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Beatrice Bernocchi

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Université Lille 2 Droit et Santé

Ecole doctorale Biologie-Santé de Lille

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Discipline : Physiologie, Physiopathologie et Biologie systémique médicale

Présentée et soutenue publiquement par

Beatrice BERNOCCHI

Le 18 Juillet 2016

**POROUS MALTODEXTRIN NANOPARTICLES FOR THE INTRANASAL
DELIVERY OF VACCINES**

Composition du jury:

<u>Rapporteurs :</u>	Pr Gillian BARRATT	Institut Galien-Paris Sud, CNRS UMR 8612
	Pr Patrick MIDOUX	Université de Orléans, CNRS UPR 4301
<u>Examineurs :</u>	Pr Pierre DESREUMAUX	Université de Lille2, Inserm - UMR 995
	Pr Philippe CHAVATTE	Université de Lille 2, Inserm - UMR 995
<u>Directeur de Thèse :</u>	Pr Didier BETBEDER	Université de Lille 2, Inserm - UMR 995

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LIST OF PUBLICATIONS

Mechanisms allowing protein delivery in nasal mucosa using NPL nanoparticles,

B. Bernocchi, R. Carpentier, I. Lantier, C. Ducournau, I. Dimier-Poisson, D. Betbeder,

Journal of Controlled Release 232 (2016) 42-50

Nasal Nanovaccines,

B. Bernocchi, D. Betbeder

Submitted to Biomaterials

Endocytosis of Nanoparticles,

B. Bernocchi, R. Carpentier, D. Betbeder

Submitted to Nanoscale

ABBREVIATIONS

(except of publications)

16HBE14o-, human bronchial epithelial cell line

ADP, adenosine diphosphate

APC, antigen presenting cell

API, active pharmaceutical ingredient

B cell, lymphocyte B

BM2, ion channel influenza B virus

BSA, bovine serum albumin

cAMP, cyclic adenosine monophosphate

CD4, cluster of differentiation 4

CD8, cluster of differentiation 8

CM2, ion channel influenza C virus

CME, clathrin mediated endocytosis

CTA1, cholera toxin subunit A1

CTL, cytotoxic T lymphocytes

DAS181, sialidase inhibitor of influenza virus attachment

DC, dendritic cell

DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, C-type lectin receptors

DD, dimer of the D-fragment of the protein A from *Staphylococcus aureus*

DPPG, dipalmitoylphosphatidylglycerol

EMA, European Medicine Agency

FDA, Food and Drug Administration

FITC, fluorescein isothiocyanate

GCN4, transcriptional activator protein

GM1, monosialotetrahexosylganglioside

GRAS, generally recognized as safe

Gs α , G-protein α -subunit

GTMA, glycidyltrimethylammonium chloride

GTP, guanosine triphosphate

HA, hemagglutinin

hAEpC, primary human alveolar type I-like cells

HBc, hepatitis B virus core protein

HBsAg, hepatitis B virus antigen

HLA-DR, human leukocyte antigen, antigen D related

HSP70, heat shock protein 70

IFN- γ , interferon gamma

Ig, immunoglobulin

IIV, inactivated influenza virus

IL-2, interleukin 2

ISCOM, ImmunoStimulating COMplex

LAIV, live attenuated influenza virus

LD50, median lethal dose

L-SIGN, lectin-specific intercellular adhesion molecule-3-grabbing non-integrin, C-type lectin receptors

LTR192G, non-toxic form of the heat labile toxin

M1, matrix protein 1 of influenza virus	PMA: phorbol 12-myristate 13-acetate
M2, matrix protein 2 of influenza virus	QH-A, QH-B, QH-C, fraction of <i>Quil A</i>
M2e, matrix protein 2 extracellular domain of influenza virus	QS-21, purified fraction of <i>Quillaja saponaria</i>
M-cells, microfold or membranous cells	RNA, ribonucleic acid
MDCK, Madin-Darby canine kidney cells	RNPs, ribonucleoproteins
MHC, major histocompatibility complex	RSV, respiratory syncytial virus
NA, neuraminidase	sIgA, secretory immunoglobulin A
NALT, nose-associated lymphoid tissue	SMBV, supramolecular biovectors
NB, ion channel influenza B virus	T cell, lymphocyte T
NP, nucleoprotein of influenza virus	TEER, transepithelial electrical resistance
NP ⁺ , maltodextrin nanoparticles	Th, T helper
NPL, maltodextrin nanoparticles containing a lipid core	THP-1: monocyte cell line
OVA, ovalbumin	TJ, tight junction
PA, Influenza RNA polymerase subunit A	TLR, toll like receptor
PB1, Influenza RNA polymerase subunit B1	TMC, N-trimethyl chitosan
PB2, Influenza RNA polymerase subunit B2	TNF- α , tumor necrosis factor alpha
PLGA, polylactic-co-glycolic acid	WHO, world health organization

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AIM AND OUTLINE OF THE THESIS

Aim of the thesis

The aim of these studies is the evaluation of cationic maltodextrin nanoparticles with a lipid core (NPL) as drug vector. We intend to elucidate the mechanism of nanoparticles interaction with the nasal mucosa and evaluate their application as protein delivery system for a universal influenza vaccine.

The UniVacFlu Consortium

This manuscript describes the thesis work performed under the supervision of Professor Didier Betbeder, head of the laboratory of Nanomedicine. This laboratory is part of the group of Therapeutic Innovation Targeting Inflammation of the INSERM unit LIRIC-UMR 995 of the University of Lille 2.

We evaluated the mechanism implied in NPL protein delivery after intranasal administration. These studies are prerequisite for developing a new adjuvant system for vaccine application.

Hence we used this NPL technology in the project UniVacFlu, financed by the European Union Seventh Framework Programme FP7/2007/2013, part of the People Programme, Marie Skłodowska-Curie Actions. This International Training Network focuses on the development of a mucosal universal Influenza vaccine.

The UniVacFlu project is coordinated by Professor Lycke from the University of Gothenburg. Lycke and co-workers developed the non-toxic mucosal adjuvant CTA1-DD, to circumvent the safety problems of the cholera toxin. Lycke *et al.*, together with Pr. Fiers and Pr. Saelens from the University of Ghent, developed the fusion protein CTA1-3M2e-DD, where M2e is a conserved epitope of influenza virus A. Therefore to optimize the mucosal vaccine efficacy we combined CTA1-3M2e-DD with nanoparticles technology. This vaccine is currently under evaluation for the induction of protective immunity after intranasal administration in the *Consortium*.

We also investigated the oral administration of the nanoparticle vaccine in collaboration with Rescigno *et al.*, from the European Institute of Oncology in Milan. Eventually we addressed the protection from virus transmission with Pr. Staeheli and co-workers, from the University of Freiburg.

The role of our laboratory in the UniVacFlu *Consortium* is (i) to prepare and characterize nanoparticles and vaccine formulations, (ii) to analyze the interaction of these nanoparticles with

the airway mucosa and to evaluate them as delivery systems, (iii) to investigate the vaccine stability and (iiii) to supply the vaccine formulations to the partners.

Outline of the thesis

This document is organized in four principal chapters:

The PART I is a general introduction about the state of the art of the nanotechnology applied to drug delivery. This chapter is arranged in two main sections. The first section focuses on the nanoparticles interaction with cells and their use in vaccinology. A review (submitted) on the literature concerning the study of nanoparticles endocytosis is included. This section contains also a review entitled “Nasal Nanovaccines” (submitted), which discusses the types of nanocarriers studied in literature for the nasal vaccination and controversies regarding these studies. The importance of the knowledge of vector and antigen biodistribution is also introduced. In the second section, the main features of Influenza virus and vaccines are described.

