (DCs) in the airways. / **a** Upon exposure of an inhaled antigen the immature DCs migrate towards the site of attack. DCs at this stage express a wide variety of receptors (Fc, C-type lectin receptors etc.) and uptake the antigen. Simultaneously, some DCs upregulate the CC-chemokine receptor 7 (CCR7) and migrate towards the lymphatic vessels expressing CC-chemokine ligand 21 (CCL-21) where they are carried to the draining lymph node. After antigen uptake and activation, high amounts of peptide-loaded major histocompatibility complex (MHC) molecules and T-cell co-stimulatory receptors appear on the surface of DCs. The DCs then migrate to the lymph nodes and activate the antigen specific T-cells. / **b** After antigen uptake, the antigen is either processed through MHC class I (either through endogenous or exogenous pathway) or MHC class II (the antigen is degraded in endosomes and the obtained polypeptide is transported and loaded onto MHC II molecules) and DCs present it on their surface for specific T-cell activation. *ER – Endoplasmic reticulum.

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

Targeting Antigen to the DC

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

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

Nanoparticles for Inhalation

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

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

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

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

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

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	*In vitro*	(58)
t2.4	MN-decorated PLGA NPs	Mannose receptor CD206	NA	*In vitro*	(121)
t2.5	PLGA NPs	DEC-205 receptor	Ovalbumin	Mice	(122)
t2.6	PLGA NPs	Humanized targeting antibody hD1 (DC-SIGN)	FITC-TT/DQ Green BSA	*In vitro*	(123)
t2.7	PLGA NPs coated with streptavidin	gp120, ManLAM, Lex, aDC-SIGN 1, aDC-SIGN 2, aDC-SIGN 3	DQ-BSA, $gp100_{272\text{-}300}$ and FITC-TT	*In vitro*	(56)
t2.8	Carbon magnetic NPs (CMNPs)	Endocytosis	Hen egg lysozyme (HEL)	Mice	(124)
t2.9	Polystyrene and PLGA microparticles	CD40, Fcg, α(v)β3 and α(v)β5	NA	*In vitro*	(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	*In vitro*	(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

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.

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

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

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

Polymer-based Nanoparticles

Wide varieties of polymers, both natural and synthetic, have been exploited to form biodegradable NPs. In addition, some of the polymers can act as adjuvants themselves (65). Natural polymers that have been widely investigated for formulating NPs include albumin, alginate, chitosan, collagen, cyclodextrin and gelatin; synthetic polymers include polyesters, polylactides, polyacrylates, polylactones and polyanhydrides (66,67). While natural polymers have a relatively short duration of drug release, synthetic polymers can be tailored to release the drug over days to several weeks allowing the usage of a single dose rather than multiple doses (65).

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

t4.1 **Table IV** Examples Of Nanoparticles Currently Being Evaluated For Vaccine Delivery

