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Sistemas coloidais utilizados para a imunização por via intranasal

Faculdade Ciências da Saúde

Universidade Fernando Pessoa

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Assinatura

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Sumário

A vacinação é um dos métodos mais adequado para proteger a população em geral de infeções. No entanto, a contínua evolução e a resistência dos microrganismos aos métodos atualmente existentes traduz-se na necessidade de inovar nesta área.

A imunização através das mucosas, particularmente por via intranasal, apresenta diversas vantagens, relativamente à imunização por outras vias, particularmente no que concerne à potenciação da resposta imunológica, sendo um tema amplamente estudado por vários investigadores.

Na presente dissertação é analisada a influência de diversos sistemas coloidais na encapsulação de antigénios e/ou adjuvantes utilizados para a imunização intranasal. Os estudos *in vivo* mais recentes envolvendo nanopartículas, micelas, lipossomas, arqueossomas, nanotubos de carbono ou partículas '*virus-like*' são apresentados, bem como as barreiras fisiológicas, farmacêuticas e regulamentares que estes sistemas têm que ultrapassar para atingirem o sucesso no mercado e na adesão da população.

Palavras-chave: Vacina, imunização nasal, sistemas coloidais, nanopartícula de sílica, nanopartícula de ouro, lipossoma, micela, virossoma, arqueossoma, nanotubo de carbono, partícula '*virus-like'*, resposta imunológica, antigénio, adjuvante.

Abstract

Despite being one of the most effective ways to protect the world population against infections, the continuous emergence and resistance of pathogens to vaccination highlights the constant need of innovation on this field.

Immunization through the mucosa, particularly through the intranasal route, has several advantages over immunization by other routes, particularly with regard to the potentiating the immune response, being a topic widely studied by several investigators.

This dissertation provides an overview on recent studies evolving particulate systems for antigens delivery, with or without adjuvants, to the airway mucosa. The most recent and relevant *in vivo* studies with nanoparticles, micelles, liposomes, archaesomes, carbon nanotubes or virus-like particles are presented, as well as physical, pharmaceutical and regulatory barriers that have to be overtaken by these particulate systems to hit clinical and commercial success.

Keywords: Vaccine, immunization, nasal, particulate systems, silica nanoparticle, gold nanoparticle, liposome, micelle, virosome, archaesome, carbon nanotube, virus-like particle, immune response, antigen, adjuvant.

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Abreviaturas

AMVAD: *Archael lipid mucosal vaccine adjuvant and delivery*

APC: Célula apresentadora de antigénios (do inglês, *antigen-presenting cell*)

AuNP: Nanopartícula de ouro

c-di-GMP: Guanosina monofosfato dimérica bis (3,5)-cíclica (do inglês, *bis-(3,5)-cyclic dimeric guanosine monophosphate*)

CAIV-T: Vacina trivalente para o Influenza adaptada ao frio (do inglês, *cold-adapted Influenza vacine trivalente*)

CDC: *Centers for Disease Control*

Células M: Células epiteliais

CMC: Concentração micelar crítica

CpG: Citosina-fosfato-guanina (do inglês, *cytosine-phosphate-guanine*)

CpG ODN: Citosina-fosfato-guanina oligodesoxinucleótido (do inglês, *cytosine-phosphate-guanine oligodeoxynucleotide*)

CRM-197: Material reativo 197 (do inglês, *cross-reactive material 197*)

DNA: Ácido desoxirribonucleico (do inglês, *deoxyribonucleic acid*)

DOTAP: 1,2-dioleoyl-3 (trimethylammonium) propane

ELISA: Ensaio de imunoabsorção enzimática (do inglês, *enzyme-linked immunosorbent assay*)

ErbB2 ou HER2/neu (do inglês, *Human Epidermal growth factor Receptor 2*)

GRAS: Geralmente reconhecido como seguro (do inglês, *generally recognised as safe*)

HA: Hemaglutinina

HAC_1: *Influenza haemagglutinin antigen*

HBV: Vírus da Hepatite B (do inglês, *Hepatitis B virus*)

HIV: Vírus da imunodeficiência humana (do inglês, *human immunodeficiency virus*)

ICMV: *Interbilayer-crosslinked multilamellar vesicles*

IFN-γ: Interferão γ

Ig: Imunoglobulina

IL: Interleucina

LUV: Vesícula unilamelar grande (do inglês, *large unilamellar vesicle*)

MALT: Tecido linfoide associado a mucosas (do inglês, *mucosa-associated lymphoid tissue*)

MHC: Complexo principal de histocompatibilidade (do inglês, *major histocompatibility complex*)

MLA: Monofosforil lípido A (do inglês, *monophosphoryl lipid A*)

MLV: Vesícula multilamelar grande (do inglês, *large multilamellar vesicle*)

MWNT: Nanotubo de parede múltipla (do inglês, *multi-walled nanotubes*)

NALT: Tecido linfoide associado à mucosa nasal (do inglês, *nasal-associated lymphoid tissue*)

NDV: Vírus de doença de Newcastle (do inglês, *Newcastle disease virus*)

NOD_2: *Nucleotide-binding oligomerization domain containig 2*

NP-CpG: Nanopartículas conjugadas com citosina-fosfato-guanina

OVA: Ovalbumina

PC lip: Lipossoma constituído por fosfatidilcolina

PEG: Polietilenoglicol

PEG-PLL-PLLeu: poli (etilenoglicol)-b-poli (L-lisina)-b-poli (L-leucina)

PIC: Ácido polirribonusínico:polirribocitidílico (do inglês, *polyribonusinic:polyribocytidylic acid*)

PLGA: Poli(ácido lático-co-ácido glicólico) (do inglês, *poly lactic-co-glycolic acid*)

PRINT: *Particle replication in non-wetting templates*

PS lip: Lipossoma constituído por fosfatidilserina

PsP A: Proteína A de superfície do *Streptococcus pneumoniae* (do inglês, *Pneumococcal surface protein A*)

PVA: Álcool de polivinilo (do inglês, *poly(vinyl) alcohol*)

RSV: Vírus sincicial respiratório (do inglês, *respiratory syncytial virus*)

S-IgA: Imunoglobulina A secretora

SA lip: Lipossoma constituído por estearilamina

SiO_2: Dióxido de silício ou sílica

SIV: Vírus da imunodeficiência símia (do inglês, *simian immunodeficiency virus*)

SUV: Vesícula unilamelar pequena (do inglês, *small unilamellar vesicle*)

SWNT: Nanotubo de parede simples (do inglês, *single-walled nanotubes*)

T_h: $T_{auxiliar}$ (do inglês, T_{helper})

TLR: Recetor do tipo Toll (do inglês, *Toll-like receptor*)

TNF-α: Fator de necrose tumoral α

VLP: Partícula vírus-like (do inglês virus-like particles)

γ-PGA: ácido γ-poliglutâmico (do inglês poly (γ-glutamic acid))

I. Introdução

A primeira vacina, produzida há mais de dois séculos por Edward Jenner, marcou o início da era moderna da medicina. A imunização por vacinação (i.e. imunização ativa) consegue atuar contra uma grande variedade de agentes patogénicos e é responsável pela sobrevivência anual de, aproximadamente, 2,5 milhões de crianças (Kunda *et al*., 2013). A vacinação representa uma estratégia essencial no controlo da saúde pública, sendo a medida mais eficaz para erradicar e reduzir a incidência de doenças infeciosas e morte (Diniz e Ferreira, 2010).

As vacinas para uso humano são preparações contendo substâncias capazes de induzir imunidade específica e ativa contra um agente infecioso e suas toxinas e/ou antigénios. Quando administradas no organismo induzem memória, usualmente sob a forma de anticorpos produzidos contra um antigénio específico (Irvine *et al.*, 2015). A constante capacidade dos microrganismos se adaptarem e resistirem a tratamentos convencionais fundamenta a necessidade de inovação e desenvolvimento de vacinas com potencial para defender e criar uma resposta imunológica efetiva e prolongada com uma administração única (Gregory *et al.*, 2013).

A vacinação pretende proporcionar aos indivíduos uma longa proteção contra os microrganismos através da estimulação do sistema imunológico. Este pode ser dividido em inato e adaptativo (Sahdev *et al.*, 2014).

A imunidade inata inclui mecanismos de defesa que não se adaptam após contacto com os microrganismos. Traduz-se numa resposta ativada rapidamente após ocorrer a infeção, mediada por diversos mecanismos envolvendo moléculas solúveis (i.e. ativação das proteínas do sistema complemento, síntese de proteínas de fase aguda, citocinas e quimiocinas) e células efetoras (células dendríticas, macrófagos, neutrófilos, células *Natural Killer*, mastócitos, basófilos e eosinófilos) (Cruvinel *et al*., 2010). A resposta inata é ativada pela deteção de padrões moleculares associados a microrganismos (Sahdev *et al*., 2014) (i.e. lipopolissacarídeos e resíduos de manose encontrados na superfície de microrganismos), após interação com os recetores de reconhecimento

desses padrões, como os recetores do tipo Toll (TLR, do inglês *Toll-like receptor*) (Cruvinel *et al*., 2010).

A imunidade adaptativa ou adquirida, ao contrário da inata, apresenta memória e especialização de resposta ao microrganismo (Cruvinel *et al*., 2010). Pode ser dividida em dois tipos, a resposta humoral e a celular.

A resposta humoral é mediada por anticorpos como as imunoglobulinas (Ig) A, D, E, G e M, produzidas por linfócitos B (Sahdev *et al*., 2014).

A imunidade celular é mediada pelos linfócitos T, cuja ativação depende das células apresentadoras de antigénios (APC, do inglês *Antigen Presenting Cell*). Destas, as células dendríticas são as mais eficazes na captação, processamento e apresentação dos antigénios aos linfócitos T. Na resposta celular intervém os linfócitos T_h (do inglês, T_{helper}) CD_4^+ e os linfócitos $T_{citotóxicos}$ CD_8^+. Os CD_8^+ detetam os antigénios apresentados pelo complexo principal de histocompatibilidade I (MHC-I, do inglês *Major Histocompatibility Complex I*), eliminando-os. Os CD_4^+, subdivididos em T_h 1 e T_h 2, reconhecem os antigénios apresentados pelo MHC-II. Os T_h 1 produzem interferão γ (IFN-γ), que possuem um papel importante na iniciação de respostas imunológicas celulares e na produção de IgG_2. Os T_h 2 produzem certas citocinas, como as interleucinas (IL) 4, 5 e 10, que regulam a ativação e a diferenciação de linfócitos B e estimulam a produção de IgE e IgG_1 (Sahdev *et al*., 2014).

As vacinas podem ser classificadas em três gerações, de acordo com as estratégias usadas na sua preparação (Diniz e Ferreira, 2010). As vacinas de primeira geração apresentam na sua constituição o(s) microrganismo(s) patogénico(s) na sua forma completa mas submetidos a determinados tratamentos químicos que os inativam, ou no estado vivo mas atenuado, podendo ainda utilizar-se as respetivas toxinas inativadas (Sahdev *et al*., 2014). Enquanto as vacinas mortas têm incapacidade de produzir respostas celulares específicas de linfócitos T citotóxicos, as formas atenuadas de microrganismos induzem respostas humorais e celulares mais prolongadas e com elevada potência (Sharma *et al*., 2009). Contudo, com estas últimas podem surgir problemas de segurança quando o sistema imunológico está comprometido, entre os

quais, a possível reversão à forma virulenta. Com o avanço da biotecnologia, as novas gerações de vacinas procuraram ultrapassar e reduzir os riscos das vacinas atenuadas (Sahdev *et al.*, 2014). A segunda geração consiste em vacinas de subunidades, que incluem péptidos antigénicos específicos ou proteínas recombinantes e a terceira geração inovou ao incluir nas vacinas a informação genética do microrganismo responsável pela codificação de antigénios. As respostas imunológicas são induzidas pela introdução de ácido desoxirribonucleico **(**DNA, do inglês *Deoxyribonucleic Acid*) nas células e pela produção de proteínas que alertam o sistema imunológico, induzindo uma resposta (Diniz e Ferreira, 2010). Os problemas de toxicidade das gerações novas de vacinas são menores, apresentando, adicionalmente, características de síntese ou purificação mais fáceis e uma administração segura. Por outro lado, as maiores desvantagens destas vacinas incluem a fraca imunogenicidade e respostas imunológicas de curta duração (Sahdev *et al.*, 2014).

O local de imunização é um dos fatores a considerar aquando do desenvolvimento de vacinas. A imunização através das mucosas tem sido bastante explorada devido à capacidade de vários vírus e bactérias iniciar infeções nas superfícies das mucosas dos tratos respiratório, intestinal, lacrimal e urogenital (Zaman *et al*., 2013). A vacinação ao nível das membranas das mucosas apresenta vários benefícios em relação à imunização parenteral, podendo induzir uma adequada resposta local ou sistémica (Sharma *et al*., 2009). Na administração parenteral, a presença de anticorpos na circulação sistémica pode não prevenir infeções em diferentes mucosas. À melhor imunidade gerada pela imunização nas mucosas, acrescenta-se a vantagem de não necessitar de agulhas para a administração, eliminando a possibilidade de infeção local oriunda da injeção. Para além disso, é possível ocorrer uma imunização ao nível de mucosas distantes do ponto de administração, devido à interligação do sistema imunológico comum e compartimentalizado das mucosas. Estas mucosas são constituídas por tecido linfoide (MALT, do inglês *Mucosa-Associated Lymphoid Tissue*) (Lycke, 2012). Na imunização através das mucosas, a vacina pode ser administrada por via oral, intranasal, retal ou vaginal. Destas, as vias oral e intranasal são consideradas as mais acessíveis e aceitáveis para imunizações repetidas e em massa. No entanto, a via oral pode apresentar várias limitações, como a diluição da formulação no conteúdo gastrointestinal e a degradação do antigénio por exposição a ácidos e enzimas, necessitando de doses maiores de antigénio (Sharma *et al*., 2009). A imunização intranasal pode ser induzida por doses

baixas de antigénio (Amorij *et al*., 2007). Possui também capacidade de induzir uma imunização sistémica, aumentando os níveis sistémicos de imunoglobulina G e os níveis de imunoglobulina A secretada pelos linfócitos B da mucosa nasal (Dehghan *et al.*, 2014).

Atualmente, existem poucas vacinas comercializadas destinadas à administração por via intranasal nos humanos, sendo que todas apresentam ação contra o vírus Influenza. Estas vacinas são de vários tipos: vivas atenuadas, entre as quais se pode citar a FluMist®, uma vacina tetravalente, a Fluenz Tetra®, uma vacina tetravalente (Sharma *et al*., 2009) e a NASOVAC-S, uma vacina trivalente (Brooks *et al*., 2016). A FluMist® era inicialmente trivalente e indicada apenas para pessoas saudáveis entre os 5 e os 49 anos, excluindo as principais idades alvo de uma vacina para o Influenza. A FluMist® estava apenas disponível na forma congelada, o que dificultava o transporte e o armazenamento da vacina. No entanto, uma nova fórmula da FluMist® trivalente foi desenvolvida, a CAIV-T (do inglês, *Cold-Adapted Influenza Vaccine*), uma vacina trivalente que pode ser armazenada em condições de temperaturas de frigorífico, e que tem como idades alvo dos 2 aos 49 anos (Sharma *et al*., 2009). A FluMist® evoluiu para tetravalente, apresentando as mesmas vantagens da CAIV-T e oferecendo proteção a quatro estirpes do vírus Influenza.

Atualmente nenhuma destas vacinas está recomendada pelos CDC (do inglês, *Centers for Disease Control*), devido à perda de efetividade registada entre 2013 e 2016.

As principais dificuldades na formulação de vacinas eficazes traduzem-se na baixa capacidade das moléculas atravessarem as membranas das mucosas, da clearance rápida destes tecidos e da escassa disponibilidade de adjuvantes compatíveis com o organismo humano (Zaman *et al*., 2013).

De acordo com as considerações anteriores, a necessidade de desenvolver sistemas de veiculação de antigénios e/ou imunoestimuladores é uma área bastante promissora, com a finalidade de obter respostas imunológicas mais seguras e eficazes. O principal local de indução de imunidade, na administração por via intranasal, é o tecido linfoide associado à mucosa nasal (NALT, do inglês *Nasal-Associated Lymphoid Tissue*).

Enquanto nos roedores este tecido localiza-se em ambos os lados do ducto nasofaríngeo, nos humanos corresponde ao anel linfático de Waldeyer. O NALT consiste em tecido linfoide localizado na cavidade oral que protege a entrada do trato digestivo e respiratório, funcionando como um local de indução de respostas imunológicas. O anel linfático de Waldeyer é composto pelas amígdalas faríngea, palatina e lingual, compondo o tecido linfoide associado à mucosa (MALT) da faringe. Estes locais de indução de imunidade são constituídos por células dendríticas, macrófagos, linfócitos T e linfócitos B, estando sobrepostos por células epiteliais (M). As amígdalas são cobertas por camada epitelial composta por células M com criptas profundas que permitem aumentar a superfície específica de contacto e o tempo de retenção de antigénios. Enquanto os antigénios solúveis atravessam o epitélio nasal, os antigénios em forma de partículas necessitam de um transportador. À medida que estes antigénios são apresentados à mucosa nasal, são transportados ativamente por células M até às células dendríticas, aos macrófagos e aos linfócitos B (células apresentadoras de antigénio), para o seu processamento e apresentação (Figura 1).

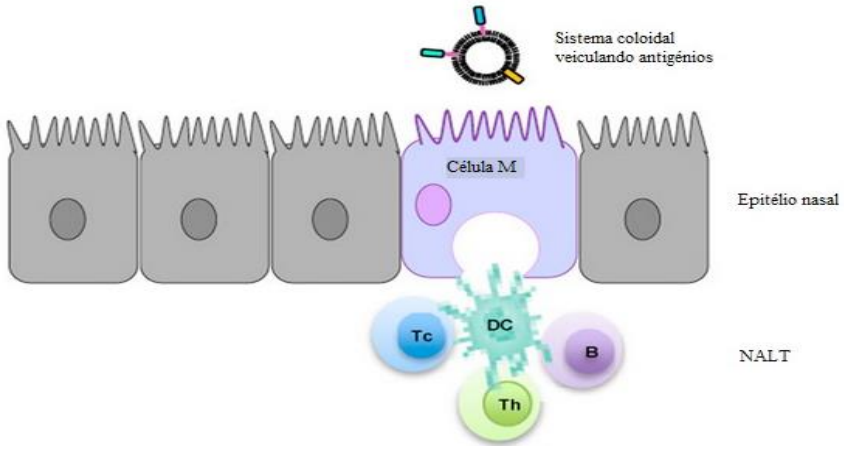

Figura 1 - Apresentação e transporte do antigénio ao NALT (adaptado de Kahki et al., 2016).

Em resposta, os linfócitos T_{helper} CD_4^+ ativados pelos antigénios interagem com os linfócitos B, formando células IgA^+ B. Estas células deslocam-se ao local de ação e diferenciam-se em células plasmáticas que secretam IgA em dímeros. Os dímeros ligam-se aos recetores transformando-se em imunoglobulina A secretora (S-IgA). A

S-IgA tem a capacidade de neutralizar a atividade de toxinas, vírus e bactérias, concedendo proteção imunológica nas mucosas (Zaman *et al*., 2013).

As respostas imunológicas induzidas pelos antigénios podem ser potenciadas através da sua incorporação em sistemas coloidais, produzindo respostas mais estáveis, completas e de longa duração apenas com uma única administração da vacina. Com a utilização destes sistemas transportadores, os antigénios estão protegidos das enzimas, são facilmente captados pelas células M e são libertados de forma controlada, aumentando o tempo de contacto com as APCs (Sharma *et al*., 2009).

A incorporação de antigénios nos sistemas coloidais tem sido uma estratégia promissora para induzir imunização por via intranasal, representando um avanço na tecnologia da vacinação quer na terapêutica (i.e. vacinação antitumoral) quer na prevenção contra microrganismos patogénicos (i.e. imunoterapia profilática). Os sistemas coloidais apresentam uma escala de tamanho semelhante ao dos agentes patogénicos (bactérias e vírus), e, desta forma, simulam um processo de infeção similar, induzindo, consequentemente, uma resposta imunológica (Zhao *et al*., 2014).

A utilização de sistemas coloidais permite ainda a co-encapsulação de antigénios e adjuvantes. Os adjuvantes são substâncias que aumentam a imunogenicidade dos antigénios, ampliando a potência e a duração das respostas imunológicas (De Magistris, 2006). O custo de produção das vacinas diminui com a incorporação de uma substância adjuvante, devido à possibilidade de diminuir a quantidade de antigénio veiculada. Deste modo, a produção de vacinas cujos antigénios derivem de péptidos e proteínas com baixa imunogenicidade (i.e. vacinas de subunidades) é beneficiada em termos de custos e eficácia com a incorporação de adjuvantes (Mota *et al*., 2006).

Os sistemas coloidais veiculando antigénios e adjuvantes permitem uma boa interação entre os adjuvantes, os antigénios e as células do sistema imunológico inato, como as APCs, facilitando a apresentação repetida de epítopos, o que proporciona um aumento da ativação de linfócitos B e linfócitos T (De Magistris, 2006). A co-encapsulação de um antigénio e um adjuvante (ex.: ligando de um TLR) ativa a imunogenicidade inata e adaptativa, aumentando a potência das vacinas (Sahdev *et al*., 2014). A obtenção de um

adjuvante potente que evite reações de toxicidade revela-se um grande desafio para a biotecnologia. É, no entanto, um desafio necessário, uma vez que são poucos os adjuvantes permitidos na vacinação em humanos. Atualmente, os adjuvantes mais utilizados são os sais de alumínio e esqualeno (ex.: MF59). Apesar de possuírem boa capacidade de indução de resposta humoral, geralmente não induzem respostas mediadas por células, pelo que não estão aprovados na vacinação das mucosas (Resende *et al*., 2004).

Os novos adjuvantes mais estudados para a vacinação intranasal incluem a citosina-fosfato-guanina contendo oligodesoxinucleótidos (CpG ODN, do inglês *Cytosine-Phosphate-Guanine oligodeoxynucleotide*) e o monofosforil lípido A (MLA, do inglês *Monophosphoryl Lipid A*) (Fujkuyama *et al*., 2012). O CpG ODN imita os efeitos imunoestimulatórios do DNA bacterial e induz a ativação e maturação das células dendríticas. O MLA surgiu da modificação de lipopolissacarídeos de origem bacteriana, apresentando menor toxicidade e a mesma potência imunoestimulatória (Zaman *et al.*, 2013).

Através de uma revisão sistemática de artigos científicos publicados no PubMed, SciELO e ScienceDirect, num período temporal de Setembro de 2016 a Setembro de 2017, foram selecionados e apresentados nas seguintes secções estudos com sistemas coloidais utilizados para induzir uma imunização intranasal. A seleção dos estudos teve como critério as datas de publicação, optando-se pelos realizados no novo milénio. As palavras-chave utilizadas incluíram vacina, imunização nasal, sistemas coloidais, nanopartícula de sílica, nanopartícula de ouro, lipossoma, micela, virossoma, arqueossoma, nanotubo de carbono, partícula '*virus-like'*, resposta imunológica, antigénio e adjuvante. Os artigos apresentados visam comprovar a vantagem da veiculação de antigénios e/ou adjuvantes pelos diversos sistemas coloidais, referindo os seus efeitos no sistema imunológico. Considerando os estudos *in vivo* mais recentes, esta tese reporta os sistemas coloidais com maior potencial clínico e sucesso comercial à vacinação, incluindo sistemas coloidais poliméricos, lipídicos e inorgânicos, referindo os adjuvantes mais promissores para a indução de uma resposta imunológica prolongada e efetiva.

II. Desenvolvimento

1. Fatores preponderantes na vacinação intranasal utilizando sistemas coloidais

i. Fatores Fisiológicos

Para se formular um sistema de veiculação adequado à imunização através do trato respiratório é necessário considerar as características fisiológicas deste, as quais podem influenciar a eficácia e o destino dos sistemas administrados.

O trato respiratório apresenta diversas características que o tornam um ótimo alvo para a aplicação de sistemas coloidais. Possui uma elevada superfície específica de absorção, o que permite um início de ação rápido. A sua mucosa é fina, facilitando a permeação de sistemas coloidais, e possui uma vascularização elevada, facilitando a absorção sistémica. Adicionalmente, a imunização por via intranasal é vantajosa pelo facto de a via respiratória possuir escassa degradação enzimática e evitar o efeito de primeira passagem hepática, ao contrário do que ocorre por exemplo com a imunização por via oral. O trato respiratório possui ainda um elevado número de APCs e pode ser usada para obter uma resposta imunológica local e sistémica pela capacidade absortiva do epitélio alveolar. Estas características tornam a via intranasal bastante promissora e eficaz para estimular o desenvolvimento de respostas imunológicas (Muralidharan *et al*., 2015).