The PART II presents the results on the NPL evaluation for nasal drug delivery. In the first part we present the published article about the mechanism of nanoparticles interaction with the nasal mucosa. In the second part the results about the development of nanoparticle formulation for mucosal influenza vaccine are described. The characterized formulations were sent to our partners of the UniVacFlu *Consortium* for evaluation.

The PART III is a general discussion concerning the obtained results and the application of the nanoparticles in vaccinology.

The PART IV reports the conclusion and the future perspectives of this work.

ABSTRACT

Nanoparticles technology for mucosal delivery of vaccines received a growing interest in the last decades. Intranasal administration owns great advantages for immune system stimulation, such as local and systemic protection against infectious diseases. However delivery systems and adjuvants are often required to efficiently trigger mucosal and systemic immune responses. In this thesis, nanoparticles (NP) have been evaluated as delivery system for a nasal universal influenza vaccine in a People Program of the European Union Seventh Framework Program FP7 called UniVacFlu. The aim of the UniVacFlu network is to develop a universal influenza vaccine administered through the mucosal route. We used porous maltodextrin nanoparticles with a lipidic core (NPL). We loaded an adjuvanted antigen named CTA1-3M2e-DD in the NPL. CTA1-3M2e-DD is composed of the A1 subunit of the cholera toxin and a conserved epitope of influenza A virus (M2e), while DD, dimer of the synthetic analogue of the *Staphylococcus aureus* protein A, targets B cells. Interestingly the antigen loading in NPL was quantitative for the antigen: NPL 1:5 mass ratio and the formulation was stable for at least six months at 4°C. We assessed the successful delivery of the antigen by NPL in airway epithelial cells and macrophages. These formulations are currently evaluated by the UniVacFlu *consortium* in mice. One of the main issues of intranasal vaccines is the toxicity that can be elicited by the nose-brain passage of one of their components. We investigated the loading of antigens in NPL and their delivery in airway mucosa. We observed a high endocytosis of NPL and an increased protein delivery into the cells. On a transwell model of the airway mucosa we assessed the absence of transcytosis and paracellular passage of the NPL. *In vivo* results confirmed the lack of nose-brain passage of the NPL, as NPL were found not to cross the mucosa. Interestingly, we observed an increased nasal residence time of the protein targeted by NPL. The particles after having delivered their payload are totally eliminated through the gastrointestinal tract, making these nanoparticles good candidates for mucosal delivery system. These results highlight the interest of NPL as vectors for mucosal delivery of drugs.

Key words: nanoparticles, intranasal drug delivery, biodistribution, vaccine, influenza

RÉSUMÉ

Au cours des dernières décennies, la technologie des nanoparticules pour la délivrance des vaccins au niveau de muqueuses a reçu un intérêt croissant. L'administration intranasale possède de grands avantages pour la stimulation du système immunitaire, telles que la stimulation d'une immunité protectrice locale et systémique. Cependant des systèmes de délivrance et des adjuvants sont souvent nécessaires pour déclencher efficacement la réponse immunitaire. Nous avons appliqué la technologie des nanoparticules en tant que système de délivrance d'un vaccin universel nasal contre la grippe dans un projet européen FP7 appelé UniVacFlu. Nous avons formulé un antigène adjuvé CTA1-3M2e-DD avec les NPL. Cet antigène est composé de la sous-unité A1 de la toxine du choléra et d'un épitope conservé du virus de la grippe A (M2e), ainsi que du dimère de l'analogue synthétique de la protéine A de *Staphylococcus aureus* (DD). Les nanoparticules utilisées sont poreuses et constituées de maltodextrines réticulées ayant un cœur lipidique (NPL). L'association de cet antigène avec les NPL est quantitative et la formulation est stable pendant au moins six mois à 4°C. Les NPL permettent également de délivrer d'une manière accrue cet antigène dans les cellules épithéliales des voies respiratoires et les macrophages. Actuellement ces formulations sont évaluées chez la souris par le *consortium* UniVacFlu.

L'un des principaux problèmes des vaccins nasal est la toxicité qui peut être provoquée par le passage nez-cerveau de l'un de ses composants. Le but de ce travail est d'évaluer le potentiel des NPL, en tant que vecteurs pour la délivrance des vaccins nasal. Ainsi, nous avons étudié le chargement d'un antigène dans les NPL et sa délivrance dans les cellules épithéliales des voies respiratoires. Notre étude révèle que les NPL interagissent fortement avec les muqueuses et délivrent d'une manière accrue les antigènes dans les cellules. Nous avons également montré l'absence de transcytose et de passage paracellulaire des NPL ou des antigènes délivrés dans un modèle de barrière épithéliale *in vitro*. Les résultats *in vivo* confirment l'absence de passage nez-cerveau des NPL et montrent qu'elles prolongent fortement le temps de résidence nasale des antigènes qui sont ensuite éliminés par le tractus gastro-intestinal.

Ces résultats mettent en évidence l'intérêt des NPL comme vecteurs pour la prochaine génération de médicaments et de vaccins.

Mots-clés : nanoparticules, délivrance nasale de médicaments, biodistribution, vaccin, grippe

RÉSUMÉ DE LA THÈSE

Préambule

Ces travaux ont été effectués sous la direction du Professeur Betbeder au sein de l'équipe de Nanomédecine de l'unité Inserm LIRIC-U995 de l'Université de Lille 2. Nous avons évalué les nanoparticules comme système de délivrance des protéines dans la muqueuse nasale. Ces études sont un prérequis pour le développement d'un nouveau système adjuvant pour application dans le domaine du vaccin, et plus particulièrement pour le développement d'un vaccin universel contre la grippe administré par les voies de muqueuses. Ces travaux sont insérés dans un vaste projet européen nommé UniVacFlu. Le projet UniVacFlu a été financé par l'European Union Seventh Framework Programme FP7/2007/2013. Le *consortium* UniVacFlu est coordonné par Professeur Lycke de l'Université de Gothenburg et il est composé par plusieurs partenaires : l'Université de Ghent, l'Université de Freiburg, l'Institut Européen d'oncologie de Milan et le Kings College de Londres. Notre rôle dans le consortium UniVacFlu est de (i) préparer et caractériser les nanoparticules et les formulations vaccinales, (ii) analyser les interactions des nanoparticules avec la muqueuse nasale, (iii) étudier la stabilité des vaccins développés et (iiii) fournir les formulations à nos collaborateurs.

Introduction

Ces dernières années la technologie des nanoparticules a suscité de plus en plus d'intérêt. Ce sont des objets ayant au moins une dimension inférieure à 100nm (International Organization for Standardization, 2011). Les nanoparticules ont des applications dans plusieurs domaines. Cependant, dans le cadre de cette thèse, nous nous intéressons à l'application de cette technologie innovante pour la délivrance de protéines par voie nasale. Dans la première partie de ce travail nous nous intéresserons aux mécanismes d'interaction des nanoparticules avec la muqueuse nasale, puis dans la deuxième partie nous utiliserons cette stratégie pour le développement d'un vaccin universel contre la grippe.

Les nanoparticules ont plusieurs avantages, comme l'amélioration de la solubilité et de la stabilité des molécules, ainsi que la possibilité de cibler certains organes et tissus une fois administrées. Concernant les vaccins, les nanoparticules peuvent avoir différentes fonctions : adjuvant ou immunomodulateur (Zazo *et al.*, 2016; Zhao *et al.*, 2014). Quand les nanoparticules agissent comme adjuvant, elles améliorent l'immunogénicité de l'antigène par action locale simultanée avec l'antigène même. Néanmoins elles peuvent agir comme immunomodulateur par stimulation directe et systémique du système immunitaire.