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

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

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

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

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

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

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

Preparation of Polymer-Based Nanoparticles

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

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

JrnlID 11095_ArtID 891_Proof# 1 - 21/09/2012

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

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

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

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

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

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

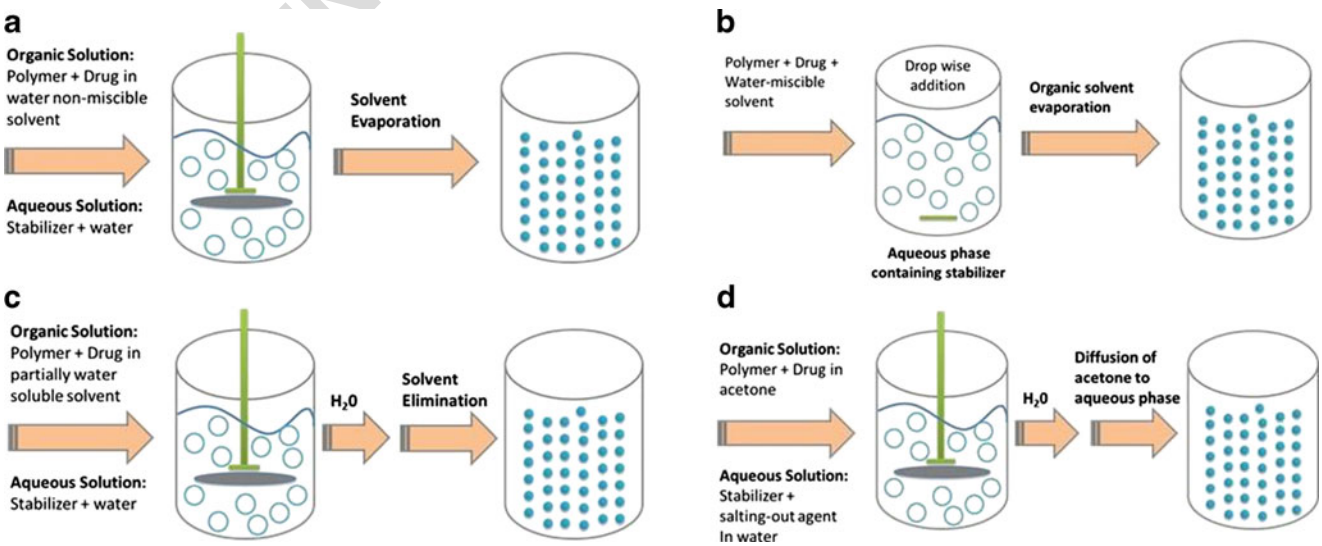

Fig. 3 Schematic representation of **a** emulsification/solvent evaporation technique, **b** emulsification and solvent displacement technique, **c** salting-out technique and **d** nanoprecipitation technique.

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

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

Konnan YN *et al.* prepared sub-200 nm NPs using a salting out method. Typically, a solution of PLGA and PLA in THF was added under mechanical stirring to an aqueous phase containing PVA and magnesium chloride hexahydrate ($MgCl_2.6H_2O$) as a salting out agent forming an O/W emulsion. To this, a large volume of water was added favoring migration of the water-miscible organic solvent into the aqueous phase forming NPs which were later purified by cross flow filtration (86).

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

Encapsulation or Adsorption

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

Table V Advantages and Disadvantages of Nanoparticle Preparation Methods

Method	Advantages	Disadvantages
Emulsification/Solvent Evaporation	Hydrophilic and hydrophobic drugs can be encapsulated	Agglomeration of nanodroplets during evaporation
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
Salting Out	High loading efficiency, Easy scale-up	Removal of electrolytes, Incompatibility of salting-out agents with drugs
Nanoprecipitation	Simple, fast and reproducible, Easy scale-up, Low surfactant concentrations required	Less polymer in the organic phase

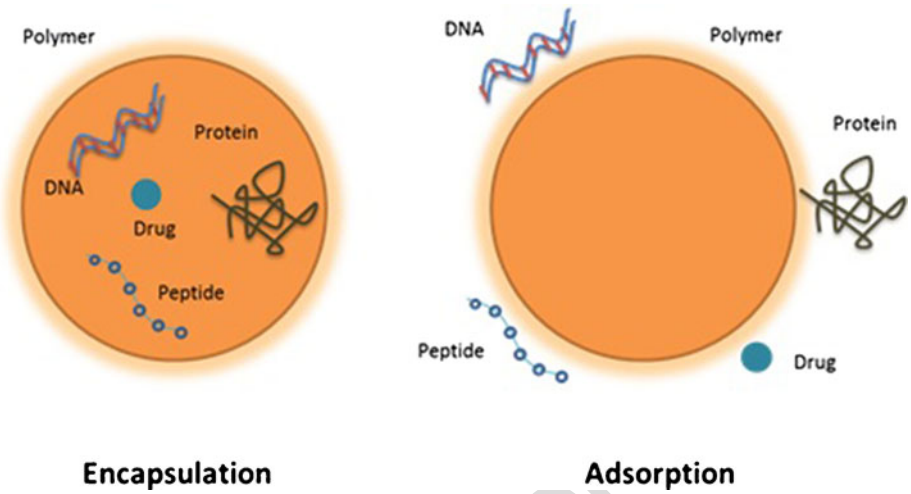

Fig. 4 The molecule of interest (DNA/Drug/Peptide/Protein) is either encapsulated *(Left)* within or surface adsorbed *(Right)* onto the polymer-based nanoparticle.