Em termos anatómicos ou funcionais, o trato respiratório pode ser dividido em diferentes regiões (Wang *et al*., 2014). Anatomicamente, pode ser dividido em trato respiratório superior e inferior (Figura 2). O superior inclui os órgãos localizados fora do tórax, como o nariz, a faringe e a laringe. O inferior inclui todos os órgãos do tórax, entre os quais a traqueia, os brônquios, os bronquíolos, o ducto alveolar e os alvéolos.

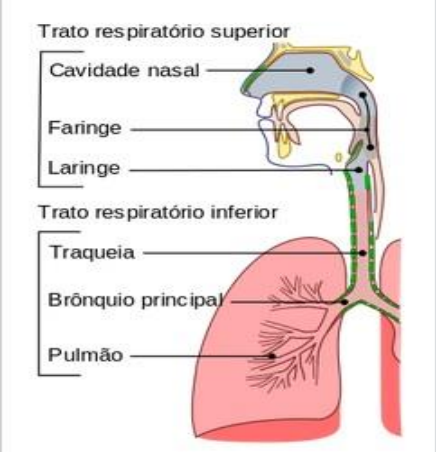

Figura 2 - Regiões anatómicas do trato respiratório.

Funcionalmente, segundo o modelo de Weibel representado na Figura 3, o sistema respiratório é constituído pela zona de condução e pela zona respiratória.

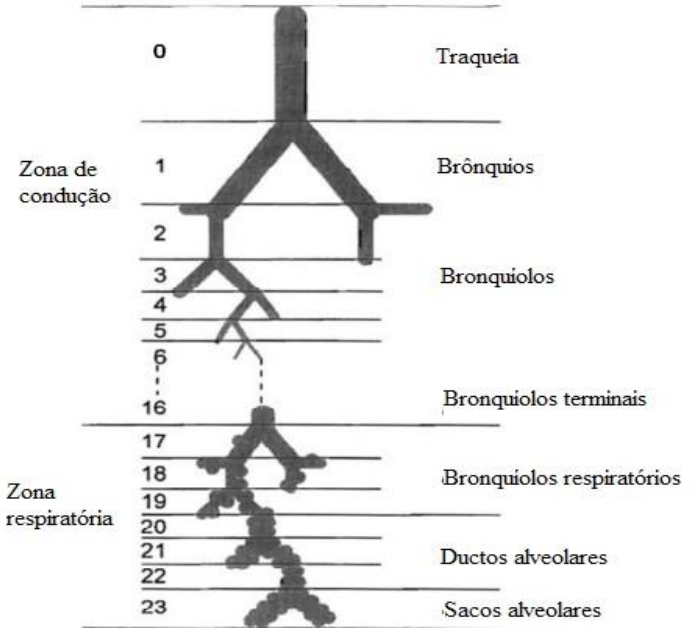

Figura 3 - Modelo do trato respiratório descrito por Weibel (adaptado de Wang et al., 2014).

A primeira zona, que se estende desde o nariz aos bronquíolos, consiste nos órgãos respiratórios que conduzem o ar inalado até às zonas de troca de gases. A zona respiratória permite a troca de gases entre os capilares e os alvéolos.

Na zona de condução não ocorre trocas de gases devido à constituição da sua parede, que não permite a difusão. Estas paredes são constituídas por cartilagem, tecido conjuntivo e músculo liso que confere suporte, flexibilidade e extensibilidade, condições perfeitas à condução de ar. Esta região tem um volume de aproximadamente 150 ml num humano (Wang *et al*., 2014).

A zona respiratória possui um comprimento de poucos milímetros, no entanto representa a maior parte do pulmão com um volume de 2,5 a 3 L. Os alvéolos possuem uma parede muito fina que permite a difusão entre o dióxido de carbono dos capilares sanguíneos e o oxigénio inspirado. Existem aproximadamente 350 milhões de alvéolos no pulmão, apresentando uma superfície de difusão entre os 60 e os 80 m^2 (Weber *et al*., 2014).

Estas regiões possuem diferentes epitélios pseudoestratificados e mecanismos de clearance que interferem na quantidade absorvida de partículas inaladas. Adicionalmente, o epitélio nasal é constituído por células pseudoestratificadas ligadas por desmossomas de pequeno diâmetro que limita a via paracelular de transporte de vacinas. Desta forma, é a via transcelular (Figura 4) a mais usada pelos sistemas coloidais para alcançar o tecido linfoide da nasofaringe (Weber *et al*., 2014).

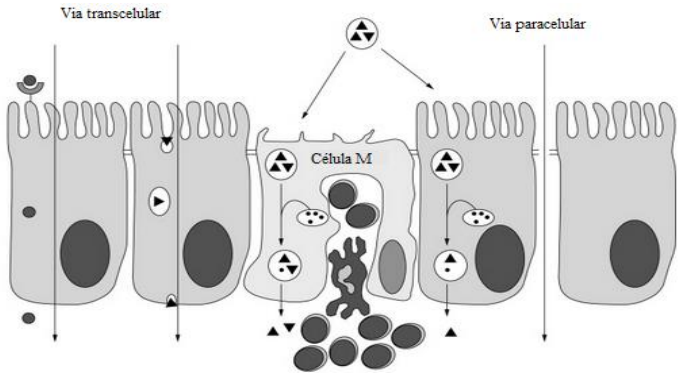

Figura 4 - Transporte paracelular e transcelular no trato respiratório (adaptado de Fasano, 1998).

A clearance das partículas e o movimento dos cílios são outros fatores fisiológicos que também influenciam a absorção das partículas ao nível do trato respiratório. Se a produção de muco pelas mucosas e células caliciformes aumentar, e se o movimento ciliar tornar-se mais rápido, as partículas não conseguem alcançar tão eficazmente as

regiões inferiores do trato respiratório. O processo de clearance mucociliário é um sistema natural de defesa do trato respiratório que, em condições saudáveis, é eficaz contra as partículas e as bactérias inaladas, as quais aderem à camada de muco e são transportadas até à garganta, onde podem ser deglutidas ou expelidas (Trindade *et al*., 2007).

A superfície do epitélio nasal apresenta uma camada espessa de muco que, em conjunto com as microvilosidades existentes, limitam a aderência e a captação dos sistemas coloidais. Consequentemente, as formulações administradas no epitélio respiratório podem ser removidas da cavidade nasal com um tempo de meia-vida de clearance de 15 minutos (Kraehenbuhl e Neutra, 2000). Um ser humano saudável produz cerca de 10-20 ml de muco por dia, sendo que a sua produção pode variar devido a diversos fatores. A clearance de muco diminui com a idade e, em alguns casos, na presença hipertensão, pela diminuição da drenagem linfática. Por outro lado, um doente com bronquite crónica ou fibrose cística pode apresentar uma produção de muco dez vezes superior (Ramos *et al*., 2014).

Após deposição na zona de condução, a maior parte das partículas insolúveis com diâmetro superior a 6 µm são removidas pela clearance do muco. As partículas mais pequenas tendem a ser absorvidas pela mucosa, atingindo o epitélio (Trindade *et al*., 2007). Consequentemente, as vacinas encapsuladas em sistemas nanoparticulados têm menor probabilidade de sofrerem clearance, quando comparados com sistemas microparticulados (Sharma *et al*., 2009).

A clearance pulmonar afeta não só o tempo de retenção das partículas como também a distribuição das mesmas. É, portanto, essencial considerar os modelos de clearance na preparação de um sistema coloidal. Apenas deste modo se consegue prever o destino exato da formulação inalada (Wang *et al*., 2014).

ii. Fatores Farmacêuticos

A eficácia de uma vacina é, em grande parte, influenciada pela natureza do sistema coloidal utilizado para a sua veiculação. As características do sistema

farmacêutico são fatores essenciais para aumentar o efeito da vacina, envolvendo diversos parâmetros que incluem o tamanho, a carga das partículas e outras modificações à sua superfície.

Diâmetro das partículas

O sucesso da deposição dos sistemas coloidais no trato respiratório está limitado pelo diâmetro das partículas. Partículas de tamanho superior a 5 µm depositam-se no trato respiratório superior, onde a velocidade do ar é elevada, por não serem capazes de seguir o fluxo de ar. As partículas com tamanho entre 1 e 5 µm conseguem passar o trato superior e depositam-se nos bronquíolos, onde a velocidade do ar é mais baixa, por sedimentação. Partículas com diâmetros menores que 0,5 µm chegam aos alvéolos por difusão (Wang *et al*., 2014), que ocorre de áreas de maior concentração para menor concentração. A difusão é mais comum nos alvéolos, onde o fluxo de ar é negligível ou ausente (Karhale *et al*., 2012).

Para além do diâmetro, as características da superfície das partículas representam propriedades com impacto direto na deposição e ação dos sistemas coloidais.

Carga

A carga de superfície das partículas influencia a bioadesão às membranas mucosas e a estabilidade da própria formulação. Considerando que a mucosa apresenta carga aniónica a pH 7 e que as células M e as células epiteliais também são carregadas negativamente, a presença de grupos com carga positiva na superfície das partículas induz uma interação entre estas e o muco, resultando numa maior adesão das partículas e, consequentemente, numa maior captação do antigénio veiculado. Os polímeros catiónicos mais usados em vacinas incluem o quitosano e o ácido hialurónico, os quais conferem uma redução da clearance ao nível da cavidade nasal (Sharma *et al*., 2009).

A carga da partícula pode ainda ter um papel essencial na estabilidade de uma vacina de DNA. Devido à sua carga negativa, esta geração de vacinas possui estabilidade geralmente baixa, pelo que a adição de compostos catiónicos à formulação parece ser

uma estratégia atrativa. Illum *et al*. (2001) estudaram a interação entre o quitosano (i.e. polímero catiónico) e o DNA veiculado em nanopartículas (20 a 500 nm) e demonstraram que as formulações sem carga tendem a agregar. Por outro lado, as nanopartículas contendo uma razão de 5:1 a favor do polímero catiónico não revelaram qualquer instabilidade nem tendência para agregar. Contudo, existem alguns autores que ressalvam a necessidade de controlar a quantidade de grupos catiónicos presentes na formulação com o intuito de minimizar a toxicidade, enquanto se obtém respostas imunológicas elevadas (Sharma *et al*., 2009).

Mucoadesividade

Os polímeros mucoadesivos possuem vários grupos hidrófilos, sofrendo hidratação e intumescimento quando em contacto com uma solução aquosa. Os grupos hidrófilos estabelecem ligações por pontes de hidrogénio com o muco, permitindo uma adesão mais prolongada (Varum *et al*., 2008).

A presença de polímeros hidrófilos com capacidade mucoadesiva à superfície das partículas, como o polietilenoglicol (PEG) ou o quitosano, permite aumentar o tempo de retenção da partícula na mucosa nasal, resistindo à clearance nasal, proporcionando uma maior eficácia da vacina (Chatudervi *et al*., 2011).

Meenach *et al*. (2013) comprovaram que a conjugação de PEG à superfície dos lipossomas resultava num tempo de residência superior do lipossoma, induzindo uma forte resposta imunológica. Shen *et al*. (2015) comprovaram ainda que a introdução de grupos PEG em nanopartículas constituídas por monoacrilato de tetraetilenoglicol e metacriiato de aminoetila, preparadas pelo método PRINT (do inglês, *Particle Replication in Non-wetting Templates*) aumentou o tempo de residência destas partículas e permitiu a sua distribuição homogénea no pulmão.

Para além das características mucoadesivas do quitosano, alguns autores relataram que este polímero pode ter propriedades adjuvantes que potenciam as respostas celulares e humorais (Zaman *et al.,* 2013). Alpar *et al*. (2005) demonstraram que lipossomas contendo quitosano à sua superfície apresentaram efeitos de abertura nos desmossomas,

favorecendo o transporte de antigénios e contribuindo para os altos níveis de respostas detetadas após a administração da formulação.

2. Sistemas coloidais na imunização intranasal

Uma grande variedade de compostos pode ser utilizada para a preparação destes sistemas coloidais. No entanto, a seleção dos compostos deve ser criteriosa, considerando a melhor resposta imunológica possível aos antigénios e/ou adjuvantes, uma vez que influencia as características do sistema coloidal, a estabilidade da molécula encapsulada, a toxicidade no organismo e a o processo de produção.

i. Base polimérica

Vários compostos de origem polimérica são usados na produção de sistemas coloidais. Os polímeros mais usados são os de origem sintética, como o poli (ácido lático-co-ácido glicólico (PLGA, do inglês *Poly Lactic-co-Glycolic Acid*), um polímero biodegradável e biocompatível, e os polímeros naturais, como o quitosano e o alginato, que diminuem o processo de clearance mucociliário e aumentam o tempo de contacto da formulação na passagem nasal (Sharma *et al*., 2009).

Nanopartículas

As nanopartículas são sistemas coloidais com tamanho que pode variar entre os 10 nm e os 1000 nm. A substância a veicular pode apresenta-se na forma dissolvida ou suspensa, sendo adsorvida ou conjugada à superfície da nanopartícula, ou encapsulada no seu interior (Mudshinge *et al*., 2011).

Dependendo do método de preparação, obtêm-se dois tipos de nanopartículas. As nanoesferas possuem uma estrutura do tipo matricial, onde as substâncias são dispersas no seu interior, enquanto as nanocápsulas possuem um núcleo que contém a substância a veicular, rodeado por uma membrana de libertação polimérica (Figura 5) (Mainardes *et al.*, 2006).

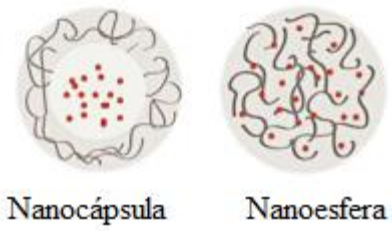

Figura 5 - Tipos de nanopartículas poliméricas (adaptado de Monteiro et al., 2015).

As nanopartículas poliméricas são os sistemas coloidais mais usados para a veiculação de agentes imunoestimulantes e/ou antigénios com o objetivo de induzir imunização através da via intranasal.

O quitosano, um polímero catiónico não tóxico, é o polímero de origem natural mais usado em estudos de imunização intranasal. Este polímero possui características que lhe confere mucoadesividade e capacidade de alteração dos desmossomas, aumentando o transporte paracelular. Slutter *et al*. (2010) prepararam nanopartículas de trimetil quitosano e provaram que, após a administração por via intranasal, estas aumentaram o tempo de residência da ovalbumina (OVA), o antigénio encapsulado, na cavidade nasal. Os resultados demonstraram ainda níveis elevados de S-IgA no soro e maturação de células dendríticas.

Pela capacidade de alteração de desmossomas, nanopartículas de quitosano também foram desenvolvidas para encapsular vacinas de DNA (Xu *et al*., 2011). Estes autores reportaram que, após a imunização intranasal de ratos, os níveis de respostas humorais e celulares foram bastante elevados nas nanopartículas expressando o antigénio superficial pneumococo A (Psp A, do inglês *Pneumococcal Surface Protein A*), um antigénio da espécie *Streptococcus pneumonia*. Os resultados mostraram igualmente níveis elevados de Anti-Psp A IgG no soro e anti-IgA nas mucosas, secreções elevadas de IFN-γ e IL-17 nos linfócitos da medula e nas mucosas. Estes resultados comprovam uma indução eficaz de respostas imunológicas sistémicas e na mucosa, protegendo da infeção do pneumococo. Quando comparado com a administração de DNA não veiculado em nanopartículas de quitosano, os níveis de anticorpos sistémicos e na

mucosa nasal demonstraram ser muito superiores. De acordo com estes resultados, os autores consideram promissor a utilização das nanopartículas de quitosano na prevenção de infeções pneumocócicas.

Considerando as propriedades das nanopartículas de quitosano, Figueiredo *et al.* (2012) tentaram potenciar a resposta imunológica de um extrato enzimático de *Streptococcus equi.* Após imunização intranasal em ratos, as nanopartículas induziram uma resposta imunológica mediada por linfócitos Th_1, dada a elevada produção de IFN e IgG2a, e por linfócitos Th_2, caracterizada pela produção de IL-4 e IgG1. Os autores constataram que as nanopartículas aumentavam a resposta imunológica do extrato enzimático, a nível humoral e celular, e que o quitosano induziu com sucesso uma forte imunização nas mucosas, registando um aumento dos níveis de S-IgA nos pulmões.

Em Portugal, na Universidade de Coimbra, Lebre *et al*. (2016) desenvolveram uma nova vacina intranasal para a Hepatite B, de modo a criar uma alternativa credível às formulações parenterais. Os autores encapsularam plasmídeos em nanopartículas de quitosano, adsorvendo soro de albumina à superfície das nanopartículas para facilitar a libertação intracelular do plasmídeo de DNA. Após administração intranasal em ratos, os autores verificaram uma indução de IgA específica ao vírus da Hepatite B (HBV, do inglês *Hepatitis B virus*) em secreções nasais e vaginais. Ao contrário da imunização com o plasmídeo livre, a nova vacina intranasal induziu uma forte imunização nas mucosas, demonstrando o seu potencial como nova plataforma para proteção do HBV.

Estes estudos demonstram o potencial das nanopartículas de quitosano para imunização nasal. A sua biodisponibilidade, mucoadesividade e biodegradabilidade posicionam estas nanopartículas como um dos sistemas de veiculação ideal para a vacinação da mucosa nasal.

O poli (γ-ácido glutâmico) (γ-PGA, do inglês *poly (γ-glutamic acid)*) é um polipéptido hidrófilo e biodegradável, de elevado peso molecular. É um polímero natural, produzido por certas estirpes de *Bacillus subtillis* (Sahdev *et al*., 2014). Matsuo *et al*. (2011) estudaram a eficácia antitumoral de nanopartículas constituídas por γ-PGA. Os autores veicularam o antigénio OVA nas nanopartículas e vacinaram ratos por via intranasal,

comparando os resultados da OVA livre com a OVA veiculada nas nanopartículas de γ-PGA. Os resultados demonstraram que a vacinação com as nanopartículas induziu uma estimulação superior dos linfócitos T citotóxicos e das células secretoras de IFN-γ específicas à OVA, nos nódulos linfáticos. Pela superior indução de linfócitos T citotóxicos (CD_8^+), as nanopartículas de γ-PGA revelaram ser sistemas de transporte promissores para a vacinação antitumoral não invasiva.

O PLGA é um polímero sintético bastante usado na área da biotecnologia. Este polímero tem sido explorado extensivamente na conjugação com sistemas coloidais, como as nanopartículas, devido à sua segurança, biocompatibilidade e biodegradabilidade. O PLGA sofre metabolização *in vivo,* formando moléculas biocompatíveis e metabolizáveis (i.e. ácido lático e glicólico) (Sahdev *et al*., 2014). Muttil *et al*. (2010a) desenvolveram nanopartículas compostas por PLGA com o objetivo de encapsular o antigénio CRM-197 (do inglês, *cross-reactive material 197*), uma forma não-tóxica de uma toxina da difteria. Após a administração intranasal destas nanopartículas em porcos, os autores detetaram valores de IgG e IgA mais elevados comparativamente com a administração intramuscular de uma vacina de alumínio-antigénio. Noutro estudo, os mesmos autores (2010b) revestiram as nanopartículas de PLGA com uma cápsula constituída por PEG, na qual foi adsorvido um antigénio de superfície da Hepatite B. O objetivo deste estudo foi determinar se os efeitos da administração desta vacina se sobrepunham aos da vacina contra a Hepatite B disponível por via intramuscular, a qual possui alumínio como adjuvante. Os porcos imunizados por via intranasal revelaram níveis mais elevados de IgA nas mucosas, enquanto os animais imunizados por via intramuscular apresentaram níveis superiores de IgG no sangue. No entanto, os autores concluíram que os aerossóis de vacina são vantajosos por não necessitarem dos adjuvantes utilizados nas vacinas intramusculares (como por exemplo, o hidróxido de alumínio).

A utilização de imunoestimulantes, como o CpG, tem apresentado um papel importante na investigação na área dos alérgenos aéreos. O CpG é conhecido por aumentar o recrutamento de células dendríticas e provocar respostas imunológicas do tipo Th1. Desta forma, a sua potenciação tem sido alvo de estudo por parte de vários investigadores. Ballester *et al.* (2015) realizaram um estudo em ratos para comprovar se

a encapsulação do oligodesoxinucleótido CpG em nanopartículas de polissulfato de propileno apresentava um efeito de prevenção (i.e. profilático) e de tratamento mais significativo do que a administração de CpG livre. Para tal, os autores sensibilizaram os animais com ácaros do pó e compararam as respostas imunológicas do CpG livre com o CpG veiculado nas nanopartículas. Quando as nanopartículas conjugadas com CpG (NP-CpG) foram administradas anteriormente à sensibilização ao alérgeno, demonstraram reduzir significativamente os níveis de IgE, eosinofilia, produção de muco e citocinas libertadas pelos Th2 (IL-4, IL-5 e IL-13), enquanto a imunização com CpG livre apresentou efeitos mais moderados. Quando as NP-CpG foram administradas após sensibilização aos ácaros, os autores reportaram os mesmos efeitos na diminuição de eosinofilia e de IgE, no entanto verificaram uma redução mais significativa de citocinas produzidas pela Th2 no pulmão. As nanopartículas demonstraram terem um papel importante na potenciação dos efeitos do CpG, demonstrando o seu efeito promissor quer na terapia quer na profilaxia de alergias.

A combinação de antigénios e imunoestimulantes no mesmo sistema coloidal é suscetível de demonstrar efeitos de potenciação do sistema imunológico. Vários investigadores basearam-se neste facto e realizaram estudos para comprová-lo. Stano *et al*. (2011) encapsularam OVA em nanopartículas de polissulfato de propileno, administrando-as em ratos por via nasal. Posteriormente, os autores adicionaram uma flagelina de um ligando do TLR5, que atua como imunoestimulante do sistema imunológico, à superfície das nanopartículas, comparando os seus efeitos com a administração de flagelina livre. A co-encapsulação da OVA e do ligando do TLR5 potenciou as respostas humorais na mucosa nasal, vaginal e retal, induzindo respostas celulares do tipo Th_1, ao contrário da flagelina livre. Os autores verificaram que as nanopartículas facilitavam o transporte da OVA pela mucosa epitelial da cavidade nasal do rato, induzindo respostas celulares no pulmão e no baço, bem como respostas humorais nas mucosas das vias respiratórias. Stano *et al*. (2011) sugerem que as nanopartículas de polissulfato de propileno são um ótimo sistema de veiculação de antigénios e adjuvantes, devendo esta estratégia tecnológica ser mais explorada para a vacinação na mucosa nasal.

Dehghan *et al*. (2014) associaram vírus completos de Influenza com dois adjuvantes, o oligodesoxinucleótido CpG ou a saponina de *Quillaia*, em nanoesferas de quitosano. Estas nanopartículas poliméricas, com comprovada eficácia no transporte do vírus Influenza pelas propriedades mucoadesivas e pela biocompatibilidade do quitosano, foram administradas por via intranasal em ratos nos dias 0, 45, 60 e 75. Quanto à ação dos adjuvantes, o CpG teve um papel mais significativo na indução de respostas imunológicas humorais e celulares, principalmente do tipo Th1, relativamente à saponina de *Quillaia*, sendo o adjuvante mais adequado para a coadministração do vírus Influenza. Os autores verificaram ainda que o grupo de ratos administrados com nanopartículas de vírus e CpG apresentou níveis mais elevados de IgG no sangue e de anticorpos de inibição da hemaglutinação, verificando-se igualmente uma estimulação de secreção de IL-2 e IFN-γ, pela ação imunoestimulatória do CpG.

As nanopartículas poliméricas são os sistemas mais utilizados na produção de potenciais vacinas nasais. Este fato deve-se à sua fácil produção, tamanho e capacidade de veiculação de antigénios e adjuvantes. A adição de polímeros naturais ou sintéticos com determinadas características potenciam os efeitos *in vivo* das nanopartículas, potenciando as respostas imunológicas. No entanto, são necessários mais estudos para a evolução destes estudos para ensaios clínicos.