Différents types de nanoparticules ont été utilisées pour délivrer des vaccins dans la muqueuse nasale. Nous avons groupé ces types des nanoparticules sur la base des matériels utilisés : polysaccharidiques, polymériques, lipidiques et protéiques (Publication «Nasal Nanovaccine »). Toutefois des systèmes plus complexes, qui mélangent différents matériaux, ont été aussi développés.

Des nanoparticules cationiques poreuses ont été utilisées comme vecteur nasal d'antigènes. Celles-ci sont constituées de maltodextrines, des polysaccharides dérivés de l'hydrolyse de l'amidon. Les maltodextrines sont hydratées et réduites, puis réticulées grâce à l'épichloridrine jusqu'à obtenir un gel. Le gel est ensuite rendu cationique par addition de Glycidyl Tri-Méthyl Ammonium chloride (GTMA) qui fixe les groupes d'ammonium quaternaire sur le polymère. Ce gel est broyé par homogénéisation à haute pression et les particules obtenues (NP^+) sont filtrées pour éliminer les résidus de la synthèse (Castignolles *et al.* 1994). Il est possible d'introduire des phospholipides dans la structure des NP^+ , afin d'obtenir des nanoparticules lipidées (NPL). Dans le cadre de ce travail le dipalmitoyl phosphatidyl glycérol (DPPG) a été introduit dans le cœur des NP^+ .

La voie nasale est très avantageuse pour l'administration des vaccins, car le système lymphatique associé au nez (NALT) est directement accessible. L'interaction des antigènes avec la muqueuse nasale, plus précisément le NALT, peut déclencher une réponse immunitaire locale et systémique, ainsi qu'une protection contre les agents infectieux. Cette voie est facile d'accès et plus confortable pour les patients (aucune aiguille n'est nécessaire). La cible principale des vaccins à délivrance nasale est l'anneau de Waldeyer, qui est composé par l'ensemble des amygdales, situées autour des cavités nasales et buccale (Gizurason, 2012). Le tissu épithélial de la cavité nasale se compose principalement de cellules pseudo-stratifiées. Au niveau de la partie supérieure de la cavité nasale se trouve la région olfactive, constituée par l'épithélium olfactif, qui peut être une voie directe vers le cerveau grâce aux neurones olfactifs insérés dans l'os cribiforme.

Le NALT est un organe lymphatique secondaire, site inductif du système immunitaire muqueux. A ce niveau l'administration d'antigènes déclenche l'initiation de la réponse immunitaire. Le système d'échantillonnage de l'antigène (par exemple, les cellules M) capte l'antigène dans l'épithélium associé aux follicules et le transfère aux cellules présentatrices d'antigène (APC), telles que les cellules dendritiques (DC). Les DC induisent la réponse de cellules T naïves CD4⁺ et CD8⁺.

Les lymphocytes T CD8⁺ mûrissent en cellules T cytotoxiques (CTL). Le rôle des CTL est de tuer les cellules infectées et de lutter contre l'infection virale.

Grâce à la présentation de l'antigène par les DC aux cellules T CD4⁺, les réponses Th1, Th2 et Th17 sont activées ainsi que la commutation des immunoglobulines de classe IgA et l'hypermutation de cellules B dans les centres germinaux. Les cellules B IgA⁺ migrent vers les sites effecteurs à travers les ganglions lymphatiques cervicaux et le sang périphérique. Dans le cas d'antigènes administrés par voie nasale, les sites effecteurs sont la lamina propria des voies respiratoires supérieures, de l'intestin et de l'appareil génital.

Une fois atteint le site effecteur, les cellules B sécrètent des IgA (sIgA). Les sIgA sont transcytosées sur le côté luminal de l'épithélium par le récepteur polymérique d'IgA ce qui permet de bloquer l'entrée d'agents pathogènes (Kiyono *et al.*, 2004; Lamichhane *et al.*, 2014; Lycke, 2012).

Les cellules épithéliales respiratoires sont impliquées dans la régulation de la réponse immunitaire (Pichavant *et al.*, 2003). En effet ces cellules expriment le complexe majeur d'histocompatibilité de classe II (MHCII), spécifique des cellules présentatrices d'antigènes. La présence de MHC II a été observée au niveau du cornet nasal (Kalb *et al.*, 1991). Par conséquent

les cellules épithéliales respiratoires peuvent avoir une fonction de cellules présentatrices d'antigènes et constituer une cible supplémentaire pour les vaccins.

Les vaccins activant des mécanismes en cascade, ceux de transport de la formulation nanoparticulaire doivent être étudiés afin d'éviter les effets secondaires toxiques.

Des virus, comme par exemple le virus d'*Influenza* à l'origine de la grippe, infectent les animaux et les humains par voie aérienne. Le virus de la grippe peut avoir une forme sphérique avec une taille de 100nm de diamètre ou une forme filamenteuse avec une taille majeure, dans l'ordre des micromètres (Rossman *et al.*, 2012). Avec des nanoparticules de propriétés similaires et chargées des antigènes viraux, il serait possible, d'un point de vue immunologique, de mimer la forme sphérique de ce virus.

Le type majoritaire des virus de la grippe est l'*Influenza* A, à l'origine de pandémies. Le virus de la grippe est constitué de plusieurs protéines dont les hémagglutinines (H) et les neuraminidases (N) qui déterminent la classification des virus de type A.

L'association des protéines H et N est très variable et chaque année, de nouvelles combinaisons apparaissent causant les gripes saisonnières. Plusieurs inconvénients aux vaccins actuels existent: (1) Le caractère saisonnier des vaccins empêche une protection contre toutes les souches de virus grippaux de type A. (2) L'injection intramusculaire est relativement efficace mais ne reproduit pas la voie d'entrée naturelle du virus. Les vaccins muqueux présentent de meilleures réponses immunitaires (cellulaires et humorales), plus proches d'une primo-infection. (3) L'utilisation de virus vivant atténué souffre de problèmes de stabilité et peut causer une réversion vers une forme pathogénique. (4) La méthode de production prévoit l'utilisation d'œufs et la formulation vaccinale finale peut être contaminée par l'ovalbumine, un allergène reconnu.

Plusieurs nouveaux vaccins sont en phase de développement. Ceux-ci sont produits par incubation du virus dans des cellules ou par technologie recombinante.

Pour les vaccins muqueux et notamment pour les vaccins recombinants, il est nécessaire d'utiliser des adjuvants afin d'obtenir une réponse immunitaire plus efficace, spécifiquement une réponse cellulaire. Les adjuvants acceptés ne sont pas nombreux mais restent nécessaires surtout pour les vaccins muqueux et recombinants. Parmi les adjuvants les plus puissants, on trouve des toxines bactériennes que nous avons modifiées afin de limiter leur toxicité. La sous-unité A1 de cette toxine a été conjuguée au canal ionique de *Influenza* (M2e), une protéine très conservée du virion et au fragment de la protéine A du *Staphylococcus aureus* (D), qui lie les cellules B

(Agren *et al.*, 1997; Eriksson *et al.*, 2004; Lycke, 2004a). Cette protéine recombinante CTA1-3M2e-DD a été synthétisée pour être utilisée comme vaccin adjuvanté contre la grippe.

But de la thèse

L'objectif de cette thèse est l'évaluation des NPL comme vecteur de médicament. Nous étudierons les mécanismes d'interaction des NPL avec la muqueuse nasale et évaluerons leur application en tant que système de délivrance des protéines pour un vaccin universelle contre la grippe.