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

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

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

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

DRY POWDER PREPARATION TECHNIQUES

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

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

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

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

Freeze-Drying

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

Spray-Drying

Spray-drying is a one-step preparation of dry powders. It is a process that converts liquid feed (solution, suspension or colloidal dispersion) into dry particles (109). The process can be divided into four parts (110): atomization (1), spray-air contact (2), drying (3) and separation (4). The liquid feed is atomized *(1)* to break the liquid into droplets and this spray form comes into contact with a hot gas *(2)*, causing rapid evaporation of the droplets to form dry particles *(3)*. The dry particles are then separated from the hot gas with the help of a cyclone *(4)* (105). Compared to particles obtained from micronization using milling, spray-dried particles are more spherical and have a homogenous size-distribution resulting in a higher respirable fraction which is advantageous for pulmonary delivery (105). In addition, spray-drying has the advantage of being; simple, easily scalable, cost-effective, suitable for heat-sensitive products and enables high drug loading (110). An economically acceptable yield can now be achieved with the fourth and newest generation of laboratory-scale spray dryer developed by Büchi, the Nano Spray Dryer B-90. This nano spray dryer can generate particles of size ranging from 300 nm to 5 μm for milligram sample quantities at high yields (up to 90%) (111). However, there is a chance of degradation of macromolecules during the process due to high shear stress in the nozzle and thermal stress while drying (105). Fourie PB *et al.* (21) describes the challenges such as thermal stress, osmotic stress, and scalability involved with spray-drying of vaccines. Fourie PB *et al.* formulated a dry powder TB vaccine for delivery to the lung by preparing *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) spray-dried particles which, when administered into *M. tuberculosis* infected guinea-pigs, resulted in enhanced immunogenicity levels compared to an equal dose injected subcutaneously into control animals (21).

Spray-Freeze Drying

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

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

Supercritical Fluid Technology

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

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

There are two major principles for particle precipitation with supercritical fluids. One employs SCF as a solvent and the other as an antisolvent (115). In the first, the drug is dissolved in the SCF followed by sudden decompression, after which the solution is passed through an orifice and rapidly expanded at low pressure. Rapid Expansion of a Supercritical Solution (RESS) employs this principle (114). In the second process, the solute is insoluble in SCF and hence utilizes SCF as an antisolvent. A solute is dissolved in an organic solvent and then precipitated by the SCF (antisolvent). Precipitation occurs when the SCF is absorbed by the organic solvent followed by expansion of the liquid phase and a decrease in the solvation power leading to particle formation. The Gas Anti-Solvent (GAS), Aerosol Solvent Extraction System (ASES), Supercritical Fluid Antisolvent (SAS), Precipitation with Compressed Antisolvent (PCA), Solution Enhanced Dispersion by Supercritical Fluids (SEDS), and supercritical fluid extraction of emulsion (SFEE) are the processes that employ this second principle (114). Using these techniques particles can be formed in a well-ordered fashion to achieve the desired morphology and any negative effects on the macromolecules can be minimized (105,113). Thorough discussions of these techniques including their advantages and disadvantages have been recently published by Al-fagih I *et al.* (114) and elsewhere (105,113,115–118).