Micelas

As micelas são sistemas coloidais de tamanho compreendido entre os 10 e os 200 nm, formadas por associação espontânea de polímeros anfifílicos em solução aquosa, quando a sua concentração está acima da concentração micelar crítica (CMC). Estes sistemas contêm cabeças hidrófilas e caudas hidrófobas (Figura 6), que se associam espontaneamente formando um núcleo que pode veicular moléculas insolúveis em água (Owen *et al*., 2012).

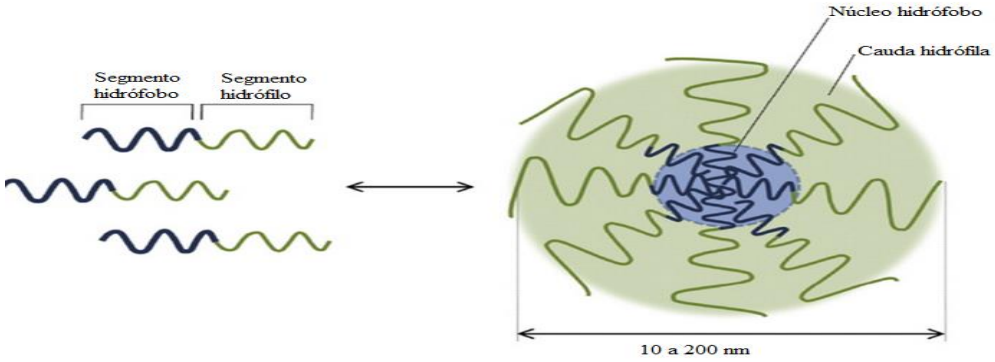

Figura 6 - Formação e estrutura de uma micela (adaptado de Owen et al., 2012).

Noh *et al*. (2013) utilizaram γ-PGA conjugado com grupos hidrófobos de colesterol e modificados com aminas, formando micelas de tamanho nanomolecular (20 nm) com capacidades mucoadesivas. Para testar a eficácia do sistema de transporte, os autores encapsularam OVA marcada com iodo e administraram as micelas por via nasal a ratos, comparando os resultados com a administração de OVA livre. Nos ratos imunizados com as micelas foram encontrados níveis superiores de IgA específicos de OVA aos encontrados nos ratos imunizados com OVA livre, indicando uma boa resposta imunológica das mucosas. As nanomicelas também estimularam a secreção de IFN-γ em níveis superiores à administração de OVA livre. Para além da resposta humoral e celular, não foi encontrada toxicidade ou inflamação na mucosa nasal dos ratos imunizados. Estes autores também utilizaram as micelas para encapsular o PR8, um antigénio do vírus Influenza A. Os ratos apresentaram respostas semelhantes às detetadas no estudo anterior, e quando submetidos a doses letais de vírus, todos os ratos imunizados com as micelas sobreviveram. Estes resultados demonstraram a indução de imunidade humoral e celular quando os ratos foram submetidos a uma dose letal do vírus H1N1, comprovando o benefício de vacinação com a micela de γ-PGA como sistema veiculador do PR8.

Luo *et al*. (2013) prepararam micelas com base no polipéptido poli(etilenoglicol)-b-poli(L-lisina)-b-(L-leucina) (PEG-PLL-PLLeu), para veicular antigénios. Com o

objetivo de verificar se as micelas desenvolvidas eram promissoras como indutores de resposta imunológica, os autores encapsularam o antigénio OVA, estudando os seus efeitos *in vivo*. As micelas demonstraram uma capacidade espontânea de encapsular a OVA, bem como uma excelente estabilidade. Para além disso, as micelas aumentaram a maturação das células dendríticas, a captação e a apresentação da OVA demonstrando uma produção aumentada de anticorpos. Os autores investigaram ainda a capacidade destas micelas na co-encapsulação da OVA e um agonista do recetor TLR3, o ácido poliribonosínico-poliribocitidílico (PIC, do inglês *polyribonusinic:polyribocytidylic acid*) para aumentar sinergicamente a resposta dos linfócitos T citotóxicos específicos de tumores. As vacinas não só demonstraram ser estáveis como aumentaram moderadamente a produção de IFN-γ e a resposta dos linfócitos T citotóxicos específicos da OVA, uma vez que as micelas facilitaram a captação do PIC (i.e, ácido ribonucleico de cadeia dupla) pelas células dendríticas, aumentando o nível da resposta imunológica em comparação com a administração de PIC livre. Os autores concluíram que as micelas PEG-PLL-PLLeu apresentam grande potencial para o desenvolvimento de futuras vacinas.

Devido ao seu pequeno tamanho, as micelas são facilmente captadas pelas células M, aumentando o transporte do antigénio às APCs. Esta característica conjugada com a capacidade de veiculação de antigénios e adjuvantes permitem às micelas constituir futuras vacinas intranasais. Apesar de apresentarem estas vantagens, não existem muitos estudos para a administração nasal, devido à possibilidade de destabilização por diluição em contato com fluidos biológicos, o que pode resultar na libertação imediata do antigénio encapsulado.

i. Base lipídica

Vários investigadores avaliaram o efeito de partículas lipídicas administradas por via intranasal, analisando as respostas *in vivo* e referindo as vantagens e desvantagens destes sistemas. Os estudos apresentados neste subcapítulo avaliam as respostas imunológicas induzidas por sistemas coloidais de base hidrófoba, a nível profilático e terapêutico. Os lipossomas são os sistemas de transporte mais utilizados na veiculação de antigénios e/ou adjuvantes. A partir da modificação de lipossomas surgiram outros

sistemas lipídicos que também demonstraram apresentar capacidade de estimular o sistema imunológico.

Lipossomas

Os lipossomas são vesículas esféricas constituídas por uma ou mais bicamadas de fosfolípidos (i.e. lípidos anfifílicos), cujo compartimento interno é aquoso. Estas características permitem os lipossomas possuírem a capacidade de encapsulação de compostos lipossolúveis, na bicamada lipídica, e hidrossolúveis, na cavidade interna aquosa. Os antigénios e adjuvantes podem ainda ser quimicamente ligados ou adsorvidos à superfície do lipossoma (Figura 7).

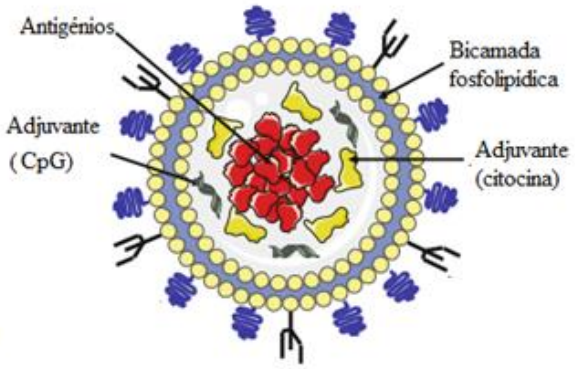

Figura 6 - Estrutura do lipossoma (adaptado de Heegaard et al., 2011).

Os lipossomas são constituídos por lípidos de elevada compatibilidade que apresentam a classificação GRAS (do inglês, *Generally Recognised as Safe*), os quais podem ser de origem natural (e.g. colesterol, lecitina do ovo e da soja), ou de origem semissintética (e.g. fosfatidilcolina, fosfatidilserina, fosfatidilglicerol e esfingomielina).

Os lipossomas podem ser classificados em três tipos, de acordo com o seu tamanho e número de camadas. O tamanho das vesículas varia entre pequeno (25 nm) a grande (2,5 µm). Quanto ao número de camadas, estas vesículas podem possuir uma, sendo

referidos como unilamelares, ou múltiplas, designadas por multilamelares. Deste modo, a classificação dos lipossomas divide-se em vesículas unilamelares pequenas (SUV, do inglês *small unilamellar vesicles*), vesículas unilamelares grandes (LUV, do inglês *large unilamellar vesicles*) e vesículas multilamelares grandes (MLV, do inglês *large multilamellar vesicles*) (Akbarzadeh *et al*., 2013).

A utilização de lipossomas como sistema coloidal de veiculação impede a degradação ou neutralização dos antigénios e/ou adjuvantes veiculados, permitindo uma libertação controlada dos mesmos. Devido ao maior controlo na libertação das moléculas veiculadas, a apresentação às APCs torna-se mais eficaz (Sharma *et al*., 2009). Os lipossomas possuem ainda a capacidade de abertura dos desmossomas, promovendo a via paracelular de transporte das moléculas veiculadas.

Adicionalmente, modificações à superfície destes sistemas coloidais podem contribuir para uma resposta imunológica mais significativa. O uso de polímeros mucoadesivos, como o alginato ou o quitosano, conferem aos lipossomas a capacidade de diminuir a clearance mucociliária, aumentando o tempo de retenção dos lipossomas (Mainardes *et al*., 2006). Chen *et al*. (2013) compararam o poder de mucoadesão do alginato e do quitosano, quando incorporados à superfície de lipossomas preparados com fosfatidilcolina de soja e dimiristiol. Os autores verificaram que o quitosano possui um poder de mucoadesão superior ao alginato, permitindo aos lipossomas um maior tempo de retenção e, consequente, uma resistência à clearance mucociliária.

Even-Or *et al*. (2011) comprovaram que a utilização de lipossomas em vacinas intranasais induziu respostas imunológicas potentes, reduzindo a gravidade da infeção pelo vírus Influenza. Neste estudo, os autores prepararam lipossomas contendo na sua composição colesterol e um esfingolípido policatiónico, a ceramida carbamoil-espermina, administrando-os intranasalmente a ratos infetados com o vírus Influenza. Os lipossomas induziram respostas humorais locais e sistémicas, com elevada produção de anticorpos inibitórios da hemaglutinação, e respostas celulares, com elevada secreção e proliferação de INF e IL-2 (tipo Th-1) e de IL-5 (tipo Th-2). Em adição, os estudos revelaram que os lípidos e os antigénios tiveram um tempo de retenção elevado na

mucosa nasal e no pulmão, o que promoveu a intensidade da resposta imune obtida no rato.

As propriedades físico-químicas dos lipossomas, como a carga de superfície, o tamanho, a composição e a fluidez, influenciam diretamente os seus efeitos imunológicos. A temperatura de transição de fase é um aspeto muito importante na fluidez do lipossoma, influenciando a sua estabilidade *in vivo* (Tseng *et al*., 2010).

Tseng *et al*. (2010) avaliaram as respostas imunológicas de três MLV com composição e carga de superfície diferentes: lipossomas com carga neutra, compostos por fosfatidilcolina (PC-Lip), lipossomas carregados negativamente, compostos por fosfatidilserina (PS-Lip) e lipossomas carregados positivamente, compostos por estearilamina (SA-Lip). Para avaliar os efeitos de cada um dos sistemas, encapsularam o vírus inativado da doença de Newcastle (NDV, do inglês *Newcastle disease virus*) e compararam as respostas com a administração de NDV livre em frangos saudáveis. Os autores verificaram que as respostas imunológicas foram potenciadas por todos os tipos de lipossomas, quando comparadas com a administração de NDV livre, no entanto o PC-Lip demonstrou ser captado mais facilmente por macrófagos do que PS-Lip e SA-Lip, induzindo valores superiores de S-IgA nas lavagens broncoalveolares e de IgG no sangue. Apesar de, teoricamente, os lipossomas neutros serem menos estáveis, uma vez que podem sedimentar e agregar, a estabilidade *in vivo* dos PC-Lip é influenciada mais significativamente pela temperatura de transição de fase. Para a formulação PC-Lip, esta temperatura é de 43 ºC, a mais baixa dos 3 tipos de lipossomas e a mais próxima da temperatura corporal do frango. Assim, quando em contacto com a mucosa, os PC-Lip tornam-se mais flexíveis, o que facilita a captação e a apresentação dos antigénios. Quando os frangos foram submetidos à infeção com o vírus, os animais imunizados com a formulação PC-Lip apresentaram uma taxa de sobrevivência de 90%, em comparação aos 0% dos animais do grupo controlo. Os autores sugerem que os lipossomas PC-Lip podem ser um sistema de veiculação promissor para a vacina contra o NDV.

Zhou *et al*. (2010) testaram a encapsulação do oligodesoxinucleótido CpG DNA como terapêutica no controlo da proliferação das células do tumor pulmonar. Nesse estudo, os

autores administraram lipossomas com o CpG DNA em ratos, comparando os resultados com o controlo (CpG DNA livre). Os ratos foram injetados com colon26/Luc (i.e. uma linha celular do adenocarcinoma) e B16F10 (i.e. linha celular do melanoma). Os autores concluíram que os ratos administrados com lipossomas apresentavam tempo de sobrevivência superior quando comparados com os animais do grupo controlo, verificando níveis de produção de IFN-γ elevados. Os autores salientaram a importância dos lipossomas nos resultados desta vacina antitumoral.

Tai *et al*. (2011) basearam-se em lipossomas para desenvolverem duas formulações que promovessem uma resposta imunológica na mucosa. Os lipossomas possuíam na sua composição dilauril-fosfatidilcolina, veiculando posteriormente moléculas diferentes. Os autores encapsularam dois adjuvantes (monofosforil lípido A e trealose 6,6 dimicolato) em parte dos lipossomas e os restantes veicularam péptidos miristilados derivados do vírus Influenza. As duas formulações de lipossomas foram administradas em ratos por via nasal, após exposição prévia dos animais à infeção com o vírus Influenza. As respostas diferiram no tipo de substâncias encapsuladas nos lipossomas. Os lipossomas que encapsularam adjuvantes atingiram preferencialmente os macrófagos pulmonares, induzindo a produção de efeitos antivirais e a secreção de citocinas, promovendo uma proteção imediata mas de curta duração. Os lipossomas que encapsularam os péptidos induziram elevada imunidade local com resposta duradoura inata e específica dos linfócitos T. Os autores concluíram que ambas as formulações devem ser investigadas com maior profundidade para a utilização futura contra o vírus Influenza.

Kakhi *et al*. (2016) estudaram o poder imunoterápico de uma vacina encapsulada em lipossomas e administrada por via intranasal em ratos que apresentavam tumor no pulmão com expressão elevada do antigénio tumoral ErbB2, também conhecido como HER2/neu (do inglês *Human Epidermal growth factor Receptor 2*). Os investigadores exploraram igualmente fatores que influenciam a eficácia dos lipossomas, como a dose de antigénio e adjuvante veiculado, a estrutura do lipossoma, o tamanho e a flexibilidade. No estudo, os autores desenvolveram 3 formulações de lipossomas, entre os quais SUVs, MLVs e SUV ultraflexíveis. Estes últimos, alterados na sua produção pela adição de um tensioativo aniónico, também são denominados de transferossomas.

Possuem elevada elasticidade, capacidade de se deformarem e de atravessar poros com dimensões inferiores às suas. Nas formulações, os autores encapsularam um epítopo de ErbB2 T-citotóxico, um epítopo derivado de uma hemaglutinina (HA) do vírus influenza e um lipopéptido adjuvante, o Pam2CAG (agonista do TLR). Apesar de se verificarem atividades antitumorais em todos os lipossomas, os resultados obtidos demonstraram que as respostas imunológicas não foram afetadas pelas características dos lipossomas, mas pela dose de antigénios e adjuvantes presentes na formulação. Este facto deve-se à elevada capacidade de imunoestimulação, que compensaram as diferenças que os vários lipossomas pudessem apresentar. Os autores concluíram que a seleção criteriosa do adjuvante é essencial para o desenvolvimento de uma vacina baseada em lipossomas.

Fan *et al*. (2015) produziram um sistema coloidal baseando-se na ligação iónica entre lipossomas catiónicos produzidos com o 1,2-dioleoil-3-trimetilamónia-propano (DOTAP, do inglês *1,2-dioleoyl-3 (trimethylammonium) propane*) e o ácido hialurónico. Neste estudo, os autores não só demonstraram a estabilidade do sistema coloidal desenvolvido como testaram a capacidade de co-encapsulação de antigénios e adjuvantes imunoestimulatórios. Os autores encapsularam a OVA, o antigénio modelo mais utilizado, com o agonista do TLR4, o MLA. Após imunização de ratos por via nasal, os resultados *in vivo* demonstraram uma boa resposta celular, com níveis elevados de linfócitos T CD_8^+ específicos da OVA, e humoral, com níveis elevados de IgG específicos da OVA. No entanto, os autores verificaram uma resposta de Th2 superior à Th1. Estes resultados permitiram concluir que a encapsulação do antigénio F1-V com MLA como candidato resulta numa vacina bastante promissora contra a *Y. pestis*. Um período de 77 dias após a imunização dos ratos, Fan e colaboradores detetaram respostas humorais com níveis de IgG superiores à vacina com F1-V solúvel, mas respostas balanceadas de Th1/Th2, o que sugere que a seleção do antigénio influencia as respostas imunológicas. Com base nestes resultados, os autores concluíram que a vacina desenvolvida contra a *Y. pestis* é bastante promissora.

Os lipossomas possuem uma grande vantagem sobre os sistemas coloidais poliméricos e inorgânicos por apresentarem elevada biocompatibilidade, devido à sua composição GRAS. A sua capacidade de veiculação de antigénios e adjuvantes potencia a indução

de respostas imunológicas prolongadas, possibilitando a formulação de vacinas promissoras nas áreas terapêuticas e profiláticas. No entanto, em condições fisiológicas, os lipossomas podem apresentar alguma instabilidade resultando na libertação das moléculas veiculadas. Deste modo, surgem sistemas provenientes de várias modificações nos métodos de produção e estrutura dos lipossomas, que visam uma maior estabilidade *in vivo* e consequentemente uma vacina mais segura e eficaz.

Para além dos transferossomas, referidos anteriormente, outros lipossomas modificados foram desenvolvidos para a imunização intranasal. Os arqueossomas foram alvo de vários estudos por Patel *et al*. (2007), que produziram uma vacina baseada em lípidos da família *Archaea,* com função adjuvante e de encapsulação de antigénios, denominada AMVAD (do inglês, *archael lipid mucosal vaccine adjuvant and delivery*). Estes sistemas possuem uma estabilidade superior aos lipossomas a elevadas ou baixas temperaturas, promovem a interação com as APCs e induzem respostas humorais (Th1 e Th2) e celulares (CD_8^+) específicas ao antigénio encapsulado. Os autores formularam o AMVAD pela adição de $CaCl_2$ a arqueossomas encapsulando OVA, após verificar que estes não induziam resposta dos anticorpos anti-OVA IgA nas mucosas. Os OVA/AMVAD não só induziram respostas celulares (linfócitos T CD_8^+) e humorais (anti-OVA IgG, IgG1 and IgG2A e anti-OVA IgA) significativas, como estas perduravam por vários meses.

Partindo do mesmo raciocínio surgem os virossomas. Estes sistemas resultam de partículas extraídas das glicoproteínas ou dos fosfolípidos de vírus, seguida da solubilização com fosfolípidos comuns nos lipossomas e remoção do surfactante de solubilização. Desta forma, refere-se que os virossomas são estruturalmente semelhantes aos SUVs. Shafique *et al*. (2013) desenvolveram um estudo para comprovar a indução de respostas locais e sistémicas de uma vacina baseada num virossoma conjugada com dois adjuvantes lipófilos, um ligando do TLR2 (Pam3CSK4) e um ligando do NOD2, do inglês *nucleotide-binding oligomerization domain containig 2,* o L18-MDP. Após a administração intranasal em ratos (*in vivo*), a incorporação de Pam3CSK4 potenciou os níveis específicos de IgG ao Vírus Sincicial Respiratório (RSV, do inglês *respiratory syncytial virus*) no sangue e os níveis de IgA nas mucosas. Apesar do L18-MDP livre não possuir a capacidade de induzir respostas humorais, a

conjugação dos dois adjuvantes estimulou respostas humorais elevadas, protegendo os ratos do RSV sem induzir uma forma avançada da doença.

Nanopartículas lipídicas

As nanopartículas lipídicas surgiram para colmatar os principais problemas dos lipossomas, que incluem a baixa capacidade de encapsulação de substâncias lipófilas, a sua produção envolver o uso de solventes orgânicos (i.e. possibilidade de possuírem resíduos tóxicos) e apresentarem alguma instabilidade nos fluidos biológicos. Estes problemas são resolvidos pela produção de nanopartículas lipídicas, com tamanho inferior a 100 nm, por métodos que não envolvem o uso de solventes orgânicos. A estrutura das nanopartículas lipídicas é um híbrido entre nanopartículas poliméricas e lipossomas, devido ao seu núcleo oleoso estabilizado por surfactantes e rodeado por uma membrana rígida de tensioativos (Huynh *et al*., 2009).

Vicente *et al.* (2013) utilizaram o Miglyol® 812 como base hidrófoba para a formação do núcleo das nanocápsulas lipídicas, com o objetivo de produzir respostas imunológicas contra o vírus da hepatite B. Para potenciar os efeitos desta vacina, os autores adicionaram ao núcleo oleoso um imunoestimulante lipófilo, o imiquimod, um agonista do TLR7 (Figura 8). A cápsula à volta do núcleo é constituída por quitosano e possui capacidade de associação a antigénios. Para produzir esta vacina contra a Hepatite B, os autores associaram à nanocápsula um antigénio recombinante de superfície deste vírus. Os estudos *in vitro* demonstraram uma boa captação destes sistemas coloidais pela linha de macrófagos murinos 264.7, com consequente indução da secreção de citocinas. Após a imunização de ratos por via intranasal, as nanocápsulas lipídicas elevaram os níveis de IgG específicos ao antigénio e induziram uma resposta de memória imunológica ao vírus. Os autores sugerem que esta formulação é uma boa estratégia de proteção contra o vírus da Hepatite B, reconhecendo, no entanto, a necessidade de futuros estudos para a possível introdução no mercado.

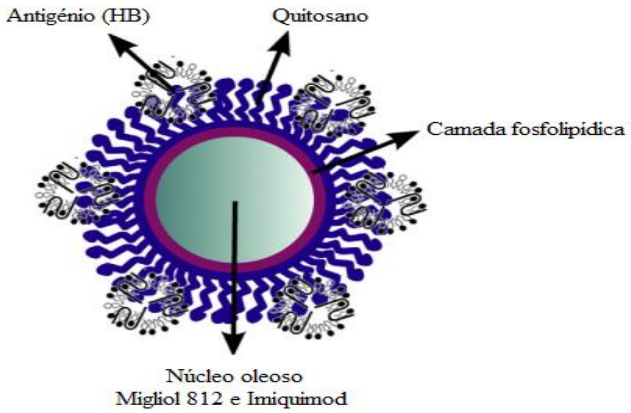

Figura 8 - Estrutura da nanocápsula lipídica (adaptado de Vicente et al., 2013).

Li *et al*. (2016) desenvolveram nanocápsulas lipídicas ao ligar grupos lipídicos adjacentes de bicamadas lipídicas em vesículas multilamelares. A este novo sistema designou-se de ICMV, do inglês interbilayer-crosslinked multilamellar vesicles. Os autores testaram a eficácia deste sistema coloidal como uma vacina intranasal que protegesse os indivíduos do vírus de imunodeficiência símia (SIV, do inglês *simian immunodeficiency virus*). Para tal, os autores co-encapsularam dois adjuvantes (MLA ou PIC) e epítopos SIV-gag, administrando-os aos ratos por via intranasal. Estes sistemas demonstraram transportar 60 vezes mais antigénio do que a vacina subcutânea equivalente, devido à vasta presença de APCs nos pulmões. As ICMV induziram respostas celulares T CD_8^+ e de memória na mucosa local e distais com maior intensidade do que uma vacina solúvel equivalente. Quando os ratos foram expostos ao vírus, as vacinas ICMV protegeram os animais, enquanto as vacinas subcutânea e solúvel demonstraram 100% de mortalidade ao fim de 5 dias.

As formulações descritas nesta subsecção demonstraram ser consideradas boas plataformas para o desenvolvimento de novas vacinas para imunização por via intranasal, por apresentarem elevada estabilidade, resistência à degradação enzimática e eficácia na indução de respostas imunológicas significativas.

ii. Base inorgânica

Vários materiais inorgânicos são utilizados para preparar sistemas coloidais de veiculação de fármacos, no entanto apenas alguns têm sido alvo de estudo como indutores do sistema imunológico num passado recente. Nesta secção, materiais, como o ouro, carbono e sílica, são apresentados como base de nanossistemas usados na imunização intranasal, explorando as vantagens por serem estruturalmente resistentes *in vivo* e por possuírem uma síntese facilmente controlada (Kalkanidis *et al*., 2006).