Résultats et discussion

Dans la première partie de ce travail nous avons cherché à mieux comprendre les interactions des nanoparticules poreuses (NPL) avec les cellules épithéliales des voies aériennes, leur capacité à traverser cette barrière, et d'analyser l'impact des lipides au sein de ces nanoparticules sur la délivrance et la transcytose d'antigènes dans les cellules épithéliales. Les études *in vivo* ont été réalisées pour suivre la délivrance de l'antigène dans les muqueuses respiratoires et sa biodistribution après administration nasale.

Les nanoparticules NP⁺ possèdent un diamètre de 70nm et un potentiel zeta de +45.9 mV. Des phospholipides, notamment le DPPG, peuvent être inclus dans la structure poreuse de ces particules, donnant les NPL. Les NPL ont une taille de 76 nm et une charge de +44.2 mV.

Il est possible de marquer, avec des marqueurs fluorescents, la partie polysaccharidique du NPL, ainsi que la partie lipidique. Suite au marquage des NPL, nous avons suivi l'endocytose des nanoparticules dans les cellules épithéliales des voies aériennes (16HBE14o-) par cytométrie en flux. Nous avons observé que les NPL sont endocytosées par les cellules épithéliales et ce mécanisme atteint un plateau après 3 h. Constatant la similitude du profil d'endocytose de la partie polysaccharidique et de la partie lipidique, nous suggérons que les lipides ne sont pas délivrés par les nanoparticules pendant l'endocytose mais restent associés à celle-ci.

Ensuite nous avons évalué la transcytose de ce vecteur à travers un modèle *in vitro* d'épithélium (Transwell®). Nous avons montré que les NPL n'ouvrent pas les jonctions serrées entre les cellules épithéliales et ne traversent pas la barrière épithéliale ni par voie paracellulaire ni par transcytose.

Afin d'évaluer la capacité des NPL à délivrer des antigènes, nous avons préparé des formulations en utilisant l'ovalbumine (OVA) comme antigène modèle. La protéine est chargée dans les NPL par simple mélange à température ambiante. Différentes quantités de NPL ont été utilisées pour formuler la protéine. Nous avons observé que dans la formulation, ayant le rapport protéine : NPL 1 :3 (poids :poids), toutes les protéines sont associées et incorporées dans les NPL. Ensuite nous avons étudié la délivrance de la protéine modèle marquée dans les cellules respiratoires 16HBE par cytométrie en flux. L'OVA est efficacement délivrée dans les cellules épithéliales grâce aux NPL (14 fois plus que l'OVA seule après 24h). La transcytose de l'OVA libre ou formulée avec les NPL a été examinée sur le modèle de Transwell®. Nous avons conclu que les NPL ne favorisent pas le passage des protéines à travers la barrière épithéliale *in vitro*. Nous avons aussi étudié la biodistribution de l'OVA après administration nasale dans un modèle animal murin. L'imagerie *in vivo* montre que les NPL prolongent le temps de résidence de

l'antigène dans la muqueuse nasale. Cependant l'OVA libre est dégradée et éliminée totalement après 1.5h, tandis que l'OVA encapsulée dans les NPL reste dans le nez jusqu'à 6h et est ensuite éliminée par le tractus gastro-intestinal. Enfin nous avons vérifié la résidence dans la muqueuse de l'OVA et des NPL. L'endocytose des NPL dans les cellules de l'épithélium nasal a été confirmée *in vivo*, ainsi que l'absence de passage transcellulaire. Nos travaux précédents ont montré que ces NPL peuvent être chargées avec une grande quantité d'antigènes et induire efficacement des réponses immunitaires humorales, cellulaires et de la muqueuse après administration nasale (Dimier-Poisson *et al.*, 2015). Par ailleurs, dans la cadre de ce travail nous avons montré que les NPL ne franchissent pas la barrière nez-cerveau. Ceci supporte les résultats obtenus précédemment (Merhi *et al.*, 2012) et confirment que ces NPL ne sont pas toxiques. Les NPL sont des vecteurs idéaux pour les vaccins car ils délivrent l'antigène dans la muqueuse et sont totalement éliminés.

Dans la deuxième partie de cette thèse nous avons utilisé les NPL pour le développement d'un vaccin universel mucosal contre la grippe. Ces travaux ont été réalisés en collaboration avec les partenaires du projet Européen UniVacFlu. En conséquence nous avons étudié l'association de l'antigène CTA1-3M2e-DD aux NPL. Différents rapports en masse d'antigènes (CTA1-3M2e-DD et OVA) et NPL ont été évalués. L'antigène est complètement associé aux NPL à partir du rapport en masse testé antigène : NPL 1:3. A l'inverse, une fraction des protéines libres a été détectée en électrophorèse natif pour le rapport 1 :0.5 Antigène :NPL. En complément, nous avons étudié la stabilité des formulations 1 :0.5 et 1 :5 à 40°C pendant 3 mois et à 4°C pendant 12 mois, notamment en termes de taille, de potentiel zêta, d'association antigène : NPL et de dégradation de l'antigène. Nous avons observé que la formulation 1 :0.5 n'est pas stable contrairement à la formulation 1 :5 (4°C pendant 12 mois). Par contre, à 40°C, la dégradation partielle de l'antigène seul et associé aux NPL a été observée après 3 mois. Ensuite, nous avons vérifié la délivrance de l'antigène par les NPL dans les cellules épithéliales des voies aériennes (16HBE) et dans les macrophages (THP1). Des résultats similaires à ceux trouvés pour l'OVA ont été obtenus pour CTA1-3M2e-DD. Les NPL augmentent la délivrance du CTA1-3M2e-DD de douze et neuf fois par rapport à l'antigène libre dans les cellules épithéliales et les macrophages respectivement. Enfin nous avons évalué la transcytose des formulations et de l'antigène libre à travers le modèle Transwell® de l'épithélium nasal. Nous n'avons pas observé de passage transcellulaire des formulations ou de l'antigène CTA1-3M2e-DD qui n'ouvrent pas les jonctions serrées. Ces formulations sont en cours d'évaluation pour la stimulation de

l'immunité humorale et cellulaire ainsi que la protection contre un challenge viral dans le modèle murin de transmission.

Conclusion

Les nanoparticules sont des outils prometteurs pour la délivrance de vaccins dans les muqueuses. Elles sont utiles pour la stabilisation des protéines, augmenter la délivrance des médicaments dans les cellules et pour fournir un effet dépôt, qui permet d'éviter les administrations multiples. Nous avons étudié les mécanismes d'interaction des nanoparticules de maltodextrine poreuses et cationiques avec la muqueuse nasale. Les NPL sont des vecteurs idéaux pour l'administration des vaccins, capables d'associer une grande quantité d'antigènes, de les délivrer efficacement dans les cellules et d'être totalement bio-éliminés.

INTRODUCTION

1. Nanoparticles

A nanoparticle is defined as an object that has at least one dimension measuring between 1-100nm (International Organization for Standardization, 2011). The discovery of nanoparticles came along with the development of suitable detection techniques and technological advances. We may attribute the introduction of the nanotechnology concept to Richard Feynmann in 1959, with his remarkable speech “*There’s plenty of room at the bottom*”. However the term nanotechnology was abandoned for about a decade and introduced back in the industry of electronics by Taniguchi in Tokyo (Bassi *et al.*, 2013).

In the last 35 years we have seen a dramatic growth of nanotechnology in multiple fields. Several disciplines such as chemistry, physics, material sciences, electronics, biology and medicine have been affected by the introduction of nanotechnology. In particular in medicine, nanotechnology finds different applications in medical devices, diagnosis and imaging, radiation therapy, theranostic, tissue regeneration and drug delivery. In this thesis we will discuss about nanoparticles used as delivery systems in nanomedicine.