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

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

CONCLUSION

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

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

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

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

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

REFERENCES

1. Brown LR. Commercial challenges of protein drug delivery. Expet Opin Drug Deliv. 2005;2:29–42.
2. Sullivan VJ, Mikszta JA, Laurent P, Huang J, Ford B. Noninvasive delivery technologies: respiratory delivery of vaccines. Expet Opin Drug Deliv. 2006;3:87–95.
3. Sou T, Meeusen EN, de Veer M, Morton DAV, Kaminskas LM, McIntosh MP. New developments in dry powder pulmonary vaccine delivery. Trends Biotechnol. 2011;29:191–8.
4. Galindo-Rodriguez S, Allémann E, Fessi H, Doelker E. Physicochemical parameters associated with nanoparticle formation in the salting-out, emulsification-diffusion, and nanoprecipitation methods. Pharm Res. 2004;21:1428–39.
5. Rossi S, Sandri G, Caramella C. Buccal delivery systems for peptides: recent advances. Am J Drug Deliv. 2005;3:215–25.
6. Shojaei AH, Chang RK, Guo X, Burnside BA, Couch RA. Systemic drug delivery *via* the buccal mucosal route. Pharm Tech:70–81 (2001).
7. Kumria Rand GG. Emerging trends in insulin delivery: Buccal route. J Diabetol. 2011;2:1–9.
8. Ozsoy Y, Gungor S, Cevher E. Nasal delivery of high molecular weight drugs. Molecules. 2009;14:3754–79.
9. Patel VF, Liu F, Brown MB. Advances in oral transmucosal drug delivery. J Control Release. 2011;153:106–16.
10. Kalluriand H, Banga A. Transdermal delivery of proteins. AAPS PharmSciTech. 2011;12:431–41.
11. Carstens MG. Opportunities and challenges in vaccine delivery. Eur J Pharm Sci. 2009;36:605–8.
12. Maurice J, Davey S. State of the world's vaccines and immunization. http://www.unicef.org/media/files/SOWVI_full_report_english_LR1.pdf (accessed 17/02/2012 2012).
13. W.H. Organization. World Health Statistics 2011 in WHO Statistical Information System (WHOSIS). http://www.who.int/whosis/whostat/2011/en/index.html (accessed 17/04 2012).
14. Blank F, Stumbles P, von Garnier C. Opportunities and challenges of the pulmonary route for vaccination. Expet Opin Drug Deliv. 2011;8:547–63.
15. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med (2005).
16. Labiris NR, Dolovich MB. Pulmonary drug delivery. Part II: The role of inhalant delivery devices and drug formulations in therapeutic effectiveness of aerosolized medications. Br J Clin Pharmacol. 2003;56:600–12.
17. Sanders M. Inhalation therapy: an historical review. Prim Care Respir J. 2007;16:71–81.
18. Mack GS. Pfizer dumps Exubera. Nat Biotech. 2007;25:1331–2.
19. Onoue S, Hashimoto N, Yamada S. Dry powder inhalation systems for pulmonary delivery of therapeutic peptides and proteins. Expert Opin Ther Pat. 2008;18:429–42.
20. de Swart RL, LiCalsi C, Quirk AV, van Amerongen G, Nodelman V, Alcock R, *et al.* Measles vaccination of macaques by dry powder inhalation. Vaccine. 2007;25:1183–90.
21. Fourie P, Germishuizen W, Wong Y-L, Edwards D. Spray drying TB vaccines for pulmonary administration. Expert Opin Biol Ther. 2008;8:857–63.
22. LiCalsi C, Maniaci MJ, Christensen T, Phillips E, Ward GH, Witham C. A powder formulation of measles vaccine for aerosol delivery. Vaccine. 2001;19:2629–36.
23. Amorij JP, Saluja V, Petersen AH, Hinrichs WLJ, Huckriede A, Frijlink HW. Pulmonary delivery of an inulin-stabilized influenza subunit vaccine prepared by spray-freeze drying induces systemic, mucosal humoral as well as cell-mediated immune responses in BALB/c mice. Vaccine. 2007;25:8707–17.
24. Thomas C, Rawat A, Hope-Weeks L, Ahsan F. Aerosolized PLA and PLGA Nanoparticles Enhance Humoral. Mucosal and Cytokine Responses to Hepatitis B Vaccine. Mol Pharm. 2010;8:405–15.
25. Effros RM. Anatomy, development, and physiology of the lungs. GI Motility online. 1: (2006).
26. Kleinstreuer C, Zhang Z, Li Z. Modeling airflow and particle transport/deposition in pulmonary airways. Respir Physiol Neurobiol. 2008;163:128–38.
27. Kleinstreuer C, Zhang Z, Donohue JF. Targeted drug-aerosol delivery in the human respiratory system. Annu Rev Biomed Eng. 2008;10:195–220.
28. Gehr P. Annexe A. Anatomy and morphology of the respiratory tract. Ann ICRP. 1994;24:121–66.
29. Scheuch G, Kohlhaeufl MJ, Brand P, Siekmeier R. Clinical perspectives on pulmonary systemic and macromolecular delivery. Adv Drug Deliv Rev. 2006;58:996–1008.
30. Shen X, Lagergård T, Yang Y, Lindblad M, Fredriksson M, Holmgren J. Systemic and Mucosal Immune Responses in Mice after Mucosal Immunization with Group B Streptococcus Type III Capsular Polysaccharide-Cholera Toxin B Subunit Conjugate Vaccine. Infect Immun. 2000;68:5749–55.
31. Ballester M, Nembrini C, Dhar N, de Titta A, de Piano C, Pasquier M, *et al.* Nanoparticle conjugation and pulmonary delivery enhance the protective efficacy of Ag85B and CpG against tuberculosis. Vaccine. 2011;29:6959–66.
32. Muttil P, Pulliam B, Garcia-Contreras L, Fallon J, Wang C, Hickey A, *et al.* Pulmonary immunization of Guinea Pigs with diphtheria CRM-197 antigen as nanoparticle aggregate dry powders enhance local and systemic immune responses. AAPS J. 2010;12:699–707.
33. Alpar HO, Somavarapu S, Atuah KN, Bramwell VW. Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery. Adv Drug Deliv Rev. 2005;57:411–30.
34. Vermaelen K, Pauwels R. Pulmonary Dendritic Cells. Am J Respir Crit Care Med. 2005;172:530–51.
35. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245–52.
36. Nobelprize.org. The Nobel Prize in Physiology or Medicine 2011. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2011/ (accessed 19 Apr 2012).