Nanopartículas de sílica

O potencial das nanopartículas de sílica foi explorado por Neuhaus *et al*. (2013), que em dois estudos testaram a capacidade imunogénica destas nanopartículas encapsulando uma hemaglutinina do vírus Influenza H1N1 (HAC1, do inglês *Influenza haemagglutinin antigen*) produzida em plantas de tabaco. Num estudo, Neuhaus *et al*. (2013) testaram a toxicidade da vacina e mediram a resposta imune *ex vivo* em tecidos de pulmão humano cortado, que simula os efeitos locais pelo sistema imunológico inato induzidos pelos imunomoduladores. Os resultados não revelaram qualquer toxicidade nestes tecidos orgânicos e demonstraram que as nanopartículas induziram a secreção do fator de necrose tumoral (TNF-α) e da interleucina 1 β (IL-1β), mostrando o papel adjuvante da sílica na imunização. As nanopartículas de sílica mostraram ainda um papel na reativação de uma resposta celular de linfócitos T específicos ao antigénio.

Num outro estudo, Neuhaus *et al*. (2014) testaram a capacidade imunológica das nanopartículas de sílica (HAC1/SiO_2) com um adjuvante, a guanosina monofosfato dimérica bis-(3,5)-cíclica, ou c-di-GMP. Os autores vacinaram os ratos com três formulações, a primeira sem adjuvante (HAC1/SiO_2), a segunda apenas com adjuvante (HAC1/ c-di-GMP) e a terceira com adjuvante e sílica (HAC1/SiO_2/c-di-GMP). As respostas imunológicas específicas foram avaliadas pela presença de IgG específicos da inibição da hemaglutinina. As respostas locais foram avaliadas pela presença de IgA e IgG contidos nas lavagens broncoalveolares. Os ratos imunizados com as nanopartículas de sílica ou com o adjuvante c-di-GMP apresentaram baixos níveis de anticorpos sistémicos e locais, quando comparados com o grupo de ratos administrados com

HAC1/SiO_2/c-di-GMP. Esta vacina induziu ainda uma resposta local de linfócitos T, demonstrada por níveis elevados de IFN-α e IL-2, o que confirmaram o seu potencial como vacina contra o Influenza.

Zhao *et al*. (2016) exploraram a utilização das nanopartículas de sílica na veterinária. As aves domésticas, incluindo galinhas, patos ou perus, podem ser infetadas pelo NDV, desenvolvendo a doença sem que haja uma vacina eficiente para os proteger. Os autores desenvolveram um gene do NDV contendo DNA e encapsularam-no em nanopartículas de sílica contendo prata. Posteriormente, os autores avaliaram a capacidade de indução de uma resposta imune potente. A prata foi incluída devido à sua capacidade antibacteriana contra micro-organismos Gram + e Gram -. Os estudos *in vitro* não revelaram toxicidade das nanopartículas em fibroblastos de embriões das galinhas e o plasmídeo de DNA não perdeu a sua atividade após exposição à DNase I. Após a imunização intranasal das galinhas, registou-se elevados valores de IgA no sangue, uma elevada proliferação de linfócitos e uma elevada expressão de IFN-α e IL-2. Estes resultados comprovam a eficácia e segurança desta vacina na indução de uma forte imunidade na mucosa.

A capacidade das nanopartículas de sílica em produzir respostas alérgicas foram alvo de estudo por Yoshida *et al*. (2011), que testaram diferentes tamanhos e os seus efeitos em ratos. Formulações de nanopartículas com três tamanhos diferentes foram administradas intranasalmente a ratos, 30 nm, 70 nm e outra no intervalo micrométrico, todas elas encapsulando a OVA. As partículas de tamanho inferior induziram a produção de níveis mais elevados de IgE e IgG do que as de maior tamanho. Comparando a imunização resultante da administração de OVA livre, as nanopartículas de 30 nm secretaram níveis mais elevados de citocinas do tipo Th_2, o que indica a capacidade de indução de respostas imunológicas alérgicas *in vivo*.

Nanopartículas de ouro

As nanopartículas de ouro são vastamente utilizadas no encapsulamento de fármacos, no entanto há poucos estudos sobre a sua distribuição nos pulmões, interação com células imunológicas ou influência *in vivo* da respetiva funcionalização.

Seydoux *et al*. (2016) formularam nanopartículas de ouro (AuNPs) com álcool polivinílico (PVA, do inglês *poly(vinyl alcohol)*) e, utilizando o antigénio modelo OVA, funcionalizaram-nas com um grupo positivo NH_3^+ ou com um grupo negativo COO^-. O objetivo foi determinar se a carga da superfície das nanopartículas de ouro possuía alguma influência na resposta imune induzida pelas mesmas. Após a imunização de ratos com ambos os tipos de nanopartículas, verificou-se uma maior captação das nanopartículas carregadas positivamente, produzindo uma resposta celular de linfócitos T CD_8^+ específicos à OVA no pulmão superior à das nanopartículas carregadas negativamente. Uma justificação possível para a superioridade de resposta imune induzida pelas nanopartículas carregadas positivamente pode ser a carga negativa da membrana celular, promovendo a adesão das partículas. Com base neste estudo, os autores concluíram que a carga assume um papel determinante na captação das nanopartículas de ouro pelas APCs nos diferentes locais do trato respiratório.

As nanopartículas de ouro despertaram o interesse de Tao e Gill (2015), que testaram-nas como vacina intranasal contra o vírus Influenza A. Os autores encapsularam uma proteína da membrana do vírus H1N1 (M2e), imobilizando-a em AuNPs. No entanto, o M2e possui pouca imunogenicidade e, por isso, adicionaram um adjuvante (CpG) e um excesso de M2e solúvel à formulação (Figura 9). Os autores imunizaram ratos com diferentes quantidades de M2e solúvel, medindo os níveis de IgG, IgG1 e IgG2 específicos ao M2e por ensaio de imunoabsorção enzimática (ELISA, do inglês *enzyme-linked immunosorbent assay*). Quanto menor a quantidade de M2e solúvel menores eram as quantidades de anticorpos específicos ao antigénio detetados no sangue e, consequentemente, menor era a sobrevivência dos ratos, quando expostos a uma dose letal do vírus. Para além disso, as nanopartículas de ouro demonstraram ter um papel importante para gerar uma resposta imunológica significativa, uma vez que os ratos vacinados com M2e e CpG solúveis não foram capazes de induzir uma reposta de anticorpos específicos ao M2e tão elevada quanto a das nanopartículas.

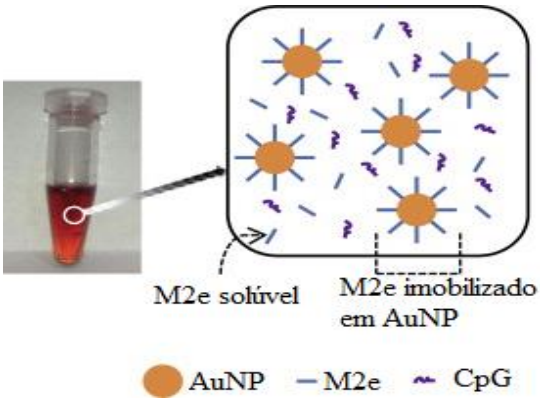

Figura 9 - Constituição da vacina baseada em AuNP (adaptado de Tao e Gill, 2015).

Nanotubos de carbono

Os nanotubos de carbono são estruturas inorgânicas de tamanho nanométrico, originalmente descobertos como um produto secundário obtido na formação de fulerenos (i.e. nanomoléculas de carbono, em que o C_{60} é a forma mais comum, possuindo estrutura de um icosaedro (Santos *et al*., 2010)). Estes sistemas podem ser divididos em nanotubos de carbono de parede simples (SWNT, do inglês *single-walled nanotubes*) ou nanotubos de carbono de parede múltipla (MWNT, do inglês *multi-walled nanotubes*), representados na Figura 10.

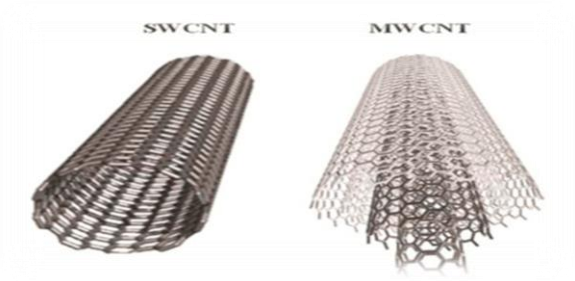

Figura 10 - Tipos de nanotubos de carbono (adaptado de Alanazi et al., 2016).

Os nanotubos de carbono possuem características que facilitam a apresentação das moléculas veiculadas às APCs. Os nanotubos são relativamente inertes, não apresentam

toxicidade e possuem elevada estabilidade *in vivo*. As estruturas destes sistemas permitem a conjugação de vários antigénios e/ou adjuvantes e a sua entrada rápida nas células, principalmente as células dendríticas, induzindo respostas imunológicas (Scheinberg *et al*., 2013). Na imunização nasal, alguns estudos têm indicado a indução de respostas alérgicas nas vias aéreas.

Nygaard *et al*. (2009) testaram a capacidade dos dois tipos de nanotubos de carbono na indução de uma resposta imunológica alérgica nos ratos. O antigénio modelo OVA foi encapsulado em SWNT, MWNT e em partículas de carbono ultrafinas (esféricas). Os autores verificaram que as duas formulações de nanotubos induziram fortemente a presença de IgE específico à OVA no sangue, sendo que o nível de indução demonstrou ser dependente da dose. Já as partículas esféricas apresentaram um limite na resposta induzida, independentemente da dose de OVA encapsulada. Ambas as formulações de nanotubos aumentaram o número de IgG1, de eosinófilos e de citocinas do tipo Th2. No entanto, apenas os MWNT e as partículas ultrafinas aumentaram significativamente os níveis de $IgG2_a$, neutrófilos e da citocina inflamatória TNF-α. Quando comparados com os resultados da administração de OVA livre, os três sistemas demonstraram aumentar o número de linfócitos no fluido de lavagem broncoalveolar, indicando presença de resposta inflamatória. Comparando os dois tipos de nanotubos com as partículas esféricas, os autores reportaram que os nanotubos induziram uma resposta alérgica superior. Esta resposta foi mais intensa e dose-dependente da OVA encapsulada. Com este estudo, os autores apresentaram dados concretos que comprovam a promoção de resposta alérgica pelos nanotubos de carbono.

A disponibilidade dos vários tipos de matérias inorgânicos permite a oportunidade de formular novos sistemas de imunização da mucosa nasal. A facilidade de funcionalização associada à maior resistência *in vivo*, são as grandes vantagens sobre os sistemas orgânicos. No entanto, as incógnitas em relação ao comportamento *in vivo* são o maior debate na evolução para os ensaios clínicos, por não existirem dados suficientes sobre a sua biodegradação, vias de excreção e toxicidade a longo termo. O desenho de mais estudos será necessário, com o objetivo de desenvolver sistemas inorgânicos perfeitamente seguros para a imunização intranasal.

iii. Partículas ‘virus-like’

As partículas “virus-like” (VLP, do inglês *virus-like particle*) são sistemas coloidais recentemente utilizados na vacinação intranasal. As VLPs são nanoestruturas multiproteicas compostas por proteínas estruturais virais com material genético não infecioso. Estas partículas apresentam grande quantidade de proteínas virais de superfície, bem como proteínas funcionais intracelulares, responsáveis pela penetração na célula e direcionamento específico, dependendo da origem viral (Figura 11). Desta forma, a sua aplicação na vacinação é promissora, por apresentar bons perfis de segurança e imunogenicidade. São partículas versáteis contendo epítopos específicos aos linfócitos B e T, induzindo, respetivamente, potentes respostas imunes humorais e celulares (Chroboczek *et al*., 2014).

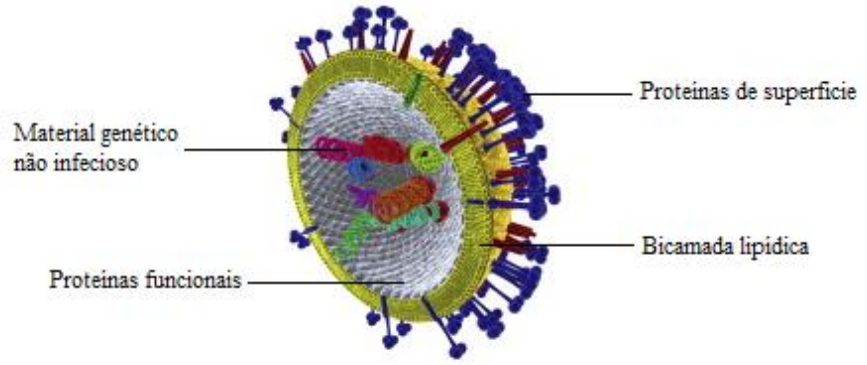

Figura 11 - Estrutura e constituição da VLP (adaptado de Krammer e Grabherr, 2010).

A imprevisibilidade e a capacidade de evolução do vírus Influenza A obriga à produção de novas vacinas anualmente para combaterem as estirpes circulantes. Schwartzman *et al.* (2015) encararam as dificuldades da vacinação anual (i.e. não conseguir proteger contra o desenvolvimento de uma estirpe não prevista, ou contra o aparecimento de um vírus com uma nova hemaglutinina (HA)) como um desafio para desenvolver uma vacina universal que oferece proteção contra vários tipos do Influenza A. A vacina produzida surgiu de uma mistura de VLPs contendo as hemaglutininas H1, H3, H5 ou H7, sendo posteriormente administrada a ratos por via nasal. Os ratos vacinados apresentaram uma proteção significativa após a exposição aos vírus Influenza, expressando os seguintes subtipos de hemaglutininas: 1918 H1, 1957 H2 e H5 aviário, H6, H7, H10 e H11.

Jiao *et al*. (2017) investigaram os efeitos *in vivo* de uma vacina baseada em VLPs para combater o RSV. Os autores produziram as VLPs ao combinar o vetor adenoviral (FGAd) com uma proteína da matriz e uma glicoproteína de fusão, formando as partículas com sucesso. Os autores imunizaram ratos por via intranasal e intramuscular, infetando-os com o RSV. Após a administração intranasal, os níveis de resposta Th1, de anticorpos neutralizantes do vírus RSV na mucosa e de linfócitos T CD_8^+ foram bastante superiores aos detetados na vacinação por via intramuscular. Nos ratos imunizados intranasalmente com as VLPs, foram detetados os anticorpos neutralizantes de RSV até 15 meses, comprovando uma resposta duradoura e eficaz da vacina nos ratos expostos ao vírus. Estes resultados comprovaram o potencial desta vacina na indução de uma resposta eficaz e segura contra o RSV.

As VLPs são consideradas os sistemas coloidais perfeitos para a vacinação, pois possuem o poder da estrutura viral dos vírus, o qual é otimizado para promover uma interação com o sistema imunológico, evitando qualquer componente infecioso. Estes sistemas possuem os bons aspetos dos vírus e evitam os maus. A sua composição e o tamanho nanométrico, de 20 a 100 nm (Wang *et al*., 2015), permitem às VLPs ter a capacidade de indução de uma resposta imunológica potente, mesmo na ausência de adjuvantes.

3. Ensaios clínicos com sistemas coloidais e perspetivas futuras

A utilização de sistemas coloidais em humanos revela-se um desafio complicado para os investigadores. A maior parte dos estudos realizados envolvem animais como modelo (rato, porco e macaco) e, apesar dos resultados promissores, é necessário encontrar o modelo pré-clínico mais favorável para a comercialização dos diferentes sistemas (Cordeiro e Alonso, 2016).

A aplicação clínica de sistemas coloidais para imunização intranasal ainda está numa fase bastante precoce, dependendo da relação entre estes e os processos biológicos do organismo humano, tais como a clearance das partículas, a absorção e o transporte intracelular. Estes processos são cruciais no desenvolvimento de formulações com perfis farmacocinéticos e farmacodinâmicos eficazes e seguros.

Seguindo estes princípios, são poucos os sistemas coloidais para indução de imunidade que estão ou foram avaliados em ensaios clínicos. Entre eles incluem-se vacinas formuladas a partir de lipossomas, virossomas ou nanopartículas.

Stephenson *et al*. (2006) conduziram um estudo clínico de fase I, cego e aleatório, em voluntários humanos, avaliando os efeitos de uma vacina nasal trivalente para o Influenza. A vacina é constituída por hemaglutininas e neuraminidases de três vírus, o Influenza A H5N3, o Influenza A H3N2 e o Influenza B. Como adjuvante, os autores utilizaram uma enterotoxina da *Escherichia coli* (LTK 63). Os antigénios e o adjuvante foram veiculados num sistema coloidal com um núcleo polissacárido carregado positivamente, contendo, à sua volta, uma bicamada lipídica de fosfolípidos e colesterol. Neste ensaio clínico, três grupos de voluntários foram vacinados com três formulações distintas. O primeiro grupo foi imunizado com a vacina possuindo o adjuvante e antigénios veiculados, o segundo com apenas o adjuvante e antigénios e o terceiro com placebo. Os resultados revelaram que o grupo vacinado com o sistema coloidal induziu mais fortemente uma resposta de IgA na mucosa, oferecendo proteção às várias estirpes do Influenza. Os autores sugerem que esta formulação é bastante promissora e necessita de investigação adicional antes da sua introdução no mercado.

Utilizando virossomas, um ensaio clínico de fase I, duplo-cego e aleatório, foi conduzido submetendo mulheres saudáveis a testes de segurança, tolerabilidade e imunogenicidade de virossomas encapsulando péptidos derivados da glicoproteína 41 do vírus da imunodeficiência humana (HIV, do inglês *human immunodeficiency virus*) (MYM-V101). Os autores reportaram que a formulação demonstrou ser segura e tolerada nas duas doses testadas, a 10 μg/dose ou a 50 μg/dose, quando administradas por via intramuscular e intranasal. Anticorpos sistémicos e de mucosa anti-gp41 foram detetados na maioria dos sujeitos com inibição da transcitose do HIV-1, o que pode contribuir para a redução do HIV-1 sexualmente transmissível (Leroux-Roels *et al*., 2013).

Testes clínicos com nanopartículas de prata têm sido conduzidos para avaliar a indução de respostas imunológicas nas células pulmonares. Ensaios de fase I foram conduzidos em adultos saudáveis e não-fumadores com idades entre os 18 e os 60. Os testes têm

como objetivo determinar se a inalação das nanopartículas provoca uma alteração no nível de macrófagos ou células epiteliais pulmonares, avaliar alterações dos níveis de citocinas em lavagens broncoalveolares, a absorção de prata no sangue e excreção pela urina. Este estudo foi verificado pelo National Institute of Environmental Health Sciences em Novembro de 2016 e é esperado terminar em Dezembro de 2017. É esperado que as nanopartículas de prata ajudem a potenciar o sistema imunológico.

Com base em respostas imunológicas potentes obtidas em estudos anteriores, Betancourt *et al*. (2007) realizaram um ensaio clínico duplo-cego e aleatório de fase I com uma vacina nasal possuindo antigénios de superfície do HBV (HBsAg) e antigénios do núcleo (HBcAg). Os participantes (adultos do sexo masculino sem marcadores serológicos de imunidade ou infeção para HBV) foram divididos em dois grupos, um imunizado com a vacina e o outro com placebo (solução salina a 0,9%). Os níveis de anti-HBs e anti-HBc foram medidos nos dias 30 e 90, demonstrando a presença de anti-HBc em todos os participantes do grupo imunizado, ao dia 30. Os níveis de anti-HBs atingiram o máximo no dia 90 em 75% dos participantes imunizados. O grupo placebo foi seronegativo durante o ensaio. Este estudo clínico provou a eficácia da vacina, revelando uma boa tolerância *in vivo*. Foram detetados alguns efeitos adversos de baixa intensidade e autolimitados, como espirros, rinorreia, congestão nasal, cefaleia e mal-estar.

O número reduzido de ensaios clínicos realizados ou em curso resulta da dificuldade em controlar a reprodutibilidade da vacina e respetivo sistema transportador, bem como, da falta de estabilidade durante os processos de produção e armazenamento. Para além destes inconvenientes, Sharma *et al*. (2009) apontaram outros obstáculos ao sucesso de imunização intranasal em humanos, tais como a falta de um animal modelo com vias aéreas similares às humanas, para uma melhor avaliação da potência, eficácia e segurança dos sistemas de transporte, a dificuldade em prever a dose de administração intranasal ou a possibilidade de indução de alergias ou doenças respiratórias devido à ligação da via intranasal ao cérebro. É de extrema importância desenvolver mais estudos de segurança pré-clínica e clínica dos sistemas coloidais para uma aplicação clínica mais extensa e segura destas formulações.

III. Conclusão

A vacinação não só traz benefícios pessoais como também benefícios para toda a comunidade, sendo a estratégia mais eficaz e segura de proteção contra as doenças. É, por isso, um dos grandes desafios a contínua melhoria da eficácia das vacinas para se obter uma melhor proteção contra doenças novas ou já existentes.

A imunização por via intranasal apresenta todos os benefícios de uma imunização nas mucosas, como a imunização em mucosas distantes devido ao sistema imunológico comum. Por não necessitar de agulha nem de elevadas quantidades de antigénio e /ou adjuvante, apresenta igualmente vantagens em termos de aceitação do público e custos de produção.

Baseados no conceito que a imunidade na mucosa é induzida no tecido linfoide, vários investigadores deduziram que sistemas de transporte, como os coloidais, que facilitassem a entrega e a apresentação dos antigénios e/ou adjuvantes às células M e NALT trariam grandes benefícios na indução de respostas imunológicas potentes.

O sistema coloidal ideal deve apresentar tamanho adequado à administração pulmonar, possuir componentes que potenciem a ação do antigénio transportado, possuir grupos hidrófilos com capacidade mucoadesiva à sua superfície, não apresentar qualquer toxicidade *in vivo*, ter capacidade de proteção e libertação do antigénio e /ou adjuvante às APCs e permitir uma fácil produção e escala.

Apesar de vários estudos apresentarem resultados prometedores, são necessárias mais investigações para se formular vacinas intranasais totalmente eficazes e seguras. Apenas um pequeno número de vacinas intranasais encontra-se em ensaios clínicos, o que corresponde à grande dificuldade da sua introdução no mercado. Para alcançar sucesso na vacinação intranasal e aumentar a quantidade de vacinas comercializadas, é essencial ter em conta aspetos regulamentares, industriais e de marketing, de modo a combater as dificuldades de manufaturação de novas formulações, potenciando a aceitação e a satisfação do público.

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V. Anexos

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REVIEW ARTICLE

Overview on Inhalable Nanocarriers for Respiratory Immunization

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Abstract: Despite the value of vaccination, the control of re-emerging infectious and non-infectious diseases remains a challenge for researchers. In this topic, mucosal immunization, in particular at airway mucosa, is receiving increased investigational focus. Innovative vaccine platforms to deliver immunogens with or without adjuvants in a safe and stable manner have been explored to improve vaccine efficacy and induce long-term and protective immunity. This review provides an overview of the features of respiratory immunization and the fate of inhalable nanocarriers in the respiratory tract. The review also highlights the most representative delivery approaches based on inhalable nanocarriers, including polymeric, lipid and inorganic-based nanosystems, which can enhance vaccine uptake by antigen-presenting cells. The review takes into consideration the most relevant and recent *in vivo* studies to provide readers a realistic insight into the potential of these technologies in the advantages and potential hurdles to clinical and commercial success of these platforms for vaccination.

Keywords: Inhalable nanocarriers, respiratory immunization, airway mucosa, polymeric nanocarriers, lipid nanocarriers, inorganic nanocarriers.

1. INTRODUCTION: RESPIRATORY IMMUNIZATION

Immunotherapy is based on drugs and/or biological agents to initiate, modulate and control an immune response. Currently, there is a wide range of immunotherapeutic strategies that are under investigation for both prophylactic and therapeutic purposes. Prophylactic immunotherapy (*i.e*. termed as vaccination process) refers to the use of specific antigens along with immunomodulators or immunostimulators (adjuvants) to produce a protective immunity against future infections. Therapeutic immunotherapy is applied after the onset of a disease, for example in certain tumor conditions [1].

Immunization through the vaccination is regarded by the World Health Organization (WHO) as the most effective approach to eradicate or reduce the occurrence of infectious diseases and death in vulnerable populations [2]. According to the WHO, around 2.5 million children's lives are saved each year due to the availability of vaccines against a variety of pathogens [3]. Nowadays, the field of immunology has concentrated significant efforts to develop effective therapeutic vaccines that are administrated to patient with an established disease [4]. Therapeutic vaccines have also achieved clinical proof-of-concept for prostate cancer management [5].