1.1 Nanoparticles as drug delivery system

Nanoparticles are suitable drug delivery as they improve drug stability, counterbalance drug solubility issues and reduce drug toxicity (W. H. De Jong *et al.*, 2008). In fact, the use of nanoparticles does not only enable the administration of poorly soluble drugs, but also the one of nucleic acids and proteins. Bioavailability of nucleic acids and proteins is improved and their degradation is slowed down. At the nanoscale a high surface area to volume ratio is observed, thus favouring the chances of drug absorption. The application of nanoparticles may also modify drug pharmacokinetics and tissue distribution leading to drug targeting. As theorized by Paul Ehrlich, the ideal “magic bullet” is able to transport active molecules to action site with no side effect. Despite the non-“magic bullet” presence, many advances have already been accomplished, especially in cancer therapy (Couvreur *et al.*, 2006). Doxorubicin (Doxil®) is the first drug licensed as liposomal formulation and it is used for AIDS associated with Kaposi’s sarcoma, ovarian cancer and multiple myeloma. Its encapsulation into stealth liposomes prolongs its half-life, enhancing tumor targeting and reducing the drug toxicity. The risk of cardiotoxicity, one of the principal adverse effects of doxorubicin, is reduced by the administration of doxorubicin-loaded liposomes (Kubecek *et al.*, 2015; Xing *et al.*, 2015).

Other nanoparticles formulations currently used are AmBisome®, amphotericin B liposomes used for fungal infections, and Abraxane®, albumin-paclitaxel conjugates for breast cancer (L. Zhang *et al.*, 2008).

However safety is the main concern of nanoparticles technology. Several materials, in particular polymers and lipids, have been used to prepare nanoparticles for drug delivery. The use of biodegradable compounds is always highly suited for pharmaceutical formulations. Hence the possible side effect of the degraded material has to be considered. Moreover the knowledge of the *in vivo* fate of the carrier, e.g. its elimination, degradation or potential accumulation in the body should be investigated.

There are many reasons to deliver drugs into cells. Firstly the drug bioavailability can be improved and the adverse effects reduced. For instance paclitaxel loaded into PLGA nanoparticles increases drug efficiency of two fold (Betbeder *et al.*, 2015; Le Broc-Ryckewaert *et al.*, 2013).

Drugs should reach a specific target localized in a defined cell compartment (e.g. endosome, cytoplasm) to be functional. Hence nanoparticles can direct drug delivery towards the targeted cell area. When cytoplasm delivery is required, pH responsive polymers can be applied. For instance polyethylenimine can induce the osmotic lysis of the endosomes through the “proton sponge effect” allowing cytoplasm drug release. Peptide modified particles have been investigated to achieve nuclear or organelle drug targeting (e.g. neurogenerative diseases) (Parodi *et al.*, 2015).

Nevertheless when the drug has to enter the cell to reach the aimed intracellular target, nanoparticles can assist its entrance into the cell using endocytosis mechanisms. In the review entitled “Endocytosis of nanoparticles” we describe the main mechanisms of endocytosis and we review the literature about the endocytosis of different vectors.

**PUBLICATION 1:
ENDOCYTOSIS OF NANOPARTICLES**

Endocytosis of nanoparticles

B. Bernocchi^{a,b,c}, R. Carpentier^{a,b,c}, D. Betbeder^{a,b,c,d}

^a Inserm, LIRIC—UMR 995, F-59000 Lille, France

^b Université de Lille, LIRIC—UMR 995, F-59000 Lille, France

^c CHRU de Lille, LIRIC—UMR 995, F-59000 Lille, France

^d University of Artois, 62000 Arras, France

Abstract

Endocytosis of nanoparticles is influenced by several factors related to the particle itself, like size, surface charge and shape or to the cell like type, phase and differentiation. The nanoparticles composition also affects the cell uptake. We briefly describe the main mechanisms of endocytosis used by different cells types, hence we review the recent findings on nanoparticles endocytosis. Besides the nanomaterial composition is just one of the parameters affecting the endocytosis, we propose here a qualitative and quantitative analysis of the current literature based on this aspect.

Key words: endocytosis, nanoparticles

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1. Introduction

Endocytosis is a class of highly regulated heterogeneous mechanisms that allows outer material to enter cells. This process is essential for cell's communication with the surrounding environment. Cells have various possibilities to interact with the external milieu, like receptors and ion channels. Unfortunately not all these complexes processes have been completely elucidated yet.

The knowledge of these mechanisms is a considerable advantage when talking about identifying new therapeutic targets and mechanisms dysfunctions (e.g. Alzheimer, Huntington) [1].

Endocytosis is also used to direct drug delivery in a specific compartment of the cell, thus improving drug efficacy. To attain this goal, drugs can be chemically modified or delivered by a suitable targeted carrier. Therefore nanoparticles are used, not only to direct drug delivery into the cell, but also to improve the amount and the kinetics of the drug released, leading to a reduction of the dose administered and limiting toxicity issues. Nanoparticles, as drug delivery systems interact with the endocytic machinery of the cell, mimicking viral pathogens, and may reach subcellular compartment (e.g. the nucleus) to deliver drugs. Additionally the use of nanocarriers can modify the intracellular fate of drugs, leading to its lysosomal degradation rather than its cytosolic delivery.

Interest should be paid also to the nanoparticle fate, once they are endocytosed by the cell. Different scenarios are possible: the carrier may be transcytosed or metabolized. Exocytosis may occur towards the luminal side of the cell, the same side of nanoparticle entry, leading to the carrier elimination [2]. Some nanoparticles may cross the cell barrier, reaching other targets present in the underlying tissue [3]. In this case we talk about transcytosis.

With this review we aim to describe general endocytosis mechanisms and possibilities of nanoparticle interactions with the cellular barrier: nanoparticles alternatives to enter the cell by endocytosis, to exit the cell by exocytosis and to eventually overcome the cell's barrier by transcytosis are reported. Moreover we describe different nanoparticles materials in order to establish general rules for nanoparticles uptake.

2. Endocytosis

Endocytosis is the transport of solid or liquid matter into a cell by means of a vesicle. It is divided into two major categories: phagocytosis (also called cell eating) that involves larger particles and pinocytosis (or cell drinking) that includes solutes and particles of smaller sizes. The phagocytosis is typically operated by specific cells (e.g. macrophages and DCs) while the pinocytosis by almost all eukaryotic cell types [4].

Macropinocytosis is considered as a pinocytosis mechanism [5] since it is operated by all the cells, however *Underhill et al.* defined it as a “triggered phagocytosis” [6].

Nanoparticles are internalized by pinocytosis or following interaction with the cell membrane. This interaction may be non-specific, caused by the charge or hydrophobic interactions, or specific, such as a receptor-mediated binding [7].

Rab GTPases have a pivotal role in the intracellular vesicle trafficking and their multiple roles in the regulation of these mechanisms are reviewed elsewhere [8].

Mechanisms of cellular uptake are typically elucidated by the use of pharmacologic inhibitors. These allows to exclude an endocytosis pathway in favor of another. However particles use often more than one internalization mechanism to enter the cell [9, 10]. This depends on several characteristics of particles and cells: the composition, shape, charge, size, elasticity, porosity of the nanoparticles, the cell's type, the medium composition, the protein corona are just some elements influencing this interaction [11].