37. Lassila O, Vainio O, Matzinger P. Can B cells turn on virgin T cells? Nature. 1988;334:253–5.
38. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu Y-J, *et al.* Immunobiology of Dendritic Cells. Annu Rev Immunol. 2000;18:767–811.
39. Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. Antigen Presentation and T Cell Stimulation by Dendritic Cells. Annu Rev Immunol. 2002;20:621–67.
40. Foged C, Sundblad A, Hovgaard L. Targeting Vaccines to Dendritic Cells. Pharm Res. 2002;19:229–38.
41. Lambrecht BN, Hammad H. Biology of Lung dendritic cells at the origin of asthma. Immunity. 2009;31:412–24.
42. GeurtsvanKessel CH, Lambrecht BN. Division of labor between dendritic cell subsets of the lung. Mucosal Immunol. 2008;1:442–50.
43. Lommatzsch M, Bratke K, Bier A, Julius P, Kuepper M, Luttmann W, *et al.* Airway dendritic cell phenotypes in inflammatory diseases of the human lung. Eur Respir J. 2007;30:878–86.
44. Ba-Omar T, Al-Riyami B. Microscopic study of human alveolar macrophages. Microsc Microanal. 2008;14:1518–9.
45. Kiama SG, Cochand L, Karlsson L, Nicod LP, Gehr P. Evaluation of phagocytic activity in human monocyte-derived dendritic cells. J Aerosol Med. 2001;14:289–99.
46. von Garnier C, Nicod LP. Immunology taught by lung dendritic cells. Swiss Med Wkly. 2009;139:186–92.
47. Demedts IK, Brusselle GG, Vermaelen KY, Pauwels RA. Identification and characterization of human pulmonary dendritic cells. Am J Respir Cell Mol Biol. 2005;32:177–84.
48. Gallucci S, Matzinger P. Danger signals: SOS to the immune system. Curr Opin Immunol. 2001;13:114–9.
49. Copland MJ, Baird MA, Rades T, McKenzie JL, Becker B, Reck F, *et al.* Liposomal delivery of antigen to human dendritic cells. Vaccine. 2003;21:883–90.
50. Burgdorf S, Lukacs-Kornek V, Kurts C. The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. J Immunol. 2006;176:6770–6.
51. Platt CD, Ma JK, Chalouni C, Ebersold M, Bou-Reslan H, Carano RAD, *et al.* Mature dendritic cells use endocytic receptors to capture and present antigens. Proc Natl Acad Sci. 2010;107:4287–92.
52. Thornton EE, Looney MR, Bose O, Sen D, Sheppard D, Locksley R, Huang X, Krummel MF. Spatiotemporally separated antigen uptake by alveolar dendritic cells and airway presentation to T cells in the lung. The Journal of Experimental Medicine (2012).
53. Foged C, Brodin B, Frokjaer S, Sundblad A. Particle size and surface charge affect particle uptake by human dendritic cells in an *in vitro* model. Int J Pharm. 2005;298:315–22.
54. Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol. 2008;38:1404–13.
55. Reddy ST, Swartz MA, Hubbell JA. Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends Immunol. 2006;27:573–9.
56. Cruz LJ, Tacken PJ, Pots JM, Torensma R, Buschow SI, Figdor CG. Comparison of antibodies and carbohydrates to target vaccines to human dendritic cells via DC-SIGN. Biomaterials. 2012;33:4229–39.
57. Caminschi I, Maraskovsky E, Heath WR. Targeting dendritic cells *in vivo* for cancer therapy. Frontiers in Immunology. 3: (2012).
58. Carrillo-Conde B, Song E-H, Chavez-Santoscoy A, Phanse Y, Ramer-Tait AE, Pohl NLB, *et al.* Mannose-functionalized "Pathogen-like" polyanhydride nanoparticles target c-type lectin receptors on dendritic cells. Mol Pharm. 2011;8:1877–86.
59. Geijtenbeek TBH, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol. 2009; 9:465–79.
60. Sung JC, Pulliam BL, Edwards DA. Nanoparticles for drug delivery to the lungs. Trends Biotechnol. 2007;25:563–70.
61. Bailey MM, Berkland CJ. Nanoparticle formulations in pulmonary drug delivery. Med Res Rev. 2009;29:196–212.
62. Smola M, Vandamme T, Sokolowski A. Nanocarriers as pulmonary drug delivery systems to treat and to diagnose respiratory and non respiratory diseases. Int J Nanomedicine. 2008;3:1–19.
63. Smyth HDC, Smyth HH, Hickey AJ. *Macro and Microstructure of the Airways for Drug Delivery* in Controlled Pulmonary Drug Delivery, Springer, 2011.
64. Heffernan MJ, Kasturi SP, Yang SC, Pulendran B, Murthy N. The stimulation of CD8+ T cells by dendritic cells pulsed with polyketal microparticles containing ion-paired protein antigen and poly(inosinic acid)–poly(cytidylic acid). Biomaterials. 2009;30:910–8.
65. Rice-Ficht AC, Arenas-Gamboa AM, Kahl-McDonagh MM, Ficht TA. Polymeric particles in vaccine delivery. Curr Opin Microbiol. 2010;13:106–12.
66. Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev. 2003;55:329–47.
67. Rytting E, Nguyen J, Wang X, Kissel T. Biodegradable polymeric nanocarriers for pulmonary drug delivery. Expet Opin Drug Deliv. 2008;5:629–39.
68. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Surf B Biointerfaces. 2010;75:1–18.
69. Bolhassani A, Safaiyan S, Rafati S. Improvement of different vaccine delivery systems for cancer therapy. Mol Cancer. 2011;10:3.
70. Doria-Rose NA, Haigwood NL. DNA vaccine strategies: candidates for immune modulation and immunization regimens. Methods. 2003;31:207–16.
71. Bivas-Benita M, van Meijgaarden KE, Franken KLMC, Junginger HE, Borchard G, Ottenhoff THM, *et al.* Pulmonary delivery of chitosan-DNA nanoparticles enhances the immunogenicity of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of Mycobacterium tuberculosis. Vaccine. 2004; 22:1609–15.
72. Jain JRA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials. 2000;21:2475–90.
73. Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev. 1997;28:5–24.
74. Fiore VF, Lofton MC, Roser-Page S, Yang SC, Roman J, Murthy N, *et al.* Polyketal microparticles for therapeutic delivery to the lung. Biomaterials. 2010;31:810–7.
75. Muttil P, Prego C, Garcia-Contreras L, Pulliam B, Fallon J, Wang C, *et al.* Immunization of Guinea Pigs with Novel Hepatitis B Antigen as Nanoparticle Aggregate Powders Administered by the Pulmonary Route. AAPS J. 2010;12:330–7.
76. Heffernan MJ, Murthy N. Polyketal nanoparticles: a new pH-sensitive biodegradable drug delivery vehicle. Bioconjug Chem. 2005;16:1340–2.
77. Yang SC, Bhide M, Crispe IN, Pierce RH, Murthy N. Polyketal copolymers: a new acid-sensitive delivery vehicle for treating acute inflammatory diseases. Bioconjug Chem. 2008;19:1164–9.
78. Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine Nanotechnol Biol Med. 2006;2:8–21.
79. Singh J, Pandit S, Bramwell VW, Alpar HO. Diphtheria toxoid loaded poly-(ε-caprolactone) nanoparticles as mucosal vaccine delivery systems. Methods. 2006;38:96–105.