Most vaccines are defined as a pharmaceutical preparation of a microbial antigen, that when administered elicits an immune response and creates an immunologic memory as a consequence of the long production of antibodies against the specific antigen [4]. Current vaccines are attenuated vaccines once microbial antigens are synthesized or highly purified recombinant antigens, *i.e*. composed of protein, peptide or polysaccharide antigens or of nucleic acids that express microbial antigens [6]. Despite improved safety and less virulence, their high purity makes them less immunogenic than the traditional vaccines which are composed of live-attenuated or inactivated/killed microorganisms. In this perspective, vaccines can incorporate efficient adjuvant or adjuvant combination (*e.g*. aluminum, oil-in-water emulsions, squalene) in their formulation, potentiating and modulating the immunogenicity of highly purified antigens by providing pro-inflammatory signals and prolonging the persistence of vaccine antigens [7-12]. At present, hydroxide and phosphate salts of aluminum and calcium, and among them alum, represent the only approved adjuvants for human vaccines although it is yet to be approved for inhalation administration [13, 14]. Alum is a moderate adjuvant for antibody production, not suitable for all antigens, and is not efficient for promoting cell-mediated immunity [15]. Therefore, concerning the inhalation immunization, two main challenges remain, namely the development of superior vaccine mucosal adjuvants (*i.e*. immunostimulators compounds, such as toll-like receptor ligands, bacterial toxins, saponins and cytokines) that provide immunity against infectious agents or the manufacture of effective and safe delivery carrier that enhances both antigen delivery and presentation by antigen-presenting cells [16, 17].

1.1. Airway Mucosa Immunization

The intramuscular and subcutaneous injections represent the most common routes of vaccines administration due to some limitations present by other less-invasive routes (*e.g*. oral and transdermal). For example, most of the antigens are macromolecules, usually of molecular weight greater than 10,000; so if antigens are not administered parenterally, they are not able to penetrate into the systemic circulation [2]. The drawbacks associated with parenteral administration (*e.g*. invasive and sterile devices, needle-based delivery systems conditions and injuries, cold transport and storage of most liquid vaccine formulations, need of medical trained personnel), make the search for alternative routes quite appealing. In this respect, mucosal routes are receiving important focus as a potential useful tool in immunological concept once mucosal surfaces are the main entry sites for the environmental antigens. Among mucosa, airway mucosa represents an attractive non-invasive approach (*i.e*. needleless and painless route) for vaccine delivery which is amenable to repeated administration, adequate for mass immunization due

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to the high accessibility, and is associated with high patient compliance [18]. Other advantages of immunization through the airway mucosa include the ability to use small antigenic dose to improve the mucosal immune response [19]; the ability to also induce a systemic immunity, and enhance the systemic levels of specific immunoglobulin G and the nasal secrete immunoglobulin A by mucosal B cells [9]; and the capacity to promote a distant mucosal protection due to the interconnection of mucosal tissues governed by the common mucosal immune system [20, 21]. For example, nasal immunization can stimulate immune responses in both the respiratory tract and the vaginal mucosa [22]. These mucosal membranes surfaces contain specialized mucosa-associated lymphoid tissues (MALT). The nasal-associated lymphoid tissue (NALT) has a well-organized structure, containing B- and T-cells as well as an epithelial layer of microfold M-cells [23]. Intra-epithelial lymphocytes are also found in the NALT, along with antigen-presenting cells (APCs), such as macrophages and dendritic cells that play an important role in the immune response [24]. The intranasal vaccine administration could increase the efficacy of vaccine once the residence time of the antigen in the alveoli is prolonged [14, 25]. Additionally, mucosal epithelial cells intimately collaborate with lymphoid tissue to produce cytokines and chemokines [20, 21]. The route of administration of vaccines plays a crucial role on immune responses. For example, animal model studies demonstrated that intranasal immunotherapy, which targets the mucosa directly, was more effective compared to the intradermal immunotherapy for the induction of airways allergen tolerance [26, 27]. A benefit of the intranasal over the oral route of administration is the lower antigen dose requirement as there is no considerable dilution by nasal fluids and no exposure to low pH or to abundant secreted degradation enzymes in the gastrointestinal tract [28]. Compared with subcutaneous or intramuscular injections, using animal models, influenza vaccines induced significantly higher mucosal antibody titers when delivered directly to the respiratory tract [19, 29-31].

Despite the multiple benefits and intense research associated with airways mucosal routes for immunization, only a small number of intranasal vaccine products are currently available, such as NasalFluMist® and Fluenz Tetra Nasal Spray Suspension and NASOVAC™ (a nasal H1N1 vaccine). All these products were against virus influenza. This fact is probably due to the difficulties of the molecules to come across the mucosal barriers put forth by the tissues, their rapid clearance and lack of human compatible mucosal adjuvant [18]. Other limitations are related to the encapsulation of antigens such as regulatory and industrial issues (*e.g*. manufacturing process, stabilization of antigen and delivery systems), higher cost of manufacturing, limited release of encapsulated antigen, risk of transporting encapsulated antigens into the brain *via* the olfactory route [32-34]. Therefore, delivery platforms that can facilitate the delivery of molecules more efficiently to the immune system in the tissues and obtain greater immunostimulating effects are required.

Intranasal vaccine formulations should have some requirements such as [18]: maintain the antigen in a stable form; remain in the nasopharyngeal region for enough time that allows the interaction between antigen and the lymphatic system; stimulate the immune system - with or without additional adjuvants; and avoid toxic effects. The association or incorporation of specific antigens to nanocarriers (*e.g*. micelles, nanoparticles, liposomes) has been explored as a promising strategy for providing local immunization by the respiratory route and represent an advance in vaccine technology either for therapeutic purposes (*e.g*. antitumor vaccination) or for vaccination against microorganisms (*i.e*. prophylactic immunotherapy). The use of nanocarriers in vaccine formulations improves antigen stability, augments the generated immune response upon uptake by immunocompetent cells, target and modulates the delivery and release of the antigen [35]. Due to the identical size range of natural pathogens (*e.g*. bacteria and viruses), the use of nanocarriers allows the simulation of an identical infection process perhaps they are better recognized by the immune systems compared to soluble antigens and, consequently, eliciting an immune response [17, 36].

Despite the strategies that have been considered to achieve an effective and safe inhalable system for delivery of the antigens to the mucosal immune systems, this trend remains a substantial challenge for pharmaceutical scientists. The present review deals with the most important issues that should be addressed during the development of an effective inhalable nanocarrier for vaccine administration and compile the recent improvements in this subject.

2. NASAL INHALATION ADMINISTRATION

In recent years, the respiratory tract has attracted great interest among the scientific community as a delivery target due to its unique characteristics, namely [37-41]: a large absorptive specific surface area, allowing a rapid onset of action; an extremely thin absorptive mucosal membrane, enhancing the permeability of molecules, macromolecules and colloidal particles; an excellent vascularization to obtain a systemic therapeutic effect; a low enzymatic activity for degradation; avoidance of the first-pass hepatic metabolism; presence of lymph nodes and an important number of intervening antigen presenting cells (*i.e*. dendritic cells and macrophages); achievement of either a local or a systemic effect at therapeutic concentrations due to the absorptive capacity of the alveolar epithelium and improvement of the drug bioavailability. All these advantages make the inhalation route particularly attractive for immunization purposes. Despite its advantages, the inhalation administration has also shortcomings, namely: oropharyngeal deposition of the antigen, the difficulty in the correct use of devices, and physiological, pharmacological and physical (*e.g*. mucous) barriers [40].

For the conception of a successful inhalable nanocarrier, a brief knowledge about the anatomy and physiology of the human respiratory system as well as the transport and deposition of inhaled material is required. In structural terms, the respiratory system is divided into upper respiratory tract (nasal cavity, pharynx and associated structures) and lower respiratory tract (larynx, trachea, bronchial tree and lungs, alveoli) [42]. In functional terms, the respiratory system comprises two vital regions: the conducting airways that create movement of air between the outside and the areas where gas exchange occurs; and the respiratory region, which allows gas exchange with blood capillaries and consists of respiratory bronchioles, alveolar ducts and alveolar sacs. In the conductive zone, gas exchange does not occur because there are no alveoli and the walls are too thick for diffusion. In a healthy human, this region has a total volume of 150 mL and is referred as an anatomical dead space [43]. In order to ensure continuous air passage, the wall of the conductive zone is constituted by a combination of cartilage, connective tissue and smooth muscle tissue that gives structural support, flexibility and extensibility [42]. The structure of the conducting airway allows the air to enter into direct contact with the bloodstream due to extensive capillary networks of alveoli.

The respiratory region, which is considered the most relevant region for nasal delivery, has a length of only a few millimeters but represents the greatest part of the lung with a total volume of 2.5-3 L [43, 44]. The alveoli, functional units of the respiratory system, are very thin walled structures that facilitate the exchange of carbon dioxide from the blood by oxygen inspired in the air. It is estimated that there are approximately 350 million alveoli per lung with a surface diffusion between 60 - 80 m^2 (*i.e*. a large surface area) [45]. The alveoli are completely surrounded by a vast capillary network that provides an excellent environment for gas exchange and drug permeation [43].

The airways and alveoli regions have different pseudostratified epithelium and clearance mechanisms that interfere in the quantitative absorption of inhaled particles [46, 47]. Two main characteristics, specifically the smaller surface area and lower blood flow,

limit the transepithelial transport of drugs in the upper airways region [47]. Moreover, the nasal epithelium corresponds to a thin layer of pseudostratified epithelial cells connected by tight junctions with a very small diameter that limits the vaccine delivery nanosystems through the paracellular route [34]. Transcellular transport represents the most expected route for vaccine nanocarriers reach the nasopharynx associated lymphoid tissue (*i.e*. an inductive site for mucosal immunity) [44].

Additionally, the primary defense mechanism of conductive zone is the mucociliary clearance that includes ciliated cells and goblet and submucosal gland cells (*i.e*. mucus-producing cells) that entrap the foreign/inhalable particles in mucus layer before moving to the lower respiratory regions and then propel along with mucus to reach out of trachea either by coughing or swallowing [48, 49]. The apical surface of nasal epithelium presents a thick layer of mucus which is associated with the presence of closely packed microvillus and infrequent endocytic microdomains sequestered at the bases of the microvillus limit the adherence and uptake of vaccine nanocarriers [50]. Mucus is secreted by the mucous glands in the bronchial walls and the goblet cells of the epithelium [51]. A healthy human produces about 10 - 20 mL of mucus per day; however, some factors could affect its production such as disease and age [52]. The mucociliary clearance rate tends to decrease with age, diabetes and hypertension. A patient with chronic bronchitis or cystic fibrosis can produce up to 10 times higher mucus [53]. Olsson *et al*. [54] reported that β-adrenergic agonists, such as formoterol, have shown to be potent stimulant, increasing mucociliary clearance in patients with chronic bronchitis.

After deposition on the conductive zone of the respiratory system, most foreign insoluble particles with a diameter greater than 6 μm are removed by mucociliary clearance. In contrast, smaller particles tend to penetrate the mucus layer and reach the epithelium [48, 55]. Alveolar space has a large surface area containing a surfactant-lining fluid layer and is intimately in contact to the systemic circulation via the pulmonary circulation that makes this zone less well protected against inhaled materials such as nanocarriers. Additionally, material in nanoparticulate form deposited within the lungs has greater probability to escape from the clearance mechanisms (*i.e.* mucociliary clearance) and can be absorbed compared to material with a microsize dimension [56].

2.1. Fate of Nanocarriers in the Respiratory Tract

The inhalation administration is performed using aerosols, which is an effective technique to deliver therapeutic agents in the respiratory tract with a uniform distribution and high penetration. The aerosol administration can be achieved by different methods: traditional nebulizers, metered dose inhalers (MDIs) or dry powders inhalers (DPIs). The last ones contribute to patient compliance and ease of administration [57].

The site, extent and efficacy of particle deposition after inhalation depend on various formulation properties such as particle size, size distribution, surface morphology, shape, hygroscopicity, surface electrostatic charge and density [38, 58]. These authors also point out other factors that influence the mechanism of particle deposition including lung morphology, diseased state and breathing pattern (*e.g*. rate of inspiration, coordination between the production of the aerosol device and the patient's inspiration, tidal volume (*i.e.* volume of air inhaled in one inhalation).

The successful uses of nanocarriers for airway drug delivery is based on their mass mean aerodynamic diameter that determines the efficacy of nanocarriers deposition in the respiratory region. The aerodynamic diameter depends essentially on the density and size of the particle. The literature suggests that particles with aerodynamic diameters between 1 and 5 μm are expected to suffer sedimentation by gravitational force in the lung periphery [46]. Particles with aerodynamic diameter larger than 5 μm are deposited in the upper airways (*i.e.* oropharyngeal region) by inertial impaction and do not reach target sites [38, 59]. Particles with aerodynamic diameters substantially smaller, although able to reach the alveolar region, they are not able to deposit and are exhaled, that could be corresponded up to 80% of inhaled particles after inspiration because of their low inertia.

The understanding of the fate of nanocarriers in the respiratory tract is essential for their efficacy. Considering their diameter range, the nanocarriers are deposited predominantly by a diffusion mechanism based on the Brownian motion [60]. In addition, they are not suitable for deep lung delivery and can be easily exhaled or mucociliary cleared out before reaching the pulmonary epithelia [61, 62]. However, data obtained from controlled clinical studies [63, 64] demonstrated that ultrafine particles (*i.e.* with a diameter less than 100 nm) can settle effectively to the alveolar region. Other authors reported that nanoparticles in the size range of 200 nm effectively penetrated across mucus layer [65]. The probability of deposition increases with decreasing the nanocarriers' diameter below 500 nm due to the increase of diffusion mobility. Diffusion occurs in areas of higher concentration to lower concentration sites and is more common in the small airways where the air flow is low or absent (*e.g*. alveoli) [58]. Additionally, after their deposition, smaller nanocarriers can be more incorporated into the lung surfactant-lining film and increase the rate of absorption by promoting a more uniform distribution [46]. The biokinetic dissolution of inhaled nanocarriers establishes their fate, *i.e.* absorption through the epithelial membrane or non-absorptive clearance mechanisms (*e.g*. mucociliary escalator transport, macrophage phagocytosis, endocytosis by epithelial and endothelial cell depending on the respiratory tract region in which the nanocarriers have been deposited, *i.e.* from the upper airways or the alveoli, respectively) [66]. The phagocytosis depends on the size, shape and chemical characteristics of the inhaled particles. Moghimi and Hunter (2001) reported that particles lower than 70 nm are not recognized as "foreign" from the alveolar macrophages on the surface of the respiratory region; thus making them capable of absorption [67]. The particles with diameters between 1.5 - 3.0 μm are the most probable to undergo phagocytosis by phagocytic cells [48]. Therefore, nanoparticles can avoid the alveolar macrophages' clearance experienced by microparticles [68].

The exact mechanism involved in the particle uptake, transport and clearance in the alveolar region has not yet been fully clarified [48]. The absorption could occur by passive diffusion which is faster in the alveolar region than in the small airways due to its physiological and histological structure or by active transport mediated by regional receptors or transporters. Another possible route is via the transitory pores in the epithelium [46].

Besides diameter, the surface coat and the surface electrostatic charge of a nanocarrier can affect both the deposition and the cell uptake of inhaled nanocarriers. Kato *et al.* suggested that albumin or lecithin coating inhaled nanoparticles appeared to facilitate nanoparticle endocytosis [69]. Concerning the intravenous administration of nanocarriers, a common approach to avoid their macrophage uptake and to extend the circulation time is by coating the surface of nanocarriers with hydrophilic polymers such as poly(ethylene glycol) (PEG) [70-73]. In the literature, it is hypothesized that a PEG coat generates a hydrophilic and neutral shell that reduces hydrophobic adhesive interactions with mucus [74]. Several researchers have also applied the PEGylation strategy in the context of pulmonary drug delivery [57, 72, 75, 76] for immunization purposes. For example, Meenach *et al.* developed PEGylated liposomes that have proved to be effective in mucus penetration and escaping pulmonary and immune clearance [77]. Shen *et al.* reported that the surface modification of nanocarriers with PEG molecules increases their residence time in respiratory region and provides homogeneous distribution, delaying macrophage clearance [57]. In addition, nanoparticles densely coated with PEG demonstrated an enhancement of mucus penetration in the lung [78].

Charged inhaled nanoparticles present a higher deposition than neutral nanoparticles [46]. Based on the results that positive surface charge of stearylamine-PEG-polylactide nanoparticles elicited increased pulmonary side effect and the better tolerability of anionic PEG- polylactide nanoparticles, Harush-Frenkel *et al.* suggested that the latter can be useful as therapeutic drug delivery system in pulmonary administration [79].

3. INHALABLE NANOCARRIERS

In the nasal passages and airways, the mucus layer overlying the epithelium acts as a penetration barrier, the associated mucociliary clearance mechanisms actively clear away deposited molecules and the enzymatic system could contribute to the drug degradation. Ideally, the delivery system must facilitate the antigen transfer across epithelium membrane, protect molecules from physical elimination and chemical enzymatic degradation and promote uptake by the cells (*e.g*. antigen-presenting cells and specialized epithelial cells) that contribute to induce the immune response.

The development of an antigen-delivery carrier system that presents an adequate aerodynamic diameter for alveolar deposition and the molecular patterns of viral antigens seem to be a promising approach to explore the inhalation route for enhancing the host defense and regulating immune responses. Based on these considerations, the use of nanocarriers as delivery systems has been investigated since the late 1980s [27], which demonstrated that antigen specific immune responses can be elicited at levels far more potent than the existing vaccines [22, 80-82]. Todoroff and Vanbever also reported that insoluble and non-biodegradable nanoparticles easily escape from the phagocytosis and can remain in the lung tissue for a long period of time (*i.e*. several weeks) with no significant translocation across the respiratory epithelia [74]. Semmler–Behnke *et al.* demonstrated this capacity after the inhalation of 192-iridium radiolabeled nanoparticles in healthy rats [83].

In the context of immunization, nanosystems can act as delivery carriers that increase antigen processing (*e.g*. protein stabilization or controlled antigen release) or as an immunostimulant adjuvant that activates or improves immune response [35]. Some properties of nanosystems make them attractive as mucosal vaccine delivery vehicles, namely their superior uptake by antigen-presenting cells, their preferential draining to the lymphatic system rather than to the bloodstream, and, depending on size and composition, their ability to diffuse through mucus and penetrate the mucosal barriers [4]. As explored before, the presence of both bronchoalveolar lymphoid tissue (as lymph system) and antigen-presenting cells (as immune competent cells) in the respiratory tract makes possible the induction of the immune response [39]. The M-cells, located on NALT, have been referred as the principal transporting cells used by antigens to reach the lymphatic system [84] due to the presence of variable microvilli or microfolds interspersed with large plasma membrane subdomains exposed to the luminal environment in their apical surface. In addition, M cells present antigen retaining crypts that provide longer contact time of antigen with M-cells [85]. The functionality of biologically derived materials can be explored using nanosystems in order to develop superior platforms with better shelf life and thermal stability [86].

Several strategies have been researched to develop an effective respirable vaccine/immunotherapy treatment using nanocarriers such as dry inhalable nanoparticulate powders. Inhalable nasal dry powder vaccine formulations provide a real opportunity to improve antigen stability compared with the traditional liquid formulations currently available in the market that require low temperature storage or the addition of preservatives [38, 87]. Additionally, the enhancement of immunity induced after vaccination and the feasibility of dry powder aerosol vaccines have been investigated in several studies.

The composition of nanocarriers may influence the effect on the size of carrier as well as the interaction of the carrier with the biological environment [17]. In the next sections, we will explore some of the most relevant studies on the topic of inhaled polymer, lipid and inorganic-based nanocarriers used in respiratory immunization (Fig. **1**). The delivery system whose design has been inspired by the structure of bacteria and viruses is out of scope of this review article.

3.1. Polymer-Based Nanocarriers

Polymer-based nanocarriers are characterized by their polymeric nature and present colloidal dimensions, *i.e*. size range from 1 nm to 1000 nm, although sizes less than 250 nm are desirable for vaccine delivery purposes in the respiratory tract [88]. Both natural and synthetic polymers have been used to prepare different types of nanocarriers (*e.g*. polymeric nanoparticles and polymeric micelles) for inhalation vaccines. The polymers are selected based on their functional properties to prepare good physicochemical stability nanosystems and on their biological and toxicological behavior, namely biocompatibility, biodegradability, low toxicity and low immunogenic response [74]. Additionally, some biodegradable polymers can suffer degradation and produce molecules with adjuvant activity [89]. For example, chitosan prolongs the residence time of the nanoparticles in the lungs due to the mucoadhesive properties and PEG creates a neutral and hydrophilic layer which reduces lung mucus interactions [90, 91]. Despite the large amount of available polymers with potential for biomedical application, not many have regulatory approval by the U.S. Food and Drug Administration (FDA) and other worldwide authorities [92, 93]. The potential of polymer nanotoxicity is critical and should be attained to develop safe inhalable dry powder inhalation formulations [38]. Table **1** summarizes the information presented in this article related to the *in vivo* studies, using polymeric-based nanocarriers for respiratory immunization. The interested reader can find more detailed information about these studies in the next sections.

3.1.1. Polymeric Nanoparticles

Polymeric nanoparticles consist of solid colloidal carrier systems that can encapsulate, absorb or chemically link molecules to their surface. The polymeric nanoparticles include nanospheres (*i.e*. a matrix-type carrier) and nanocapsules (*i.e*. a reservoir-type carrier containing a core with drug surrounded by a polymeric coating). Polymeric nanoparticles emerged in the 1970s to overcome some of the shortcomings of the liposomes. These carriers are more stable than other colloidal carriers (*e.g*. liposomes) and can better control the release of the drug. Additionally, reproductive physicochemical properties and surface modification can be easily obtained [94, 95]. Most studies in the context of inhalable nanocarriers for vaccine respiratory immunization are based on polymeric nanoparticles using different polymers.

3.1.1.1. Immunostimulant-Loaded Polymeric Nanoparticles

Ballester *et al.* demonstrated that vaccination via pulmonary administration of pluronic-stabilized poly(propylene sulfide) nanoparticles-conjugated with the immunostimulatory unmethylated cytosine-phosphate guanine (CpG) oligodeoxynucleotide was significantly more effective than the administration of free CpG in reducing allergy symptoms [96]. The authors used both prophylactic and therapeutic animal models using one of the main sources of allergens, *i.e.* the common aeroallergen house dust mite-allergic mice. CpG is used as a potent adjuvant for shifting immune responses to the Th1 type [97]. When administered as allergen-free immunomodulatory prophylaxy in mice model (*i.e.* before allergen sensitization), the polymeric nanoparticles-conjugated CpG produced a significant reduction in various immunologic parameters such as eosinophilia, IgE levels, mucus production and Th2-related cytokine production compared to the moderate effects observed in the case of free CpG administration. However, when CpG was administered after sensitizing the mice (*i.e.* therapeutic setting), both free CpG and nanoparticles-CpG led to similar

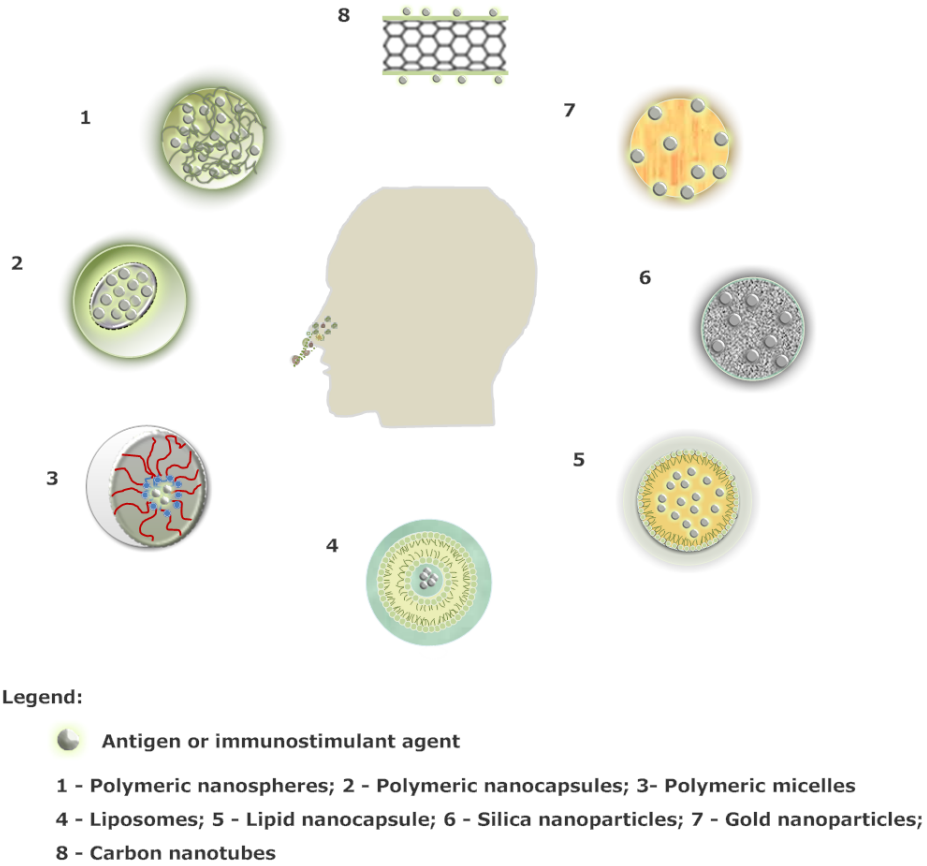

Fig. (1).