2.1 Phagocytosis

Phagocytosis occurs primary in professional phagocytes such as macrophages, neutrophils and DCs for particles of bigger sizes and cell debris. Particles are opsonized predominantly by immunoglobulins (IgG and IgM) and complement proteins to induce phagocytic recognition [12]. Phagocytosis is also enhanced by surface receptors. This interaction induces actin rearrangement and the consequent particle engulfment into phagosome. The phagosome undergoes maturation and it is finally merged to a lysosome to form a phagolysosome and next a late endosome [5, 13]. This pathway is used for the uptake of large particles and bacteria [14]. However, opsonin-independent phagocytosis was observed in alveolar macrophages due to the relatively low presence of opsonins in the airways [11, 15].

2.2 Pinocytosis

This mechanism is subclassified on the basis of the protein or lipid involved. The mechanisms of pinocytosis differ in the composition of the coat of the endocytic vesicle (if present), the size and the fate of the internalized particles [16]. On the other hand a common feature of these mechanisms is the involvement of the actin cytoskeleton, necessary for the vesicles to move in the cytoplasm and reach the targeted cell compartment [4, 16-18].

2.2.1 Clathrin-dependent endocytosis

Clathrin-coated vesicles are the first vesicles identified able to mediate the sorting and the transport of membrane-bound protein [19].

The recycling of activated G-coupled receptors is the most known mechanism of clathrin-mediated endocytosis (e.g. β_2 adrenergic receptor) [20, 21].

Clathrin-mediated endocytosis (CME) may be either receptor-dependent or independent [22].

Clathrin is a protein, firstly identified in 1975 by Pearse, composed by a triskelion. Each leg of the triskelion is formed by a heavy chain and a light chain. Three legs are linked at the c-terminal domain of the light chains by a central hub [23, 24].

Briefly, in case of agonist-receptor binding, when the endocytosis is receptor-dependent, the beta arrestin is bound to the G-coupled receptor. Then the adaptor protein 2 (AP2) bounds the beta arrestin [25](or directly the plasma membrane, for a receptor-independent stimuli [26]). The AP2-beta arrestin complex recruits and assembles a clathrin lattice at the level of the plasma membrane to form a clathrin-coated pit. The clathrin-coated pit is finally detached from the membrane thanks to the action of the dynamin that assembles at the neck of invaginated coated pits causing constriction. Subsequently GTP hydrolysis causes a conformational change necessary to generate the force required for membrane fission and release of the vesicle in the cytoplasm [27]. Afterwards the vesicle evolves into an early endosome thanks to the clathrin depolymerization.

Early endosomes develop into different intracellular paths, depending on the nature of the cargo. The payload of early endosome leads either to degradation or to plasma membrane recycling. Early endosomes may fuse with each other forming mature endosomes that, following further fusion, result into lysosomes. During this process the pH drops from neutral to 6 in early endosomes, then to 5 in late endosomes and lysosomes [16, 28]. In the endosomes, the cargo is sorted to different cellular compartment like lysosomes for degradation, the Golgi network, the nucleus or the plasma membrane for recycling. Retrograde trafficking from early or late endosomes to the trans-Golgi network (TGN) has been also observed as an alternative to the degradation [1, 29].

Cargo recycling to the plasma membrane is mediated by Rab4. This process may be direct and fast from the early endosome to the membrane [1]. Alternatively, the payload may be deviated from the early endosome to the endocytic recycling compartment before ending up to the plasma membrane [30].

2.2.2 Caveolae-dependent endocytosis

Caveolae are flask-shaped invagination of the plasma membrane enriched in cholesterol and glycosphingolipids [31]. Caveolin are a family of membrane protein present in almost all mammalian cells but more abundantly in adipocytes, endothelial cells and fibroblast as well as in

pneumocytes. These proteins, named Caveolin 1, 2 and 3, are differently distributed in tissues where their abundance is variable [32].

Caveolae-dependent endocytosis is generally recognized for its ability to avoid the lysosomal degradation [33-35].

Caveolae, once they engulfed the extracellular material, are detached by the membrane by the action of the dynamin. They then fuse with caveosomes or multivesicular bodies, having a neutral pH, or with the early endosome, in a Rab5-dependent manner [36]. Caveosomes are then transferred to the endoplasmic reticulum (ER) while endosomes are transported to the Golgi. The caveolar unit are then recycled back to the plasma membrane [36, 37].

2.2.3 Clathrin- and caveolae-independent endocytosis

These pathways are included in a heterogeneous class and have been classified in four main categories, considering the effector proteins: RhoA-dependent, Arf6-dependent, flotillin-dependent and CDC42-dependent [4, 18]. Mayor and Pagano gave a noteworthy classification of the clathrin- and caveolae-independent mechanisms. They distinguished these diversified categories in pathways that use a dynamin-mediated scission mechanism (dynamin-dependent) and those that require other processes to separate the vesicle from the plasma membrane (dynamin-independent). RhoA-mediated endocytosis is dynamin-dependent while Arf6-, flotillin- and CDC42-dependent mechanisms instead, are dynamin-independent. All these pathways seem to require specific lipid compositions and are dependent on cholesterol [38]. Studying the pathway responsible for the interleukin-2 receptor internalization, it was shown that the GTPase RhoA35 regulates a clathrin- and caveolae-independent endocytosis [16, 38]. While the cell division cycle 42 (CDC42) has a role in the internalization of glycosphingolipids anchored proteins. This pathway is associated with the formation of tube-like invagination of the plasma membrane that has been observed for the cholera toxin B [4, 39]. The role of the ADP-ribosylation factor 6 (Arf6) and flotillin in endocytosis needs to be further clarified [4]. Arf6 is found in clathrin vesicles and interacts with the dynamin [40]. Flotillin role in endocytosis is controversial. Meister and Tikkanen suggested a flotillin-assisted mechanism, instead of a flotillin-driven mechanism, since the presence of an actual pathway has not been proved yet [41].

When internalization by these pathways occurs, the cargo merges into early endosomes and here it is sorted to the different intracellular compartments [38].

2.2.4 Macropinocytosis

Macropinocytosis is a route for the non-selective endocytosis of solutes macromolecules [16, 17]. This mechanism may be triggered by the transient activation of receptor tyrosine kinases by growth factors but also by virus and bacteria [42, 43]. The activation of receptors induce membrane ruffles that are described as actin-driven membrane protusions, similarly to phagocytosis [35]. Depending on cells type and activation pathway, the ruffles can have different shapes: ruffles can be planar folds (lamellipodia-like), circular cup-shaped extensions (circular ruffles) or large plasma extrusions (blebs) [42, 44]. These protusions fuse with the plasma membrane and form large vesicles called macropinosomes (0.5–10 μm) [17, 22, 42]. Macropinosomes have no coat and do not concentrate receptors [16]. The intracellular fate of macropinosomes depends again on the cell type but often they acidify [38]. They may also fuse with lysosomes or recycle the cargo to the plasma membrane [22, 45].

Macropinocytosis mediates antigen sampling by antigen-presenting cells of the innate immune system: macrophages and activated dendritic cells operate extensive and prolonged macropinocytic activity [17, 43, 46].

3. Exocytosis

Exocytosis is the opposite mechanism of endocytosis, used to actively export molecules outside the cell. These two systems are perfectly balanced in the cell and are employed as intercellular communication tool. During this process the membrane of intracellular organelles fuses with the cellular membrane. Exocytosis can be secretory, when neurotransmitters and proteins use this pathway to be released in the extracellular medium, or non-secretory, when membrane-anchored receptors are transferred on the cell surface [47, 48]. Freshly synthesized proteins in the cell are translocated in the endoplasmic reticulum. Consequently they are transported to the Golgi apparatus in COPII-coated vesicles, to be finally sorted at the TGN. The sorting is necessary to address the vesicle cargo to the right cellular compartment or for secretion. However this mechanism of TGN sorting remains unclear. Proteins can follow different paths as being transported to the lysosomes through a clathrin-mediated pathway, stored into secretory granules or transported outside the cell [47].