80. Mahapatro A, Singh D. Biodegradable nanoparticles are excellent vehicle for site directed *in-vivo* delivery of drugs and vaccines. J Nanobiotechnology. 2011;9:55.
81. Dinarvand R, Sepehri N, Manoochehri S, Rouhani H, Atyabi F. Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents. Int J Nanomedicine. 2011;6:877–95.
82. Lee JS, Hwang SJ, Lee DS, Kim SC, Kim DJ. Formation of Poly (ethylene glycol)-Poly(ε-caprolactone) Nanoparticles via Nanoprecipitation. Macromol Res. 2009;17:72–8.
83. Ranjan AP, Zeglam K, Mukerjee A, Thamake S, Vishwanatha JK. A sustained release formulation of chitosan modified PLCL: poloxamer blend nanoparticles loaded with optical agent for animal imaging. Nanotechnology. 2011;22:1–10.
84. Allémann E, Gurny R, Doelker E. Preparation of aqueous polymeric nanodispersions by a reversible salting-out process: influence of process parameters on particle size. Int J Pharm. 1992;87:247–53.
85. Muthu M. Nanoparticles based on PLGA and its co-polymer: An overview, 2009.
86. Konan YN, Gurny R, Allémann E. Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles. Int J Pharm. 2002;233:239–52.
87. Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. J Control Release. 2001;70:1–20.
88. Bivas-Benita M, Romeijn S, Junginger HE, Borchard G. PLGA–PEI nanoparticles for gene delivery to pulmonary epithelium. Eur J Pharm Biopharm. 2004;58:1–6.
89. Bivas-Benita M, Lin MY, Bal SM, van Meijgaarden KE, Franken KLMC, Friggen AH, *et al.* Pulmonary delivery of DNA encoding Mycobacterium tuberculosis latency antigen Rv1733c associated to PLGA–PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice. Vaccine. 2009;27:4010–7.
90. Pulliam B, Sung JC, Edwards DA. Design of nanoparticle-based dry powder pulmonary vaccines. Expet Opin Drug Deliv. 2007;4:651–63.
91. Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. Science. 2004;303:1818–22.
92. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol. 2004;82:488–96.
93. O'Hagan DT, MacKichan ML, Singh M. Recent developments in adjuvants for vaccines against infectious diseases. Biomol Eng. 2001;18:69–85.
94. Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, Narasimhan B. Vaccine adjuvants: current challenges and future approaches. J Pharm Sci. 2009;98:1278–316.
95. Amorij J-P, Kersten GFA, Saluja V, Tonnis WF, Hinrichs WLJ, Slütter B, Bal SM, Bouwstra JA, Huckriede A, Jiskoot W. Towards tailored vaccine delivery: Needs, challenges and perspectives. Journal of Controlled Release.
96. Sekaly R-P. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? J Exp Med. 2008;205:7–12.
97. Lewis DJM, Huo Z, Barnett S, Kromann I, Giemza R, Galiza E, *et al.* Transient facial nerve paralysis (Bell's Palsy) following intranasal delivery of a genetically detoxified mutant of *Escherichia coli* heat labile toxin. PLoS One. 2009;4:e6999.
98. Vajdy M. Immunomodulatory properties of vitamins, flavonoids and plant oils and their potential as vaccine adjuvants and delivery systems. Expert Opin Biol Ther. 2011;11:1501–13.
99. Skountzou I, Quan F-S, Jacob J, Compans RW, Kang S-M. Transcutaneous immunization with inactivated influenza virus induces protective immune responses. Vaccine. 2006;24:6110–9.
100. Nottenburg C. Types of adjuvants: In Introduction to Adjuvant Patent Landscape. http://www.patentlens.net/daisy/adjuvants/Background/Adjuvant_types.html (accessed 14th April 2012).