Table 1. Examples of *in vivo* studies using inhaled polymeric-based nanocarriers for respiratory immunization.

Antigen or/and adjuvant	Composition of nano-carriers	Animal model	Relevant effects	References
CpG oligodeoxynucleo-tide	Pluronic-stabilized poly(propylene sulfide) nanoparticles	Aeroallergen house dust mite-allergic mice	More potent than free CpG in reducing allergy symptoms in both prophylactic and therapeutic animal models; Before allergen sensitization, reduced significantly eosino-philia, IgE levels, mucus production and Th2 cytokines com-pared to the moderate effects caused by free CpG administra-tion; After allergen sensitization, similar results in reducing eosino-philia and IgE levels for both treatment (*i.e.* nanoparticles-conjugated CpG and free CpG) and higher reduction of Th2 cytokines compared to free CpG.	(Ballester *et al*., 2015)
Ovalbumin	Maltose lipidated nanoparticles	Swiss OF1 mice	Lipids are not released from the nanoparticles in the cells during their endocytosis; Nanoparticles did not open the tight junctions nor cross the airway epithelial barrier *in vitro* ((16HBE) human bronchial epithelial cell line) or *in vivo;* Nanoparticles remained in mucosa cells of the nose (at least for a 3 hours) probably due to an enhanced cellular uptake compared to free ovalbumin and potentially protected oval-bumin from degradation (*in vivo*).	(Bernocchi *et al*., 2016)

(Table 1) Contd....

Antigen or/and adjuvant	Composition of nanocarriers	Animal model	Relevant effects	References
Total extract *Toxoplasma gondii*	Porous maltodextrin-based with lipid core nanoparticles	Female CBA/J mice	Nanoparticles efficiently delivered the antigen into airway epithelial cells, macrophages and dendritic cells (*in vitro* studies); Increased immunogenicity by induction a strong and specific Th1/Th17 cellular immune response; Protected mice against a lethal dose of wild parasite (in acute toxoplasmosis); Significant decreased of brain cysts in chronic toxoplasmosis.	(Dimier-Poisson *et al*., 2015)
Ovalbumin	N-trimethyl chitosan (TMC) - nanoparticles poly(lactic-co-glycolic acid) nanoparticles (PLGA) – nanoparticles TMC-coated PLGA nanoparticles	Balb/c mice	Non-toxic effect in isolated nasal epithelium TMC nanoparticles increased the residence time in nasal cavity and stimulated the dendritic cell maturation compared to ovalbumin solution; Quick antigen-releasing from TMC nanoparticles presented high sIgA levels and serum antibody titres; Both PLGA nanoparticles and TMC-coated PLGA nanoparticles presented slow antigen release and did not induce detectable antibody titers.	(Slutter *et al*., 2010)
Pneumococcal surface protein A – Psp A	Chitosan-DNA nanoparticles expressing (Psp A)	Female BALB/c mice	Elevated significantly anti-Psp A IgG antibody in serum and anti-IgA antibody in mucosal lavages after intranasal immunization; Generated mucosal and systemic immune responses and prevented pneumococcal nasopharyngeal colonization.	(Xu *et al*., 2011)
Influenza antigens	Poly-gamma-glutamic acid-chitosan nanoparticles	Mice	Increased antibody titers compared to alum-adsorbed antigen vaccine formulation; 100% animal survival after viral challenge.	(Sung *et al*., 2012)
Streptococcus equi enzymatic extract	Chitosan nanoparticles	Female BALB/c mice	Elicited mucosal, humoral and cellular immune responses; Increased sIgA levels in the lungs; Induced a Th1-mediated immune response characterized by IFN-γ production and high IgG2a antibody titers; Induced a Th2 immune response characterized mainly by IL-4 production and IgG1 antibodies.	(Figueiredo *et al*., 2012)
Plasmid DNA nucleocapsid (N) protein of severe acute respiratory syndrome coronavirus (SARS-CoV)	Surface functionalized biotinylated chitosan nanoparticles with bifunctional fusion protein (bfFp) vector	Female BALB/c mice	Following incubation with DNAse I, nanoencapsulated of plasmid DNA resisted to nuclease digestion; Enhanced magnitude of mucosal IgA as well as systemic IgG against N protein; Higher level of IFN-γ (*i.e*. a Th1 cytokine) with the targeted formulation compared to the non-targeted formulation; Higher N protein specific serum IgG antibodies and an increased of IFN-γ levels, when the targeted formulation was co-administered with dendritic cell maturation/ activation stimuli (anti-mouse-CD40 mAb).	(Raghuwanshi *et al.*, 2012)
Recombinant hepatitis B surface antigen (rHBsAg)	Poly(lactic-co-glycolic acid) (PLGA)/polyethylene glycol (PEG) nanoparticles	Male guinea pigs	Dry powder to the lungs elicited a higher Ig A titers and smaller IgG titers immune response compared to alum adsorbed hepatitis B surface antigen administered by the intramuscular route.	(Muttil *et al*., 2010a)
Diphtheria CRM-197 antigen (CrmAg)	Poly(lactic-co-glycolic acid (PLGA) nanoparticles	Guinea pigs	Dry powder to the lungs elicited a higher Ig A titers and smaller IgG titers immune response compared to alum adsorbed hepatitis B surface antigen administered by the intramuscular route.	(Muttil *et al*., 2010c)

(Table 1) Contd....

Antigen or/and adjuvant	Composition of nano-carriers	Animal model	Relevant effects	References
F1-V antigen	Polyanhydride nanopar-ticles	Female C57BL/6 mice	Administered as a single intranasal dose successfully induced long-term protection against the pathogenic agent *Y. pestis*; High titer and high avidity IgG1 anti-F1-V antibody response.	(Ulery *et al*., 2011)
Ovalbumin	Poly(γ-glutamic acid) (γ-PGA) nanoparticles	C57BL/6 mice and BALB/c nude mice	Significantly suppressed tumor cells and lung metastasis with three intranasal doses; Total serum anti-ovalbumin IgG titer was similar in both immunized groups (*i.e*. ovalbumin entrapped in γ-PGA nanoparticles and ovalbumin solution); Induced cytotoxic T lymphocytes (CTLs) and interferon-γ-secreting cells specific for antigen protein in the spleen and lymph nodes; γ-PGA nanoparticles were rapid taken up by nasopharyngeal-associated lymphoid tissue and delivered to the lymph nodes.	(Matsuo *et al*., 2011)
Ovalbumin and PR8 antigen (*i.e*. an influ-enza A viral antigen)	Poly(γ-glutamic acid) γ-PGA conjugated with hydrophobic cholesterol groups and modified with amines nanomi-celles	Mice	Increased the residence time of co-delivery antigen in the mucus layer and controlled antigen release for the nasal epithelial cells High levels of ovalbumin-specific IgA antibody titers in the nasal tissue and IFN-γ-producing cells compared with the immunization with ovalbumin only; High levels of PR8-specific IgG titers in the serum and PR8-specific mucosal IgA antibody titers in nasal washes com-pared to the immunization with PR8 antigen alone	(Noh *et al*., 2013)
Ovalbumin (antigen) and muramyldipeptide or lipopolysaccharide or CpG (adjuvant)	N-trimethyl chitosan nanoparticles	Female BALB/c mice	Co-encapsulation of an additional immunopotentiator with the ovalbumin into N-trimethyl chitosan nanoparticles improved the immunogenicity; The strength and quality of the response depended on the immunopotentiator as well as the route of administration; Muramyldipeptide and lipopolysaccharide were effective nasally as immunopotentiators, while CpG and lipopolysac-charide were the most effective for intradermal vaccine.	(Bal *et al.*, 2012)
Ovalbumin (antigen) and unmethylated CpG DNA (adjuvant)	N-trimethyl chitosan crosslinked nanoparti-cles	Female Balb/c mice	Modulated the immune response towards a Th1 response after nasal vaccination, while maintaining the strong systemic and local antibody responses observed with N-trimethyl chitosan crosslinked with tripolyphosphate nanoparticles.	(Slutter and Jiskoot, 2010)
Influence whole virus (antigen) and CpG oli-godeoxynucleotide or *Quillaja* saponin (adju-vant)	Dry powder form of chitosan nanospheres	New Zealand albino female rabbits	Stimulated both humoral and cellular immune response; CpG was more potent in induction humoral immune response (both local and systemic), as well as a Th1 type response than *Quillaja* saponin. Rabbit serum IgG titers significantly augmented in animal vaccinated groups with highest response in animal administer-ing nanospheres loaded both antigen and CpG adjuvant; CpG adjuvant also stimulated the secretion f IL-2 and IFN-γ cytokines.	(Dehghan *et al*., 2014)
Dermatophagoides ptero-nyssinus-2 antigen (Der p2) and CpG (adjuvant)	Poly(lactic-co-glycolic acid (PLGA) nanoparti-cles	Male C3H/HeBFeJ mice	Airway hyper responsiveness and eosinophilia accumulation in lungs were decreased after Der p2 exposures; Increased in the secretion of Der p2-specific IgG2a antibodies; Encapsulation of CpG produced aTh1-dominant immunity.	(Joshi *et al*., 2014)

(Table 1) Contd....

Antigen or/and adjuvant	Composition of nano-carriers	Animal model	Relevant effects	References
Ovalbumin (antigen) and CpG oligodeoxynucleotide (adjuvant)	Pluronic-stabilized poly(propylene sulfide) nanoparticles	C57BL/6 and OT-I ova-transgenic mice	Induced a threefold enhancement of splenic antigen-specific $CD8^+$ T cells displaying increased CD107a expression and IFN-γ production compared with immunization with unconjugated ovalbumin with CpG; Potent Th17 cytokine profile in $CD4^+$ T cells; Recruitment to the lung of a long-lasting pool of protective effectors memory cytotoxic T-cells by disulfide-linked antigen-conjugated nanoparticles formulation.	(Nembrini *et al*., 2011)
Ovalbumin (antigen) and TLR5 ligand flagellin (adjuvant)	Polypropylene sulfide nanoparticles	Mice	Nanoparticles penetrated the nasal epithelium, transited via M cells, and were taken up by APCs in the nasal-associated lymphoid tissue; Induced cellular and humoral immune response at both systemic and mucosal levels; Co-conjugation of the TLR5 ligand flagellin enhanced humoral responses in the airways and in the distant mucosal compartments such as in vaginal washes and rectal washes compared to free flagellin.	(Stano *et al*., 2011)
DNA plasmid encoding foot and mouth disease virus (FMDV) (antigen) and bovine IL-6 gene (mucosal adjuvant)	Chitosan-coated PLGA nano/microparticles	Guinea pigs and Wistar rats	Bovine IL-6 effectively functioned as a mucosal adjuvant, *i.e*. significantly enhancing mucosal and systemic immune responses; Formulation that FMDV protein (and IL-6) targeting endoplasmic reticulum produced a stronger immune response and provided better protection against aerosol infections of foot and mouth disease virus.	(Wang *et al*., 2011)

outcomes in terms of eosinophilia and IgE levels, the polymeric nanoparticles generated high reduction of Th2 cytokines in the lungs of allergic mice model. The authors suggested the potential use of polymeric nanoparticles in the clinical context as a delivery platform for allergen-free therapy to improve the activity of immunomodulators administered via airway mucosal. Compared with other nanocarriers, the authors also reported that the PEG-poly(propylene sulfide) nanoparticles present advantages concerning with manufacture and safety features. For example, the preclinical and clinical studies revealed that CpG-loaded virus-like particles (VLPs) failed to achieve endpoints [98, 99].

3.1.1.2. Antigen-Loaded Polymeric Nanoparticles

The nasal immunization with free antigens usually induces weak mucosal and systemic immune responses and protection [9]. Therefore, it is important to use strategies capable of inducing the immune response. Several studies have been focused on using polymeric nanoparticles as carriers for antigen delivery [100-103].

Kunda *et al.* formulated a dry powder vaccine containing an antigen of *Streptococcus pneumonia* (pneumococcal surface protein A - Psp A) that is adsorbed onto the surface of polymeric nanoparticles, using a biodegradable polymer, poly(glycerol adipate-co-ω-pentadecalactone) - PGA-co-PDL for delivery *via* the inhalation route [101]. The nanoparticles were then encapsulated into L-leucine microparticles. The Psp A is a member of metal binding lipoproteins family and has shown cross-reactivity amongst all pneumococcal serotypes [104]. The PGA-co-PDl nanoparticles were internalized within 1 h when co-incubated with dendritic cells. The functional part of the antigen was active in the polymeric nanoparticles and maintained its stability and integrity. The *in vitro* release data indicated a burst release of 40% with complete release (94%) within 48h. Although the aerosol properties suggested a broncho-alveolar lung deposition, ideal for nanoparticles uptake by dendritic cells, further *in vivo* investigations should be focused on determining the immunogenicity of the release Psp A.

Bernocchi *et al*. explored the use of supramolecular nanoparticles, prepared by loading phospholipids in maltodextrin nanoparticles (*i.e*. polysaccharide nanoparticles) core, as potential vaccine carriers in airway mucosa [105]. *In vitro* transcytosis studies using 16HBE14o-(16HBE) human bronchial epithelial cell line demonstrated that these polysaccharidic lipidated nanoparticles which contain an anionic lipid in their core did neither open tight junctions nor modify the *in vitro* epithelial permeability, demonstrating no potential toxicity associated to the nose-brain passage. The authors also demonstrated that nanoparticles loading ovalbumin (OVA), a well-known vaccine model antigen, did not cross the epithelial barrier transcytosis. After nasal administration, *in vivo* biodistribution studies on nostrils of mice demonstrated that polysaccharidic lipidated nanoparticles prolonged the nasal residence time of OVA and increased its cellular uptake in airway epithelial cells. Additionally, no nanoparticle formulation was presented in the tissues below the epithelial barrier, supporting *in vitro* results and excluding the chances of adverse effects. Although this was the promising observation, the authors suggest the performance of *in vivo* studies to understand the real fate of the polysaccharidic lipidated nanoparticles and the encapsulated protein within nasal mucosa. In other study, the same authors vaccinated mice via intranasal route with porous cationic maltodextrin-based with lipidic core nanoparticles loaded with the total extract of *Toxoplasma gondii* antigen [36]. The lipid core contained DPPG (1,2- dipalmitoyl-sn-glycero-3-phosphatidylglycerol) which can facilitate the cytoplasmatic delivery of proteins/antigens [106]. The authors used *in vivo* acute and chronic toxoplasmosis mouse models to prove the nasal vaccine efficiency. A delayed humoral response and a strong and specific Th1/Th17 cellular immune response were induced. A protection effect was also reported once only mice vaccinated with antigen-

loaded porous nanoparticles formulation survived to acute *Toxoplasma gondii* infection.

One of the promising approaches for nasal vaccination is the encapsulation of antigen into mucoadhesive and biocompatible particles, once antigens present diminutive affinity for the nasal epithelium and tend to be rapidly removed by mucociliary clearance [41, 107]. The co-administration of antigen with mucoadhesive polymers (*e.g*. as polylactide-co-glycolide (PLGA) and chitosan) to the mucosae allows the enhancement of their absorption and extends the nasal residence time increasing the interaction with the immune system [107]. The hydrophilic bioadhesive polymers generally absorb water on the surface of mucosa, swell and acquire a gel-like aspect, increasing the residence time of the antigen on the mucus layer [9].

Chitosan is a well-known cationic, biodegradable and mucoadhesive polymer. The positive charge of chitosan allows it to establish bonds with negatively charged sialic residues in the mucus lining of the nasal epithelial cells and, consequently, retarding the nanoparticles clearance [108]. Additionally, some studies have demonstrated the ability of chitosan-based nanocarriers to facilitate antigens overcome mucosal barriers and to elicit systemic and mucosal immune responses against different antigens upon nasal immunization [109-113]. In a patent, Sung *et al.* reported the development of poly-gamma-glutamic acid-chitosan nanoparticles for the delivery of influenza antigens [114]. When administered to mice, by subcutaneous or intranasal routes, the nanoparticles elicited higher antibody titers than alum-adsorbed antigen vaccine. Additionally, the authors reported a 100% animal survival after viral challenge, while mice receiving the alum-adsorbed antigen demonstrated a survival rate of only 50%. The inventors also suggested that the order of addition of the polymers (poly-gamma-glutamic acid and chitosan) is important to the surface charge and, consequently, to the establishment of the ratio of bonds between the antigen and the nanoparticles. Slutter *et al.* demonstrated that nasal immunization with N-trimethyl chitosan (TMC) nanoparticles, *i.e.*, positively charged nanoparticles, in mice increased the residence time of ovalbumin in the nasal cavity [115]. Regarding immunogenicity, rapid antigen-releasing TMC nanoparticles led to high sIgA levels and serum antibody titers. These nanoparticles also stimulated the maturation of dendritic cells. In this study, authors also developed PLGA nanoparticles, negatively charged nanoparticles, and TMC-coated PLGA nanoparticles. Both formulations provided slow release of antigen from particles and did not induce measurable antibody titers. Figueiredo *et al.* encapsulated the low immunogenic *Streptococcus equi* enzymatic extract into two positively charged nanoparticulate carriers (phosphatidylcholine-cholesterol-stearylamine liposomes and chitosan nanoparticles) aiming to potentiate the immune response [113]. After intranasal immunization of mice, both formulations induced a Th1-mediated immune response characterized by the production of IFN-γ and high titers of IgG2a antibody, as well as, a Th2 immune response characterized mainly by the production of IL-4 and IgG1 antibodies. Based on these effects, the authors concluded about the potential use of both nanosystems as antigen carriers to stimulate mucosal, humoral and cellular immune responses. However, nanoparticles induced a more successful mucosal stimulation compared to liposomes due to the mucoadhesive properties of chitosan which was confirmed by increased sIgA levels in the lungs.

Chitosan has also been widely investigated as a non-viral gene delivery system due to their cationic nature. This polymer has also the ability to modulate the tight junction integrity, which can increase the transport by the paracellular route. This makes chitosan an ideal carrier for the delivery of DNA vaccines through mucosal tissues [108, 116]. Therefore, DNA-based vaccines using cationic components such as chitosan to form complexes with the DNA antigen in the form of nano/microsystems have been investigated for intranasal mucosal immunization [117]. Xu *et al.* investigated the immunization of mice with chitosan-DNA by intranasal application of nanoparticles expressing pneumococcal surface antigen A (Psp A) [102]. Besides the mucoadhesive property, chitosan has adjuvant properties enhancing both humoral and cellular immune responses [115, 118, 119]. Immunized mice with chitosan-DNA nanoparticles presented high levels of anti-Psp A IgG antibody in serum and anti-IgA antibody in mucosal lavages. The intranasal administration of these nanoparticles also induced a more balanced IgG1/IgG2a antibody ratio in serum, enhanced IFN-γ (interferon gamma, *i.e*. a Th1 cytokine), as well as, IL-17 A levels (*i.e.* a cytokine that mediate pro-inflammatory response) in spleen lymphocytes and mucosal washes, suggesting the stimulation of both mucosal and systemic immune responses and preventing pneumococcal nasopharyngeal colonization. The results suggested that chitosan-DNA nanoparticles produced stronger mucosal and systemic antibody titers compared to levels in mice immunized with naked DNA which can be explored as a promising strategy for the prevention of pneumococcal infections. In other study, Raghuwanshi *et al.* selected a plasmid DNA encoding nucleocapsid (N) protein, of severe acute respiratory syndrome coronavirus, as vaccine antigen. The authors prepared plasmid DNA loaded biotinylated chitosan nanoparticles which were targeted to mucosal dendritic cells by the attachment of a recombinant bifunctional fusion protein (bfFp) vector in their surface [120]. The intranasal DNA immunization of mice, with bfFp targeted formulations, enhanced mucosal IgA levels as well as systemic IgG levels. Additionally, the administration of targeted formulation provided higher levels of IFN-γ compared to the non-targeted formulation. The results showed a higher titer of both N protein specific serum IgG antibodies and IFN-γ, when the targeted formulation was administered simultaneously with dendritic cell maturation/activation stimuli (*i.e*. using the anti-mouse-CD40 mAb).

Muttil *et al.* developed spray-dried PLGA/polyethylene glycol (PEG) nanoparticles, made of a biodegradable PLGA core and a PEG shell that encapsulated recombinant hepatitis B surface antigen (rHBsAg) [121]. The authors were able to incorporate the nanoparticles into porous microparticles by spray drying both to prevent the agglomeration of the nanoparticles. The authors prepared dry powder formulations containing nanoparticles with excellent aerosolization properties for deposition in the deep lung (*i.e*. with a mass median aerodynamic diameter between 1 and 5 μm) by spray drying the particles from a suspension containing L-leucine that improved the aerosolization and powder flowability properties [122]. The dry powder aerosol formulation was administered to the lungs of male guinea pigs, *i.e*., animal model of choice for evaluating adjuvant formulations. The immunization with dry powder of antigen nanoparticles via the pulmonary route resulted in a significant mucosal immune response characterized by a higher IgA levels in the bronchio-alveolar lavage fluid than the administration of alum with adsorbed rHBsAg formulation (*i.e*. a control group). However, the guinea pigs immunized with alum with adsorbed rHBsAg via intramuscular route presented the highest IgG antibody titer in serum. Muttil *et al*. also encapsulated diphtheria CRM-197 antigen (CrmAg), *i.e.* a non-toxic mutant of diphtheria toxin, within poly(lactic-co-glycolic acid (PLGA) nanoparticles using the same strategy described before [25] and similar results are reported. Therefore, the authors pointed out the potential of intranasal immunization to induce a high mucosal immune response in the respiratory tract with the advantages of avoiding the use of traditional adjuvants and producing sufficient neutralizing antibodies in the serum to provide protection against hepatitis B and diphtheria.

Interesting studies were devoted to nanoparticles based on hydrophilic and amphiphilic poly(γ-glutamic acid) (γ-PGA). This biodegradable polymer is attractive for immunization once in the presence of mucin layer glycoproteins: the carboxy groups of the γ-PGA present mucoadhesive properties while partial polymer modification with amine moieties allows the interaction with the anionic

epithelial cell layer [15]. Additionally, Matsuo *et al.* investigated the potential of γ-PGA nanoparticles as cancer vaccine carriers administered by the intranasal route. For that the authors studied the antitumor effects and related immune responses after immunization of a mouse tumor model via the nasal cavity with ovalbumin-loaded poly(γ-PGA) nanoparticles [123]. The previously vaccinated animals resisted to a challenge by *E.G*7-ovalbumin tumor cells. Additionally, using a lung metastasis model with B16-ovalbumin cells, the ovalbumin-loaded γ-PGA nanoparticles significantly decreased the number of lung metastasis nodules after three intranasal doses. The intranasal administration of ovalbumin entrapped in γ-PGA nanoparticles elicited cytotoxic T lymphocytes and interferon-γ-secreting cells specific for antigen protein in the spleen and lymph nodes and generated identical serum anti-ovalbumin IgG titers than the administration of ovalbumin solution (*i.e.* control group). The intranasal vaccination with γ-PGA nanoparticles exhibited antitumor efficacy which was related mainly with the antigen-specific CTL induction. The authors also reported the fast uptake of nanoparticles by the nasopharyngeal-associated lymphoid tissue which were efficiently captured by APCs after reaching the lymph nodes. However, further studies are required to comprehend the *in vivo* kinetics of the antigen-loaded γ-PGA nanoparticles.