Once arrived at the plasma membrane, SNARE (Soluble N-ethylmaleimide sensitive factor protein receptor) complexes form between the vesicle and the plasma membrane. This produces

the membrane fusion and cargo delivery [49]. Moreover the actin activity influences many steps in exocytosis [50].

Neurotransmitters, for example, are released in the synapsis by a calcium-dependent mechanism of exocytosis [51].

Materials that reach the early endosomes can be recycled back to the plasma membrane or delivered to the Golgi. Early endosomes fuse with late endosomes followed by lysosomes but some material may escape to the cytosol. In a typical exocytosis process, the substances are entrapped in lysosomes before being transported to the cell membrane for excretion [52].

4. Transcytosis and paracellular passage

There are two possible pathways to overcome biological (mainly endothelial and epithelial) barriers. The transcellular route involves both endocytosis and exocytosis processes. It enables substances to pass through the cell membrane from one side of a cell to the other. The paracellular passage instead allows molecules to pass through the tight junctions between epithelial or endothelial cells [52]. These processes may allow materials to reach the basolateral side of the barrier, but in other cases (e.g. sIgA) the inverse direction of translocation is followed, i.e. from the basolateral to the luminal axis of epithelial cells [53].

Not only the physicochemical characteristics of the materials may influence their transcytosis but also the characteristics of the barrier. The possibility of molecules transcytosis depends also on the complexity of the barrier encountered by nanoparticles. Importantly it has been observed that transcytosis is also species-specific [54].

5. Endocytosis of nanoparticles

Nanoparticles endocytosis and trafficking is dependent on several characteristics of particles and cells involved in the interaction. Concerning the particle physicochemical characteristics, the size and the charge play a pivotal role but the elasticity and the composition of the nanosized material are also important [55-57]. Moreover the type of cell is relevant, since certain cells (e.g. macrophages) are professional phagocytic cells as they are specialized in engulfment of cell debris.

5.1 Polysaccharide nanoparticles

Polysaccharides are biocompatible materials broadly applied for nanoparticles preparation. In this section we reported the main polysaccharide used in nanoparticles development and their endocytosis mechanisms in different cells types.

5.1.1 Chitosan

Chitosan is a class of co-polymers derived from chitin and it is one of the most used polysaccharide for drug delivery. It is composed by N-acetylglucosamine monomers linked by β -1,4 bonds [58]. Several forms of modified chitosans have been proposed for nanoparticles preparation and targeting.

Many nanocarriers made of chemically modified chitosan and trimethyl chitosan (TMC) are endocytosed mainly by the clathrin-mediated pathway by epithelial cells (e.g. HeLa, Caco-2) [59, 60], glioma cells [61], embryonic and transformed (COS-7) kidney cells [62-64].

Interestingly chitosan oligomer polyplexes (SBTCO) forms positive particles of 76 nm that are endocytosed to a higher extent than linear chitosan (LCO) by HeLa cells; in addition LCO polyplexes are unable to escape lysosomes while SBTCO successfully from endocytic vesicles. Garaiova *et al.* showed that different pathways, clathrin-dependent and independent, are involved in the uptake of these polyplexes [59].

Similarly to other polysaccharides and polymers, chitosans have been further functionalized to obtain receptor-mediated endocytosis. Han L. *et al.* showed that galactosylation of positively charged TMC nanocomplexes increases the receptor-mediated endocytosis up to 2.4 fold in hepatocarcinoma cells after 6 h [65, 66].

Other endocytic receptors that have been exploited for nanoparticles development are megalin and cubilin. These receptors are expressed on the apical membrane of polarized epithelial cells and binds molecules such as transferrin and vitamin B12 [67, 68]. Hence Gao S. *et al.* showed that 200nm chitosan/siRNA nanoparticles accumulates in the kidney and their uptake is megalin-mediated [69].

5.1.2 Maltodextrin

Maltodextrin is a polysaccharide derived from the partial hydrolysis of starch and consists of α -1,4 linked glucose units [70]. Maltodextrin have been used to prepare biocompatible non-toxic

nanoparticles [71]. Interestingly these particles can be modified by adding a lipid inside or outside the polysaccharide network [72, 73].

Porous positively charged maltodextrin nanoparticles are found colocalized with clathrin vesicles but not with ER and Golgi apparatus suggesting a clathrin-dependent endocytosis path in airway epithelial cells [72]. Porous positive maltodextrin nanoparticles are exocytosed by airway epithelial cells (i.e. 16HBE14o- [2]) and do not to cross the airway epithelial barrier [74]. However transport studies of polysaccharide nanoparticles across BBB in an *in vitro* model evidenced that neutral and cationic particles can cross this barrier [75].

Polycaprolactone, a biodegradable, hydrophobic polyester has been used to modify maltodextrin nanoparticles. These negatively charged vectors are internalized by different mechanisms in prostate cancer cell lines. However Korang-Yeboah and co-workers identified multiple mechanism of uptake of these carriers. Although the clathrin-independent pathway is mainly responsible for polycaprolactone maltodextrin particles endocytosis in prostatic LNCaP cells, CME is the main uptake mechanism used by other prostatic cells (PC3 and DU145). Moreover macropinocytosis is involved in the internalization of these carriers, as it is a critical mechanism in cancer cells [76].

5.1.3 Hyaluronic acid

Hyaluronic acid (HA), a naturally occurring polysaccharide composed of N-acetyl-d-glucosamine and d-glucuronic acid, is receiving a lot of attention because it has a strong affinity for cell-specific surface marker CD44, which is overexpressed on the surface of malignant cells [77]. Singh *et al.*, similarly to Zhao and co-workers, prepared hyaluronic acid modified silica nanoparticles and confirmed their CD44 receptor-mediated endocytosis in human colon carcinoma cells. These particles measure 70-80 nm of average diameter and have a surface charge (Z-potential) about -26 mV [78, 79]. In a like manner Mezghrani *et al.* showed the same mechanism of uptake for hyaluronic acid-glycyrrhetic acid conjugates in hepatocellular and breast cancer cells [80]. Receptor-mediated endocytosis was evidenced also by Yang *et al.*. They prepared positively charged hyaluronic acid/chitosan carriers and found that these nanoparticles can enter C6 glioma cells by multiple endocytosis mechanisms [81].

5.1.4 Cellulose

Cellulose is one of the most abundant polysaccharide in nature. It is composed by repeated units of cellobiose. Cellobiose is a disaccharide formed by glucose linked by a β -1,4 bond [82]. Many

chemically modified cellulose have been used as pharmaceutical excipient and are currently applied for nanoparticles preparation. Interestingly Pan-In and co-workers encapsulated *Garcinia mangostana* Linn extract in ethyl and methyl cellulose nanoparticles obtaining a 250nm negatively charged formulation for anticancer purpose. These particles are endocytosed by a clathrin-mediated mechanism by HeLa cells and take an endo-lysosomal pathway [83]. Hoang et al. showed that Cellax, a carboxymethylcellulose-docetaxel conjugate, is also uptaken by murine mammary carcinoma and human leukemic monocyte lymphoma cells through CME [84].