101. Wee JLK, Scheerlinck JPY, Snibson KJ, Edwards S, Pearse M, Quinn C, *et al.* Pulmonary delivery of ISCOMATRIX influenza vaccine induces both systemic and mucosal immunity with antigen dose sparing. Mucosal Immunol. 2008;1:489–96.
102. Vauthier C, Bouchemal K. Methods for the preparation and manufacture of polymeric nanoparticles. Pharm Res. 2009; 26:1025–58.
103. LiCalsi C, Christensen T, Bennett JV, Phillips E, Witham C. Dry powder inhalation as a potential delivery method for vaccines. Vaccine. 1999;17:1796–803.
104. Mansour HM, Rhee Y, Wu X. Nanomedicine in pulmonary delivery. Int J Nanomedicine. 2009;4:299–319.
105. Pilcer G, Amighi K. Formulation strategy and use of excipients in pulmonary drug delivery. Int J Pharm. 2010;392:1–19.
106. Abdelwahed W, Degobert G, Stainmesse S, Fessi H. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv Drug Deliv Rev. 2006;58:1688–713.
107. Anhorn MG, Mahler H-C, Langer K. Freeze drying of human serum albumin (HSA) nanoparticles with different excipients. Int J Pharm. 2008;363:162–9.
108. Hirsjärvi S, Peltonen L, Hirvonen J. Effect of sugars, surfactant, and tangential flow filtration on the freeze-drying of Poly(lactic acid) nanoparticles. AAPS PharmSciTech. 2009;10:488–94.
109. Malcolmson RJ, Embleton JK. Dry powder formulations for pulmonary delivery. Pharmaceut Sci Tech Today. 1998;1:394–8.
110. Peltonen L, Valo H, Kolakovic R, Laaksonen T, Hirvonen J. Electrospraying, spray drying and related techniques for production and formulation of drug nanoparticles. Expet Opin Drug Deliv. 2010;7:705–19.
111. Heng D, Lee SH, Ng WK, Tan RB. The nano spray dryer B-90. Expet Opin Drug Deliv. 2011;8:965–72.
112. Amorij JP, Huckriede A, Wilschut J, Frijlink H, Hinrichs W. Development of stable influenza vaccine powder formulations: challenges and possibilities. Pharm Res. 2008;25:1256–73.
113. Shoyele SA, Cawthorne S. Particle engineering techniques for inhaled biopharmaceuticals. Adv Drug Deliv Rev. 2006;58:1009–29.
114. Al-fagih IM, Alanazi FK, Hutcheon GA, Saleem IY. Recent advances using supercritical fluid techniques for pulmonary administration of macromolecules via dry powder formulations. Drug Deliv Lett. 2011;1:128–34.
115. Okamoto H, Danjo K. Application of supercritical fluid to preparation of powders of high-molecular weight drugs for inhalation. Adv Drug Deliv Rev. 2008;60:433–46.
116. Byrappa K, Ohara S, Adschiri T. Nanoparticles synthesis using supercritical fluid technology – towards biomedical applications. Adv Drug Deliv Rev. 2008;60:299–327.
117. Kenji M. Biodegradable particle formation for drug and gene delivery using supercritical fluid and dense gas. Adv Drug Deliv Rev. 2008;60:411–32.
118. Pasquali I, Bettini R, Giordano F. Supercritical fluid technologies: an innovative approach for manipulating the solid-state of pharmaceuticals. Adv Drug Deliv Rev. 2008;60:399–410.
119. Amidi M, Pellikaan HC, Hirschberg H, de Boer AH, Crommelin DJA, Hennink WE, *et al.* Diphtheria toxoid-containing microparticulate powder formulations for pulmonary vaccination: preparation, characterization and evaluation in guinea pigs. Vaccine. 2007;25:6818–29.
120. W.H. Organization. Pneumonia. http://www.who.int/mediacentre/factsheets/fs331/en/index.html (accessed 17/04 2012).
121. Ghotbi Z, Haddadi A, Hamdy S, Hung RW, Samuel J, Lavasanifar A. Active targeting of dendritic cells with mannan-decorated PLGA nanoparticles. J Drug Target. 2011;19:281–92.