Other biodegradable polymers use for intranasal immunization purposes are polyesters. Ulery *et al*. prepared a polyanhydride nanoparticle-based vaccine formulation that encapsulated recombinant protein F1-V for administration as a single intranasal dose [7]. Using mice models, the polymer-based nanoparticle vaccination successfully elicited long-term protection against respiratory *Yesinia pestis* infection that correlated with a high titer and high avidity IgG1 anti-F1-V specific antibody response. The authors suggested that this nanoparticulate platform can be effectively used for intranasal immunization due to the versatility of the polyanhydride chemistry in stabilizing encapsulated proteins, the ability to activate antigen-presenting cells and to provide extended release of antigens from nanoparticles.

3.1.1.3. Co-Delivery of Antigen and Adjuvant

Co-delivery of an antigen and an adjuvant agent by the same nanosystem is an interesting approach that has been explored by various researchers. Using the same delivery route, the combination of antigens and immunostimulants (*e.g*. CpG DNA, muramyldipeptide and lipopolysaccharide) in the same nanocarrier has been reported to potentiate systemic immunization [111, 112].

Dehghan *et al*. developed chitosan nanospheres loaded with influenza whole virus and adjuvants (CpG oligodeoxynucleotide or *Quillaja* saponin) [9, 124]. A dry powder form of chitosan nanospheres co-encapsulating adjuvants demonstrated to be an appropriate carrier and immunostimulator for nasal immunization of the virus influenza, due to the nanometer size range of the delivery system, the mucoadhesive property of the chitosan to adhere to mucosal membranes, the suitable release profile of the contents and biocompatibility with the mucosae [124]. For the intranasal delivery, the authors used the rabbit as *in vivo* model [9] due to their similar nasal immune system to humans [125]. The rabbits received three doses of nanospheres vaccine on days 0, 45 and 60, followed by a last booster injection on day 75. The authors reported the function of the chitosan nanospheres in stimulating the immune system. Regarding the role of the adjuvants, CpG induced strong humoral and cellular immune response, as well as, a Th1 type response than *Quillaja* saponin, demonstrating the efficient of this adjuvant for mucosal immunization against the influenza virus. In all immunized groups receiving loaded nanospheres with virus and CpG, the hemagglutination inhibition antibody titer was higher than the control group. Moreover, rabbit serum IgG titers significantly augmented in the animal vaccinated groups, with the highest response in animal administered nanospheres loaded with both antigen and CpG adjuvant. The CpG adjuvant also stimulated the secretion of IL-2 and IFN-γ cytokines.

Joshi *et al*. have shown that biodegradable poly(lactic-co-glycolic acid) (PLGA) particles carrying both the antigen and the CpG produced a potent stimulation of antigen-specific immune responses when compared to the vaccination with antigen and CpG in solution [126, 127]. The extent and type of immunization were correlated with the size of PLGA particles and the co-delivery of CpG. For example, dendritic cells better internalized PLGA particles when their size was 300 nm [127, 128]. The encapsulation of CpG in PLGA particles preferentially stimulated a Th1-type immune response. These authors developed a biodegradable PLGA nanoparticle-based vaccine to treat house dust mite allergies in mice. The immune-modulating carriers contained a strong immunogenic allergen, *Dermatophagoides pteronyssinus*-2 antigen (Der p2), and a potent Th1 adjuvant, CpG. After intranasal vaccination, the nanosized particles, produced a significantly lower airway hyper response and lower IgE antibody levels, compared to the control group.

In another study, Nembrini *et al*. conjugated the model antigen ovalbumin and CpG oligodeoxynucleotide to pluronic-stabilized poly(propylene sulfide) nanoparticles by reversible disulfide bonds [103]. The authors administered the nanoparticles through the nostrils in the lung of mice. The results demonstrated the specifically target delivery of antigen designed to pulmonar dendritic cells and increase uptake of antigen and its transport to the draining lymph node. Additionally, the disulfide-linked antigen-conjugated nanoparticles also promoted a higher $CD8^+$ T-cell immune response compared to non-conjugated ovalbumin with CpG, followed by a potent Th17 cytokine profile in $CD4^+$ T cells. The authors suggested the usefulness of this reduction-reversible nanosystem as a vaccine delivery platform for targeting intracellular pathogens infecting the lung. Stano *et al.* prepared similar ovalbumin and immunostimulatory adjuvant loaded degradable polymer nanoparticles and evaluated their efficacy in mucosal vaccination after intranasal administration to mice [129]. The authors used toll-like receptor (TLR)-ligands as the immunostimulatory adjuvant. The ovalbumin-conjugated nanoparticles efficiently crossed the nasal mucosal epithelial barrier and induced cellular responses (*i.e*. cytotoxic T lymphocytic response) in lung and spleen tissues, as well as the humoral response in mucosal airways. Additionally, the surface conjugation of the TLR5 ligand flagellin into the nanoparticles improved humoral responses, not only in the airways, but also at distal mucosal compartments, including the vagina and the rectum, and induced cellular immune responses, in opposition to what happened with free flagellin. In conclusion, the authors suggest the use of polypropylene sulfide nanoparticles platform for inducing extensive mucosal immunization by intranasal administration.

An approach to avoid the enzymatic degradation of DNA plasmid vaccines due to the presence of DNases in the mucosal surfaces is their adsorption onto chitosan-coated PLGA particles [130]. This strategy can also increase the penetration of the encapsulated gene material at mucosal surfaces [131]. Based on these considerations, Wang *et al.* manufactured chitosan-coated PLGA nanoparticles that entrapped plasmid DNA encoding the foot and mouth disease virus (FMDV) capsid protein [132]. The authors also studied the effect of bovine IL-6 gene as a mucosal cytokine adjuvant. Guinea pigs and rats were intranasally vaccinated with microparticles formulated by freeze-drying the chitosan-coated PLGA nanoparticles with mannitol. According to published data, the mannitol dissolves in physiological conditions and the microparticles quickly convert into nanoparticles [133]. In animals immunized with plasmid DNA loaded chitosan-coated PLGA nanoparticles with IL-6 inserted, the levels of antigen-specific sIgA (secretory IgA) in vaginal and nasal washes were enhanced, allowing the correlation with the higher IgA expression levels in mucosal tissues, compared to the IgA responses observed in rats immunized with a formulation without IL-6 in-

serted. Moreover, animals vaccinated with a formulation containing IL-6 generated stronger FMDV specific antibody responses as well as stronger FMDV neutralizing antibody responses. The authors suggested that the presence of IL-6 as molecular adjuvant significantly contributed to induce both mucosal and humoral immune responses. Additionally, the authors proved that magnitude in immune responses was related to the composition of DNA vaccines formulation (*i.e*. expression plasmids and the location of the IL-6 gene in the FMDV protein) which affected the targeted delivery to the endoplasmic reticulum. The formulation that FMDV capsid protein can be targeted to endoplasmic reticulum induced a significantly stronger immune response (*i.e*. group with highest antigen-specific T-cell proliferation and expression levels of IFN-γ) and provided better protection than animals immunized with the formulation that produces FMDV capsid protein that was not target to endoplasmic reticulum.

3.1.2. Polymeric Micelles

Polymeric micelles are colloidal carriers with sizes within a range of 5-100 nm which are generated by spontaneous self-assembly in water of individual amphiphilic (hydrophobic/hydrophilic segments) block copolymers. They typically present large solubility differences. These systems contain a hydrophilic head-group and a hydrophobic tail. Micelle systems have been investigated as valuable adjuvants for vaccine delivery [15, 134]. Due to their small size, micelles favor the antigen delivery to antigen presenting cells in the draining lymph nodes [135]. These colloidal systems can also display suitable surface properties, such as nature and charge, which can have a crucial role in the induction of immune responses [136, 137]. Attending to the chemical design of the hydrophobic and hydrophilic blocks that constitute the micelle structure, these nanocarriers can encapsulate or surface coupled additional immunostimulatory molecules (*e.g*. Toll Like Receptor ligands, Nod-like receptors ligands) to induce a superior activation of the dendritic cells [138]. Despite these advantages, polymeric micelles are prone to dissociate upon dilution, which can result in prompt release of their load.

3.1.2.1. Antigen-Loaded Polymeric Micelles

Noh *et al*. designed and synthesized a mucosal vaccine delivery based on biosynthetic mucoadhesive polymer nanomicelle using poly(γ-glutamic acid) (γ-PGA) conjugated with hydrophobic cholesterol groups and modified with amines [134]. γ-PGA is a hydrophilic and high anionic polymer. Ovalbumin labeled with iodine (^{123}I) was added to the nanomicelles and administered intranasally. The results suggest that γ-PGA nanomicelles increased the residence time of co-delivery antigen in the mucus layer and controlled antigen release for the nasal epithelial cells. Moreover, nasal mice immunization with nanomicelles induced higher levels of ovalbumin-specific IgA antibody titers in the nasal tissue than the immunization with ovalbumin alone, indicating a strong mucosal immune response. These polymeric nanocarriers also stimulated higher IFN-γ-secreting cells than ovalbumin alone. Based on the experimental results, the authors reported that γ-PGA nanomicelles can act as an effective adjuvant that potently induces both humoral and cellular immunity, in contrast with various types of emulsions and particulate system which are mostly able to induce a high antibody immune response with little stimulation of the cellular immune response. After immunization, the histological analysis of the nasal tissues also proved no perceptible toxicity and inflammatory responses. The authors also studied the adjuvant function of γ-PGA nanomicelles in the presence of PR8 antigen (*i.e*. influenza A virus antigen). The immunization with PR8 antigen loaded in γ-PGA nanomicelles elicited high levels of functional antibodies and IFN-γ-producing cells. The authors also investigated the induction of protective immunity against a lethal dose of influenza virus infection. The immunized mice with PR8 antigen, encapsulated in γ-PGA nanomicelles, exhibited 100% of protective immunity against a lethal PR8 virus challenge. The mice immunized with non-loaded PR8 virus only revealed a 50% of survival rate.

3.2. Lipid-Based Nanocarriers

Some lipid-based carriers have shown promise as inhaled products delivering vaccines either for prophylactic or therapeutic vaccination [85, 139, 140]. The most reported studies in literature used liposomes-based systems.

3.2.1. Liposomes

Liposomes are defined as nearly spherical vesicles consisting of an aqueous core enclosed by natural or synthetic phospholipids bilayers. The structural properties makes liposomes versatile and promising carriers, which can entrap substances with different solubilities, *i.e*. water soluble compounds (inside the aqueous cavity or in the external water phase), poorly water-soluble compounds (into the lipid bilayer) and compounds with intermediary water-solubility readily partition between the lipid bilayer and in the aqueous compartment [141]. In their composition, liposomes can contain diverse lipids that present GRAS (*Generally Recognized as Safe*) status such as natural and cell-like membrane amphiphilic lipids (*e.g*. cholesterol, egg lecithin and soy lecithin), and/or semi synthetic phospholipids**,** which could be based on the pulmonary surfactant lipids (*e.g*. sphingomyelin, phosphatidylcholine, phosphatidylethanolamine). In addition, antigens molecules can be also attached to the liposome surface by adsorption or chemical linking [142]. Depending on their production process and phospholipid composition, liposomes comprise uni-, oligo- or multi-lamellar vesicles, with sizes ranging from 20 nm up to few hundred μm, and can have different surface charges (anionic, cationic or neutral).

In immunization topic, liposomes have been reported as effective immunological adjuvant or vaccine delivery system which can enhance the adaptive immune responses after endocytosed by APCs [143, 144] and the immunogenicity of weak protein antigens or synthetic peptides [142, 145-148]. Allison and Gregoriadis were the first authors to report the ability of liposomes to induce immune responses in the seventies [149]. However, the instabilities of these phospholipids' vesicles and their high cost of manufacturing process are examples of limitations for their clinical application.

Different authors reported *in vivo* studies that demonstrated the possibility to trigger mucosal and systemic humoral or cytotoxic immune responses after nasal administration of liposome-based vaccines that could be effective in giving protection against local and distant infections (*i.e*. prophylactic immunotherapy) as well as in protecting and/or inhibiting tumor development (*i.e*. therapeutic immunotherapy) [113, 139, 140, 145, 147, 150, 151] (Table **2**).

The physicochemical properties of liposomes influence their immunogenicity such as charge, size, composition, fluidity (*i.e*. phase transition temperature [Tc] of the lipid components) [152].

3.2.1.1. Antigen-Loaded Liposomes

Tseng *et al*. referred that antigens entrapped within the liposomes can resist degradation or neutralization in the tissues and the antigens can be released over a prolonged period (*i.e*. depot effect) *in vivo* [151]. These authors evaluated the adjuvant effect of liposomes formulated with three phospholipids including phosphatidylcholine-liposomes - neutral charged liposomes - phosphatidylserine-liposomes - negatively charged liposomes, and stearylamine-liposomes - positively charged liposomes - and compared them with the inoculation of virus alone. The authors used inactivated Newcastle disease virus (NDV) as a model antigen. The formulations were inoculated intranasally in specific pathogen-free chickens. Despite all liposomal formulations, the immunogenicity of NDV has increased; the neutral charged liposomes were taken up more efficiently by macrophages than positively or negatively charged liposomes, and induce a significant higher anti-NDV s-immunoglobulin A (s-IgA) levels in tracheal lavage fluid and serum

Table 2. Examples of *in vivo* studies using inhaled lipid-based formulations for respiratory immunization.

Antigen or adjuvant	Composition of nanocarriers	Animal model	Relevant effects	Reference
Ovalbumin	Targeted galactosylated-modified liposome consisting of galactose and 1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine	BALB/c female mice	Increase the uptake and production of cytokines by macrophages; A significant increase in mucosal s-IgA and serum IgG antibody responses compared to unmodified liposomes.	(Wang *et al*., 2013)
Ovalbumin	Liposomes coated with a neoglycolipid consisting of mannotriose and dipalmitoylphosphatidylcholine	BALB/c mice	High levels of OVA-specific IgG and IgA antibodies in serum and no significant serum antibody responses with the administration of uncoated liposomes or OVA alone; Presence of antigen-specific secretory IgA in nasal washes and produced interferon-gamma secreting cells in NALT.	(Ishii and Kojima, 2010)
Inactivated Newcastle disease vírus (NDV)	Phosphatidylcholine-liposomes (PC-Lip) Phosphatidylserine-liposomes (PS-Lip) Stearylamine-liposomes (SA-Lip)	Specific pathogen-free White Leghorn chickens	Immunogenicity was significantly enhanced by all liposomal-based formulations; PC-Lip is taken up more efficiently by macrophages and induces a significant higher s-IgA in tracheal lavage and serum IgG antibody titers.	(Tseng *et al*., 2010)
Inactivated Newcastle disease vírus (NDV)	Phosphatidylcholine-liposomes	Specific pathogen-free chickens	High mucosal anti-NDV s-immunoglobulin A (IgA); High serum IgG; High hemagglutination inhibition titer; High survival rate.	(Lin *et al*., 2011)
Influenza A/New Caledonia/20/99-like (H1N1) and A/Panama/2007/99-like (H3N2) strains	Polycationic sphingolipid (ceramide carbamoyl-spermine) / cholesterol liposomes	Specific pathogen-free female BALB/c mice and healthy female ferrets	Significantly higher hemagglutination inhibition antibody titers compared with controls; Reduce the severity of influenza virus infection in ferrets; Long retention both lipids and antigens in the nose and lung; Increased cytokine levels and expression of co-stimulatory molecules.	(Even-Or *et al*., 2011)
Double-stranded RNA	Cationic liposomes prepared using phosphatidylcholine: cholesterol and stearylamine	Female BALB/c mice infected with inactivated H5N1 virus	Significant reduction in pulmonary viral titres; Improved survival rate of infected mice; Improved formulation in terms of its efficacy, toxicity and *in vivo* stability compared to poly-ICLC	(Li *et al*., 2011)
Ovalbumin, bovine serum albumin, cell extracts from *L. monocytogenes, Francisella tularensis* and *Helicobacter pylori*, pneumococcal surface antigen and a BSA-conjugate of *Escherichia coli*	Archaeal lipid mucosal vaccine adjuvant and delivery formulation	Mice	For ovalbumin and bovine serum albumin - stronger adjuvant potential, in terms of IgG and IgA levels, than the conventional archaesomes; For cell extracts - higher antibody levels compared to free antigen.	(Patel and Chen, 2013)
Gp41-subunit antigens	Virus-like particle (virosome) derived from influenza that is formed by a lipid bilayered vesicle into which molecules of the influenza virus hemagglutinin (HA) and neuraminidase (NA) are inserted	Female *Macaca mulatta* of Chinese	Four out of five vaccinated animals remained virus-negative, and the fifth was only transiently infected; None of the five animals seroconverted to p27*gag*-SIV; All placebo-vaccinated animals became infected and seroconverted. All protected animals showed gp41-specific vaginal IgAs with HIV-1 transcytosis-blocking properties and vaginal IgGs with neutralizing and/or antibody-dependent cellular-cytotoxicity activities; Plasma IgGs totally lacked virus-neutralizing activity.	(Bomsel *et al*., 2011)

(Table 2) Contd....

Antigen or adjuvant	Composition of nanocarriers	Animal model	Relevant effects	Reference
Influenza hemagglutinin derived peptide and ErbB2 TCD8þ peptide epitope and Pam2CAG adjuvant	Small unilamellar vesicles (SUV), multilamellar large vesicles (MLV), reverse-phase evaporation (REV) and ultraflexible small unilamellar vesicles (Uf-SUV), also named transferosomes,	BALB/c mice (intravenous or subcutaneous implantation of ErbB2-overexpressing cancer cells)	All the liposomal formulations had anionic charges (between -33 mV to -88 mV); Nasal vaccination with the SUV vaccine produced a significant antitumoral activity against lung tumors and a non-significant protection against subcutaneous tumors; Physicochemical characteristics had not impact in the immunostimulatory activity and antitumor efficiency against lung tumor animal model, in contrast to the total dose of vaccine or the dose of adjuvant (*i.e*. Pam2CAG); Intranasal immunization with the Uf-SUV vaccine triggered a high local immunostimulatory response.	Kakhi *et al*., 2016)
Monophosphoryl lipid A and trehalose 6,6′ dimycolate (adjuvants)	Dilauroylphosphatidylcholine liposomes (MIT) and MIT incorporated short, highly conserved influenza-derived synthetic myristylated peptides (MITpep)	C57BL/6, BALB/c, and CBA/J female mice	MIT provided robust, but short-lived, protection against multiple, highly lethal strains of influenza; MITpep provided equivalent, but more durable, protection compare to MIT.	(Tai *et al*., 2011)
Ovalbumin or F1-V (antigens). and monophosphoryl lipid A (MPLA) (adjuvant)	Cationic liposome-hyaluronic acid (HA) hybrid nanoparticle	C57BL/6 female mice	Improved colloidal stability and prolonged antigen release; *In vitro* promoted bone marrow dendritic cells maturation and upregulation of co-stimulatory markers, including CD40, CD86, and MHC-II; *In vivo* robust ovalbumin-specific $CD8^+$ T cell and antibody responses; F1-V and MPLA induced potent humoral immune responses and induced balanced Th1/Th2 humoral immune responses, compared with the lack of sero-conversion in mice immunized with soluble F1-V vaccine.	(Fan *et al*., 2015)
Hepatitis antigen and H1N1 antigen and TLR7 receptor agonist (imiquimod – adjuvant)	Miglyol® 812, squalene, vitamin E (core); phosphatidylcholine, PEG stearate and/or sodium cholate (surfactants); protamine, poly-D-glucosamine or chitosan (corona); imiquimod or polyI:C (additional adjuvants)	Female BALB/c mice	Efficient and balanced antibody response; Earlier onset of the immune response (with imiquimod); Induced a protective immune response.	(Alonso *et al*., 2014; Alonso *et al*., 2013)
Recombinant hepatitis B surface antigen and TLR7 receptor agonist (imiquimod –immunostimulant)	Miglyol® 812 core an chitosan corona nanocapsules	Female BALB/c mice	Increased IgG levels over time and specific immunological memory; Induced a protective immune response.	(Vicente *et al*., 2013)

IgG antibody titers. In addition to the charge, the authors explained the difference in immunogenicity of liposomal formulations based on the phase transition temperature [T_c] of the lipid components. This parameter affects the *in vivo* stability of liposomes and influences the ability to trigger both humoral and cell-mediated immune responses [153]. At 43 ºC, the Tc of neutral liposomes is close to the chicken's body temperature, while that of anionic liposomes and cationic liposomes (62 ºC and 58 ºC, respectively) is relatively higher and the formulations are more rigid in their liquid crystalline state, which renders them more resistant to particle adsorption at the nasal epithelial layer. Therefore, when in contact with the mucosal membrane, neutral liposomes become more flexible and fluid facilitating their attachment to the cell's surface and, consequently, the antigen delivery to the nasal cavity. In response to viral confront, the animals included in control group died, while 90% of animals which received intranasal neutral liposomes survived. Lin *et al.* also investigated the encapsulation of inactivated Newcastle Disease Virus (NDV) in liposomes [147]. The intranasal administration of these liposomes in specific pathogen-free chickens resulted in high levels of mucosal anti-NDV s-immunoglobulin A (IgA) and serum IgG, high hemagglutination inhibition titer, and high survival rate. The authors demonstrated that macrophages were stimulated by phosphatidylcholine-liposomes via the extracellular regulated kinase (ERK) 1/2 and nuclear factor (NF)-κB activation pathways.

In other study, Even-Or *et al.* reported the effectiveness of lipid assemblies comprised of a novel polycationic sphingolipid (cera-

mide carbamoyl-spermine), used as adjuvant/carrier, when complexed with cholesterol and encapsulated influenza HA antigen [154]. The authors demonstrated that, ferrets immunized intranasally with liposomal formulations, produced a higher hemagglutination inhibition antibody titers (local and systemic humoral response) compared to the control group (*i.e.* ferrets immunized intramuscularly with the unadjuvanted influenza vaccine). Additionally, the intranasal liposome-based vaccine also elicited strong cellular (proliferation, Th1-secretion of INF and IL-2, and Th2-secretion of IL-5) response and reduced significantly the severity of influenza virus infection. Pharmacokinetic and biodistribution studies demonstrated the long retention of both lipids and antigens in the nose and lung.

The use of mucoadhesive liposomal preparations is also evaluated to improve the capture and transport of antigens on mucosal surfaces and effectively induces immune responses [155]. Chen *et al.* developed soy phosphatidylcholine and phospholipid dimyristoyl phosphatidylglycerol liposomes that encapsulated bovine serum albumin [156]. The liposomes were coated with different mucoadhesive polymers (alginate, chitosan or trimethyl chitosan) to increase bioavailability and mucoadhesion. Polymer coating resulted in increased liposomes size and chitosan and trimethyl chitosan increased the mucoadhesion ability of liposomes compared to both alginate coated and uncoated liposomes.

In order to promote a target vaccine delivery to specific cells through intranasal administration, surface liposomes have been modified with receptor-specific ligands such as galactose which can be specifically recognized by macrophages (*i.e.* cells involved in the presentation of antigens to helper T-cells) [157] and mannose [150, 158]. Based on this fact, Wang *et al.* incorporated a targeting ligand, formed by the conjugation of galactose to 1, 2 - didodecanoyl-sn-glycero-3-phosphoethanolamine, on the surface of liposomes to form a galactosylated carrier able to encapsulate ovalbumin [150]. Further, mice were intranasally immunized. The targeting galactosylated liposomes presented a higher intake rate and induced superior production of cytokines by macrophages, as well as higher levels of tumor necrosis factor-α and interleukin-6 production then unmodified liposomes. Mice immunized with the OVA-encapsulated targeted galactosylated liposome had superior mucosal IgA levels in the nasal and lung wash fluid and systemic IgG antibody titers. In this study, the authors suggest that the intranasal immunization using a targeted galactosylated liposome for antigen delivery could be used for both antiviral and antitumor clinical applications. Ishii and Kojima [158] also reported similar data related to the target effects of oligomannose-modified liposomes delivered via the intranasal route in mice which resulted in high levels of ovalbumin-specific IgG and IgA antibodies.

Kakhi *et al*. investigated the prophylactic antitumor activity of liposomal vaccine administered into the nasal cavity of mice bearing lung or subcutaneous tumors over expressing the human tumor protein antigen ErbB2 [145]. The authors formulated different types of liposomes, namely small unilamellar (SUV), multilamellar (MLV), reverse-phase evaporation (REV) and ultraflexible small unilamellar vesicles (Uf-SUV), also named transfersomes, containing the ErbB2 T-cytotoxic epitope, the influenza derived HA T-helper epitope and the lipopeptide adjuvant Pam2CAG. All the liposomal formulations had anionic charges (between -33 mV to -88 mV) which are more favorable in terms of safety. Vaccines were administered to BALB/c mice by intranasal instillation followed by intravenous or subcutaneous implantation of ErbB2-overexpressing cancer cells. The results demonstrated that nasal vaccination with the SUV vaccine produce a significant antitumoral activity against lung tumors and a non-significant protection against subcutaneous tumors. In this study, unlike the results obtained with other studies, physicochemical characteristics, such as size, structure (unilamellar or multilamellar) and flexibility of liposomal vaccines had not impact in the immunostimulatory activity and antitumor efficiency against lung tumor animal model, in contrast to the total dose of vaccine or the dose of adjuvant (*i.e.* Pam2CAG). The authors explained this difference based on the fact that the adjuvant was powerful enough to level out any variation of activity among the different vaccine formulation. Therefore, the rational choice of adjuvant is critical in liposome-based mucosal vaccine development. Intranasal immunization with the Uf-SUV vaccine triggered a high local immunostimulatory response that resulted in a significant antitumor efficiency against lung tumors.

The archaesome is a special type of liposome that has also been investigated for intranasal vaccine delivery. Archaesomes are liposome-like structures built with polar lipids from *Archaea* species. These carriers can improve the interaction with APCs and induce T_H1, T_H2 and $CD8^+$ T-cell responses to the entrapped antigen [152] as well as prolong the immunologic effect due to their superior stability compared to liposomes [159]. Patel and Chen patented an archaeal lipid mucosal vaccine adjuvant and delivery formulation which has the capacity to carry a variety of antigens with different intrinsic characteristics such as ovalbumin, bovine serum albumin, a *Listeria monocytogenes* peptide, cell extracts from *L. monocytogenes, Francisella tularensis* and *Helicobacter pylori*, pneumococcal surface antigen and a BSA-conjugate of *Escherichia coli* O-chain antigen [160]. The structure of this mucosal vaccine, which is achieved by the interaction between the archaesomes/antigens and multivalent cations, acts as a self-adjuvanting carrier for the antigen(s) in the vaccine composition. Regarding ovalbumin and bovine serum albumin, archaeal lipid mucosal vaccine demonstrated stronger adjuvant potential, in terms of IgG and IgA levels, than the conventional archaesomes. In the case of cell extracts encapsulation, higher antibody levels were obtained upon immunization of mice with archaeal lipid mucosal vaccine compared to the vaccination with the free antigen. The authors reported the ability of archaeal lipid mucosal vaccine to elicit strong systemic and local humoral immune responses through different routes of administration including intranasal route.

Colloidal dispersions derived from microorganism species, such as virosomes, have been also used for immunization by intranasal route. Virosomes are liposomes prepared by combining phospholipids with virus envelope phospholipids, viral spike glycoproteins and other viral proteins. Bomsel *et al*. evaluated the protective efficacy of an HIV-1 vaccine made of gp41-subunit antigens grafted on virosomes against a virulent SHIV (simian/human immunodeficiency virus (SHIV)-SF162P3) vaginal challenge [161]. For this study, the authors used nonhuman primate females (Chinese-origin rhesus macaques - *Macaca mulatta*) which were immunized by intramuscular and intranasal routes. The authors reported that the association of both via, *i.e.* intramuscular and intranasal routes, offers the best protection to the animals compared to the use of intramuscular route alone, with undetectable viral load for six months and undetectable blood and mucosal antibodies against SIV p27gag antigen at 3 and 6 months after challenge. The vaccinated animals presented gp41-specific IgGs and IgAs with transcytosis-blocking and antiviral activities.

3.2.1.2. Immunostimulant Delivery

To overcome some limitations of commercial influenza vaccines, namely their protection against seasonal infection and the requirement for annual reformulation [162], Tai *et al.* proposed two liposome-based formulations to elicit antibodies production [163]. Firstly, these authors developed a mucosal immunostimulatory therapeutic (MIT) strategy that consists of dilauroylphosphatidylcholine liposomes containing the adjuvants monophosphoryl lipid A and trehalose 6, 6′ dimycolate. The authors exposed mice females to aerosolize influenza virus and after then they inoculated intranasally the liposomal formulations. The results demonstrated that liposomes: (i) induced a strong protection against diverse influenza strains although the lack of peptides epitopes; (ii) targeted essen-

tially to lung macrophages; (iii) elicited secretion of several cytokines with antiviral effects; (iv) afforded immediate but short-lived defense against multiple influenza highly lethal strains of influenza. In the other approach, the authors incorporated in the liposome-based system short, highly conserved influenza-derived synthetic myristylated peptides (MITpep). The intranasal administration of these liposomes resulted in an effective and localized immune response that provided immediate and long protection through both innate- and specific T cell-based immune responses but not neutralizing antibodies. The developed liposomal formulations represent an attractive approach to offer a universal protection to influenza.

Zhou *et al.* reported that intranasal administration of cationic liposome complexed with synthetic oligodeoxynucleotides containing CpG motifs (*i.e*. CpG DNA lipoplex) produced better therapeutic effects on pulmonary metastasis (*i.e*. prevention the proliferation of tumor cells and prolong survival time) than naked CpG DNA (used as control) in mice [140]. In other study, Li *et al*. tested the effect of double-stranded RNA-loaded liposomes (LE-PolyICLC) using a mouse infection influenza model [164]. PolyICLC is a synthetic double-stranded polyriboinosinic-polyribocytidylic acid stabilized with poly L-lysine and carboxymethylcellulose. The intranasal administration of LE-PolyICLC before or shortly after infection inhibited virus replication, reduced viral titers, prolonged survival of infected mice and, most importantly, effectively attenuated the development of pulmonary fibrosis. The LE-PolyICLC can be used as the molecular adjuvant and enhance both humoral and cellular responses after vaccination. The encapsulated PolyICLC in liposomes improved the efficacy of formulation when compared to the non-encapsulated Poly-ICLC. The authors concluded about the usefulness of LE-PolyICLC as prophylactic, therapeutic and immune enhancement agent (*i.e*. vaccination adjuvant) against highly pathogenic influenza infection and its associated complications.

3.2.1.3. Co-Delivery of Antigen and Adjuvant

Based on the ionic interactions between cationic liposomes composed of 1,2- dioleoyl-3-trimethylammonium-propane (DOTAP) and anionic hyaluronic acid (HA), Fan *et al.* manufactured lipid–polymer hybrid nanoparticles and evaluated their ability for the co-delivery of antigens and immunostimulatory agents [165]. In this study, the authors demonstrated that cationic liposomes can be readily incorporated with thiolated hyaluronic acid liposome (HA-SH) by promoting ionic complexation between DOTAP and HA-SH. DOTAP-HA hybrid nanoparticles were co-loaded with an adjuvant, monophosphoryl lipid A (MPLA), which is a TLR4 agonist, and antigens, ovalbumin or F1-V (*i.e*. a candidate recombinant antigen for *Yersinia pestis*, the causative agent of plague). The authors verified an *in vitro* promotion of bone marrow derived dendritic cells maturation and *in vivo* stimulation of antigen-specific cellular and humoral immune responses after intranasal vaccination of mice. The results of F1-V vaccination suggest that this vaccine platform is quite promising against *Yersinia pestis* and other infectious pathogens.

3.2.2. Lipid Nanocapsules

Lipid nanocapsules composed by an oily core stabilized by surfactants and surrounded by a polymeric material was also investigated for intranasal administration of antigens.

3.2.2.1. Co-Delivery of Antigen and Adjuvant

In a patent, Alonso *et al.* developed protamine-coated nanocapsules with different oily cores (Miglyol®, squalene or vitamin E) and considered them as antigen delivery system [166]. The authors studied the effect of immunostimulant agents (*e.g*. imiquimod - TLR7 receptor agonist). The authors reported the versatility of these nanocarriers for encapsulating both hydrophobic and hydrophilic compounds. After immunization in mice, the antigen-loaded nanocarriers (using hepatitis antigen and H1N1 antigen) elicited IgG levels (*i.e*. a protective immune response), particularly in the following administration scheme: one intramuscular prime and two intranasal boosts. In other patent, Alonso *et al*. prepared nanocapsules based on poly-D-glucosamine and squalene lipid core, whose surface has been associated to recombinant surface antigen of hepatitis B (rHBsAg) [167]. After nasal immunization of mice, the nanocapsules induced IgG levels against hepatitis B prolonged in time. In both patents, the authors reported the useful of imiquimod (*i.e*. a lipophilic immunostimulant) in the earlier beginning of the immune response.

Vicente *et al*. achieved similar results in terms of a protective immune response when developing nanocapsules consisting of Miglyol® 812 (an oil) nanocore that allocated the same immunostimulant agent and a chitosan corona that absorbed onto this surface an antigen - recombinant hepatitis B surface antigen [110]. The i*n vitro* studies demonstrated that chitosan nanocapsules were easily internalized into macrophages and induced the secretion of pro-inflammatory cytokines. The intranasal administration to mice induced also a specific immunological memory. Furthermore, the results suggested the ability of the nanocapsules to modulate the systemic immune response.

3.3. Inorganic Nanocarriers

Inorganic nanocarriers composed of different materials such as silica, gold and iron have been studied for intranasal vaccines administration. Although these nanocarriers are mostly no biodegradable, their rigid structure and controllable synthesis are the main advantages to immunization [168]. In accordance with Cordeiro and Alonso the immunomodulating properties of inorganic nanoparticles are related to the particulate structure itself rather than to their composition [17]. Table **3** presents the *in vivo* studies referred in this review, using inhaled inorganic-based nanocarriers for respiratory immunization.

3.3.1. Silica Nanoparticles

Silica nanoparticles have been explored as delivery system due to their easy production and possibility to perform modifications on their surface for a specific delivery location (*i.e*. targeted delivery), as well as their low cytoxicity. Despite their investigations as antigen carriers via the respiratory route, an adequate mucosal adjuvant is required to improve immune responses.

3.3.1.1. Antigen-Loaded Silica Nanoparticles

Neuhaus *et al*. developed a nanocarrier vaccine that encapsulated an antigen - recombinant H1N1 influenza hemagglutinin - that was produced in tobacco plants - into a silica nanoparticle delivery system [169]. The authors used a human PCLS (*i.e*. human precision-cut lung slices) model to investigate the local pulmonary toxicity of the inhalable influenza vaccine and its ability to stimulate an immune response. This model represents an organotypic *ex vivo* model of the human respiratory tract related to local pulmonary effects on the innate immune system by modulators [170]. The authors reported no local toxicity on human lung tissue. Moreover, the inhalable nanoparticles induced the secretion of TNF-α and IL-1β (*i.e*. potent pro-inflammatory cytokines), suggesting adjuvant properties of silica nanoparticles. The authors concluded that the developed silica nanocarriers induced an appropriate innate immune response and re-activated an established antigen-specific T cell response.

Yoshida *et al.* explored the potential of silica nanoparticles with variable sizes for inducing allergic immune response in mice [171] by their exposure to ovalbumin-loaded in silica delivery system with size in the nanometer, 30 nm and 70 nm, and in the micrometer ranges considering the intranasal route of administration. Mice immunized with ovalbumin plus smaller nanosilica particles had the higher IgE levels. The authors also analyzed antigenic-specific cytokine responses. Splenocytes from mice exposed to ovalbumin-

Table 3. Examples of *in vivo* studies using inhaled inorganic-based formulations for respiratory immunization.

Antigen or adjuvant	Composition of nano-carriers	Animal model	Relevant effects	References
Ovalbumin	Silica nanoparticles	Mice	Smaller silica nanoparticles (30 nm) had the higher levels of IgE and the higher secreted levels of Th2-type cytokines; Mice immunized with bigger silica nanoparticles (*i.e.* with size of 70 nm) and silica microparticles exhibited cytokine response.	(Yoshida *et al*., 2011)
NDV fusion gene plasmid DNA (antigen)	Silver and silica hollow nanoparticles	Chicken	Low *in vitro* citotoxicity; Maintenance of the plasmid DNA bioactivity; High titers of serum IgA, high lymphocyte proliferation and higher expression levels of IL-2 and IFN-γ in a dose-dependent manner.	(Zhao *et al*., 2016)
Ovalbumin	Gold nanoparticles coated with polyvinyl alcohol (PVA) containing either positively (NH2) or negatively (COOH) charged functional group	Female BALB/c and DO11.10 TCR transgenic mice	Preferentially uptake of cationic gold nanoparticles by all antigen presenting cells (APC) subpopulations and induced higher ovalbumin-specific CD4+ T cell stimulation than the anionic gold nanoparticles, or polymer alone.	(Seydoux *et al*., 2016)
H1N1 influenza hemagglutinin antigen and bis-(3,5)-cyclic dimeric guanosine monophosphate (mucosal adjuvant)	Silica nanoparticles (SiO_2)	Mice	High titers of systemic antibodies; High hemagglutination antigen-specific antibody response; High local IgG and IgA antibody responses in the bronchoalveolar lavage.	(Neuhaus *et al*., 2014)
Matrix-2 virus membrane protein (M2e) (antigen) and CpG (adjuvant)	Gold nanoparticles	Female BALB/c mice	Presence of free M2e antigen in vaccine formulation is important for inducing high levels of antibody response and for providing complete protection against lethal influenza A virus challenge.	(Tao and Gill, 2015)

loaded into smaller size nanoparticles secreted higher levels of Th2-type cytokines compared to mice exposed to ovalbumin alone. Furthermore, mice immunized with bigger silica nanoparticles and silica microparticles exhibited cytokine response.

In the veterinary area, and considering the need of an effective vaccine that protect poultry from Newcastle disease caused by NDV, Zhao *et al.* encapsulated a NDV fusion gene-containing DNA vaccine in $AgSiO_2$ hollow nanoparticles [172]. The authors selected hollow mesoporous silica spheres due to their high surface area, low effective density, high stability and their ability to stimulate both cellular and humoral immune responses. The main property of silver (Ag) nanoparticles in biological systems is their antibacterial activity against both gram-positive and gram-negative microorganisms. *In vitro* studies demonstrated that the $AgSiO_2$ hollow nanoparticles presented low citotoxicity and maintained the bioactivity of the plasmid DNA. The intranasal immunization of chickens with $AgSiO_2$ hollow nanoparticles induced high IgA antibody titers in serum; high lymphocyte proliferation and high expression levels of IL-2 (*i.e.* cytokine signaling molecule in the immune system) and IFN-γ. According with the results, the authors concluded about the safety and efficacy of $AgSiO_2$ hollow nanoparticles to control the delivery of NDV fusion gene plasmid DNA and to induce potent humoral, cellular and mucosal immunities.

3.3.1.2. Co-Delivery of Antigen and Adjuvant

Neuhaus *et al.* evaluated the immunogenicity of a double-adjuvanted influenza vaccine that combines a plant-produced H1N1 influenza hemagglutinin antigen (HAC1), a silica nanoparticle-based delivery system (SiO_2) and a mucosal adjuvant - bis-(3,5)-cyclic dimeric guanosine monophosphate (c-di-GMP). Mice vaccinated through intratracheal route with single-adjuvanted vaccine (HAC1/SiO2 or HAC1/c-di-GMP) presented lower titers of systemic antibodies than the group of animals receiving the double-adjuvanted vaccine which showed high hemagglutination antigen-specific antibody response [173]. Furthermore, the double-adjuvanted vaccine also induced higher local IgG and IgA antibody responses in the bronchoalveolar lavage than the single-adjuvanted vaccine.

3.3.2. Gold Nanoparticles

As delivery system, gold nanoparticles offer attractive advantages such as good biocompatibility, easy of production and stabilization. However, little information is known concerning their biodistribution on lung tissues, interaction with immune cells or influence of their functionalization on cell interactions. Therefore, this is a promising research area.

3.3.2.1. Antigen-Loaded Gold Nanoparticles

Seydoux *et al.* prepared gold nanoparticles coated with polyvinyl alcohol (PVA) and functionalized with different surface charges, positive NH_3^+ and negative COO^- [174]. After intranasally instilled in mice, the authors verified that surface charge plays a crucial role in the uptake of gold nanoparticles by antigen presenting cell (APC) subpopulations in different respiratory tract compartments and modulates the downstream immune responses. The cationic gold nanoparticles were preferentially captured and en-

hanced ovalbumin-specific CD4+ T cell (*i.e.* mature T-helper cells) stimulation in lung draining lymph nodes compared to nanoparticles charged negatively, or to polymer administration. The cationic gold nanoparticles presented high effects probably due to the attraction to the negatively-charged cell membrane, favoring their adhesion.

3.3.2.2. Co-Delivery of Antigen and Adjuvant

Tao and Gill prepared a formulation with Matrix-2 membrane protein of influenza A virus (M2e), which presents poor immunogenicity, immobilized on gold nanoparticles and used CpG as adjuvant agent [175]. After intranasal immunization of mice, the authors reported that the presence of free M2e antigen in vaccine formulation plays an important role to stimulate an adequate immune response, inducing high levels of anti-M2e antibody, and to provide a complete and long-lasting protection against lethal influenza A virus challenge. The group of mice immunized with soluble adjuvants alone (soluble M2e and sCpG) - *i.e.* not attached adjuvants to gold nanoparticles - did not induce strong anti-M2e specific antibodies, supporting the role of gold nanoparticles as antigen carrier in the vaccine formulation.

3.3.3. Carbon Nanotubes

Carbon nanotubes are inorganic nanostructures that have also been investigated as inhalable vaccine nanocarriers. Carbon nanotubes are derived from rolled graphene planes and present nanometer-sized diameters with a large specific surface area.

3.3.3.1. Antigen-Loaded Carbon Nanotubes

Nygaard *et al.* studied the potential of carbon nanotubes in promoting allergic immune responses using an intranasal model [176]. Single-walled carbon nanotubes, multi-walled carbon nanotubes and ultrafine carbon black particles together with the allergen ovalbumin were intranasally administered to BALB/cA mice. The authors demonstrated that both carbon nanotubes formulations strongly increased ovalbumin-specific IgE serum titer, the number of eosinophils and the secretion of Th2-associated. However, only multi-walled carbon nanotubes and ultrafine carbon black particles augmented the levels of various immunologic parameters: IgG2a antibody, neutrophil numbers, tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1. The authors suggest that carbon nanotubes are potent adjuvants to promote allergic responses in the airways, even more potent than spherical particles such as ultrafine carbon black particles.

4. CURRENT CLINICAL STATUS OF NOVEL CARRIERS IN INTRANASAL DELIVERY AND FUTURE PERSPECTIVE

Despite most studies related to the intranasal vaccine administration were performed using different animal models such as mice, chickens, guinea pigs and monkeys, which could result in specific immune response, researchers still face a challenge in finding the most suitable model for preclinical studies on humans [17]. Additionally, and even though numerous publications demonstrated the interest of inhalable nanocarriers for intranasal vaccine delivery, further details on their success in humans remain to be fully explored. In fact, the clinical application of inhalable nanocarriers is still in the early stage of development and understanding the relationship among nanocarriers and biological processes such as particle's clearance, cellular targeting, intracellular trafficking and absorption is crucial for the development of effective formulations that guarantee the most advantageous balance of pharmacokinetic and pharmacodynamic and safety profiles. As with other pharmaceutical products containing nanocarriers, the inhalable ones should also face severe regulatory hurdles which could justify the absence of commercially available vaccines.

A small number of nasal vaccine delivery systems are under evaluation in clinical trials. Some examples include a liposomal based intranasal influenza vaccine and a proteosome incorporating heat-labile enterotoxigenic *Escherichia coli* (LTK 63) as adjuvant for influenza [177]. In this study, the vaccines were formulated from highly purified hemagglutinin and neuraminidase from influenza viruses and inactivated with formaldehyde. The authors reported that the main immunization response was related with the route of administration. The intramuscular immunization produced the most important augment in circulation antibodies and the intranasal vaccination induced the largest mucosal immunoglobulin A (IgA) response.

In other study, a phase I, double-blind, randomized, placebo-controlled trial was conducted using healthy HIV non-infected women to investigate the safety, tolerability and immunogenicity of virosomes harboring surface HIV-1 gp41-derived P1 lipidated peptides (MYM-V101) [178]. The authors demonstrated that the virosome formulation was safe and well-tolerated at doses of 10 μg and 50 μg when administered by intramuscular and intranasal routes. Additionally, the virosomes induced systemic and mucosal anti-gp41 antibodies in the majority of subjects with HIV-1 transcytosis inhibition activity turning them into a promising strategy to reduce sexually-transmitted HIV-1.

Silver nanoparticles have also been a subject of study on clinical trials to evaluate their impact on lung cell immune response [179]. Phase I tests were conducted on healthy, non-smoking adults with ages between 18 and 60 years old. The primary outcome determines if inhalation of the nanometer silver containing particles is related to a change of the baseline, in the response of bronchoscopy-derived cultured macrophages, or if epithelial cells to challenge with a toll-like receptor (TLR) agonist. Other analysis evaluates variations in cytokine levels of bronchoalveolar lavage, silver absorption into the blood circulation and excretion in the urine. However, the authors predicted that silver nanoparticles will be responsible for a boost in the immune response.

Based on the high immunological responses in animal models, Betancourt *et al.* performed a phase I double-blinded and randomized clinical trial with a nasal vaccine candidate containing hepatitis B virus (HBV) surface (HBsAg) and core antigens (HBcAg) [180]. The participants included male adults with some exclusion criteria such as lack of serologic markers of immunity or infection to HBV. They were divided into two groups, receiving immunization or placebo (*i.e.* 0.9% saline solution) protocol. Anti-HBs and anti-HBc titers were measured at days 30 and 90, demonstrating anti-HBc seroconversion in all participants who received immunization at day 30, and anti-HBs titer reaching the maximum levels at day 90 on 75% of participants. All subjects of the placebo group were serum-negative during the trial. This phase of the clinical study proved that the immunogenic vaccine was efficient and well tolerated, reporting low intensity and self-limited adverse effects, such as sneezing, rhinorrhea, nasal stuffiness, palate itching, headache and general malaise.

Important concerns related to intranasal vaccine delivery are based on pharmaceutical aspects such as carrier and vaccine reproducibility and stability during pharmaceutical manufacturing processes and storage periods which can induce, for example, loss of immunogenicity. Sharma *et al.* also explored others issues that constitute barriers to the success of the intranasal vaccine delivery for human application [34], lack of an appropriate animal model which is similar to airways human physiology to predict the efficacy, potency and safety of the delivery system, lack of standardized methods for characterization delivery systems, difficulties in controlling inhalable accurate dosing, and the fact that nasal route has direct access to the brain and can induce some allergy and respiratory syndromes.

Therefore, adequate and appropriate regulatory requirements for pharmaceutical, preclinical and clinical safety assessment of vaccine inhalable nanocarriers will required to obtain reliable clinical outcomes. The toxicity of nanocarriers (*i.e.* nanotoxicity), polymers and other excipients is critical for the development of safe inhalable formulations and should be the focus of future research.

5. CONCLUSION

The development of more efficacious vaccines for prophylactic and therapeutic purposes has been one of the healthcare challenges of the last decades. Antigen delivery nanocarriers are gaining attention in the vaccine development, providing interesting improvements such as antigen protection, controlled release and intrinsic adjuvant potential. Additionally, they present an improved safety profile compared to the conventional vaccines. Intranasal vaccine delivery has also attracted the interest of researchers due to the accurate and repeated dispensing of small quantities of formulated vaccine and their deposition to all areas of the nasal mucosa, mainly lymphoid tissues.

Despite intensive research in this area, reflected by the number of patents granted, nowadays, none of the inhalable nanocarriers are in the market for intranasal immunization and only a few formulations are considered in clinical trials. Some reasons for this include regulatory issues, lack of adequate respiratory tract models, lack of studies on humans, stability problems. Additionally, a consistent understanding of the relation between physical particle properties (*e.g.* size, shape and composition) and biological outcomes is a crucial issue in advancing nanocarriers from a laboratory scale into clinical practice. It is crucial to know the specific properties and mechanisms of action of each component (*e.g.* synergistic effects, influence of the antigen localization in the resulting immune response) present in a vaccine carrier.

Future studies need to evaluate the potential of intranasal vaccine in a therapeutic model and identify the optimal dose concentrations, dose intervals and number of doses for generating maximum prophylactic and therapeutic efficacy.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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