JrnlID 11095_ArtID 891_Proof# 1 - 21/09/2012

122. Bandyopadhyay A, Fine RL, Demento S, Bockenstedt LK, Fahmy TM. The impact of nanoparticle ligand density on dendritic-cell targeted vaccines. Biomaterials. 2011;32:3094–105.
123. Cruz LJ, Tacken PJ, Fokkink R, Joosten B, Stuart MC, Albericio F, *et al.* Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN *in vitro*. J Control Release. 2010;144:118–26.
124. Kwon YJ, James E, Shastri N, Fréchet JMJ. *In vivo* targeting of dendritic cells for activation of cellular immunity using vaccine carriers based on pH-responsive microparticles. Proc Natl Acad Sci U S A. 2005;102:18264–8.
125. Kempf M, Mandal B, Jilek S, Thiele L, Vörös J, Textor M, *et al.* Improved stimulation of human dendritic cells by receptor engagement with surface-modified microparticles. J Drug Target. 2003;11:11–8.
126. Sheng K-C, Kalkanidis M, Pouniotis DS, Esparon S, Tang CK, Apostolopoulos V, *et al.* Delivery of antigen using a novel mannosylated dendrimer potentiates immunogenicity *in vitro* and *in vivo*. Eur J Immunol. 2008;38:424–36.
127. White KL, Rades T, Furneaux RH, Tyler PC, Hook S. Mannosylated liposomes as antigen delivery vehicles for targeting to dendritic cells. J Pharm Pharmacol. 2006;58:729–37.
128. Jain S, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral mucosal immunization. J Liposome Res. 2006;16:331–45.
129. Hao J, Kwissa M, Pulendran B, Murthy N. Peptide crosslinked micelles: a new strategy for the design and synthesis of peptide vaccines. Int J Nanomedicine. 2006;1:97–103.
130. Luand D, Hickey AJ. Liposomal dry powders as aerosols for pulmonary delivery of proteins. AAPS PharmSciTech. 2005;6:E641–8.
131. Barnier Quer C, Robson Marsden H, Romeijn S, Zope H, Kros A, Jiskoot W. Polymersomes enhance the immunogenicity of influenza subunit vaccine. Polym Chem. 2011;2:1482–5.
132. Matinkhoo S, Lynch KH, Dennis JJ, Finlay WH, Vehring R. Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. J Pharm Sci. 2011;100:5197–205.
133. Saluja V, Amorij JP, Kapteyn JC, de Boer AH, Frijlink HW, Hinrichs WLJ. A comparison between spray drying and spray freeze drying to produce an influenza subunit vaccine powder for inhalation. J Control Release. 2010;144:127–33.
134. Jin TH, Tsao E, Goudsmit J, Dheenadhayalan V, Sadoff J. Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). Vaccine. 2010;28:4369–75.
135. Garcia-Contreras L, Wong Y-L, Muttil P, Padilla D, Sadoff J, DeRousse J, *et al.* Immunization by a bacterial aerosol. Proc Natl Acad Sci. 2008;105:4656–60.
136. Lu D, Garcia-Contreras L, Muttil P, Padilla D, Xu D, Liu J, *et al.* Pulmonary Immunization Using Antigen 85-B Polymeric Microparticles to Boost Tuberculosis Immunity. AAPS J. 2010;12:338–47.

AUTHOR QUERY

AUTHOR PLEASE ANSWER QUERY.

No Query.