5.1.5 Other polysaccharides

Chondroitin sulfate is a glycosaminoglycan consisting of a protein core modified by tetrasaccharide linkers [85]. Chondroitin sulfate has been combined to other polymers such as chitosan or polyamidoamidine. Hagiwara and co-workers used chondroitin sulfate as coating agent, to ameliorate the transfection efficiency of pDNA/chitosan complex [86]. The negatively charged particles (-38 mV) are internalized by fibroblast through macropinocytosis. Similarly Imamura *et al.* prepared positively charged dendrimers-chondroitin sulfate complexes that improved the transfection efficiency of plasmid DNA in mouse melanoma cells [87].

Chen and co-workers covalently modified the heparosan, the exopolysaccharide of *E. coli*, by linking doxorubicin. These negatively charged conjugated have an average diameter of 140nm and showed to be endocytosed by multiple pathways by HeLa cells; the major pathway was CME followed by micropinocytosis [88].

Folic acid, a vitamin of the B group, has been linked to the nanoparticles surface to stimulate receptor-mediated endocytosis. Lee *et al.* fabricated chitosan-folic acid conjugates to ameliorate doxorubicin uptake by human carcinoma cells [89]. Similarly to CD44, folate receptor is highly expressed on cancer cells. Therefore Su and co-workers conjugated folic acid with carboxymethylcellulose to target folate receptor-positive tumors. These negatively charged carriers are successfully endocytosed by HeLa cells [90].

For cancer theranostic purpose Nagahama et al. conjugated curcumin with dextran, an α -1,6 glucose polymer, and showed high endocytosis of these conjugates in HeLa cells but not in normal fibroblast and epithelial cells [91].

Alginate is a linear polysaccharide containing β -D-mannuronic acid and α -L-guluronic acid. The endocytosis of alginate nanoparticles of different sizes in epithelial cells (Caco-2) was shown to

occur by different mechanisms related to the size: the biggest particles tested (750nm) entered the cell by macropinocytosis [92].

5.2 Polymer nanoparticles

Polymers are broadly investigated for nanoparticles development. The advantage of polymers use is the availability of a broad set of chemical modifications that permits to direct nanoparticles towards a specific cell or receptor type. However the application of synthetic polymers to nanomedicine raised toxicity issues.

We reported below the major classes of polymer nanoparticles remarkable for endocytosis studies.

5.2.1 Polystyrene

Polystyrene is an inert and hydrophobic polymer obtained by styrene polymerization. This polymer is broadly applied in the industry of plastics and it has been used to prepare nanoparticles. Polystyrene nanoparticles have a low polydispersity index and can be surface-functionalized. These particles are therefore ideal to study the effect of the nanoparticle size and surface characteristic on cell internalization [93]. For instance 50 nm polystyrene nanoparticles enters alveolar type I epithelial cells by passive diffusion, whereas 100 nm particles by CME and caveolae-dependent mechanism [94]. Also Firdessa and co-workers studied the effect of the particle size on the uptake. They compared different cells types: bone marrow derived macrophages, kidney epithelial cells and fibroblasts. Surprisingly they found that epithelial cells have relative uptake efficiency for large nanoparticles higher than macrophages. Macrophages take up five times more 20nm nanoparticles than fibroblasts [95].

Fazlollahi *et al.* investigated the effect of surface modification of polystyrene nanoparticles on the transcytosis by mouse alveolar epithelial cells. They concluded that amidine-modified particles cross the epithelium using a clathrin-dependent mechanism, while carboxylate-modified particles used a non-endocytic and a paracellular pathway [54].

5.2.2 PLGA

Poly(lactic co-glycolic) acid (PLGA) is a biodegradable polymer approved by the Food and Drug Administration and the European Medicine Agency. PLGA is used in nanoparticle preparation thanks to its properties of sustained release and the possibility to associate hydrophobic and hydrophilic drugs [96].

Sheng and co-workers showed that clathrin-dependent endocytosis is involved in the uptake of PLGA and N-trimethyl chitosan (TMC)-PLGA nanoparticles by intestinal epithelial cells (Caco-2) [97, 98].

The same mechanism is used by arterial smooth muscle cells [99], vascular and cochlear cells to endocytose PLGA and poly- ϵ -caprolactone-polyethylene glycol-modified PLGA respectively [100].

Recently He and co-workers found that 90nm negatively charged PLGA nanoparticles are endocytosed by MDCK cells by a GM1 caveolae-dependent mechanism [101]. Similarly Wang *et al.* showed that 274nm PLGA are endocytosed by the same cells through a caveolin mediated mechanism [102]. Conversely chitosan-coated PLGA nanoparticles, positively charged, are taken up by MDCK cells through macropinocytosis and CME.

PLGA nanoparticles exocytosis has been observed in vascular smooth muscle cells [103]. Reix *et al.* proposed the transepithelial transit of PLGA nanoparticles mechanism through Caco-2 cells considering two possible pathways the endolysosomal escape that would induce a cytoplasmic delivery of drugs and the exocytosis to the basolateral medium [98, 99, 104]. They hypothesize the nanoparticle transport from the late endosomes to the trans-Golgi network. Nanoparticles can be therefore included in secretory granules and exocytosed [98].

5.2.3 Polyacrylates

Polyacrylate and polymethacrylate are used for copolymers preparation. The characteristics of these polymers may be modulated by the polymerization with a broad variety of chemicals, such as PEG [105], phenylboronic acid [106], polycaprolactone [107], thus modifying their hydrophobicity and solubility. Acrylate derivatives can also be easily grafted to polystyrene particles [108] or used to modify the surface of metal particles [109]. Polymers of (2-(dimethylamino)ethyl methacrylate) (PDMAEMA) are the most used acrylates derivatives thanks to their water solubility and biocompatibility [110]. Nevertheless other monomers are used such as 2-hydroxyethyl methacrylate (HEMA) [105] and 3-(dimethylamino)propyl methacrylate (DMPMA) [111]. These polymers open the possibility to the preparation of a high variety of nanoparticles.

Han *et al.* prepared polyester nanoparticles grafted with mPEG and PDMAEMA for siRNA delivery. They prepared also similar particles using ϵ -caprolactone and lactic acid-modified PDMAEMA. They observed that the lactic acid modification of the polymer reduced the uptake efficiency of the nanocarriers and increased the siRNA delivery into human liver cell (Hep G2).

Caveolae-mediated endocytosis was mainly involved in the uptake of unmodified PDMAEMA; this path was used in a lower extent by lactic acid-modified particles [107].

Huang and co-workers investigated the endocytosis of pluronic F127-modified DMAEMA tert-butyl acrylate and acrylate particles. These positively charged nanoparticles enter human embryonic kidney cells by a clathrin- and caveolin-mediated endocytosis [112]. Amphiphilic glycopolymer poly(2-lactobionamidoethyl methacrylate-random-3-acrylamidophenylboronic acid)- based nanoparticles could modify protein release. These polymeric particles were endocytosed by Calu-3 cells through a CME and a lipid raft/caveolae-mediated endocytosis [106].

5.2.4 Polyethylenimine

Polyethylenimine (PEI) are a class of cationic polymers, linear or branched, used for gene delivery. These synthetic polymers are obtained by the polymerization of the aziridine [113, 114].

Hwang *et al.* investigated PEI/polyamidoamidine polyplexes endocytosis for gene delivery. These polyplexes enter HeLa cells by clathrin- and caveolin-mediated endocytosis simultaneously. Furthermore the inhibition of one pathway leads to the compensatory endocytosis in another path [115].

Similarly to polysaccharide nanoparticles, folic acid may be linked to polymers. Moreover copolymers of polysaccharides and synthetic polymers can be synthesized. Hence Lo *et al.* prepared folic acid-chondroitin sulfate-PEI. These carriers are endocytosed by A549 and U87MG cells through multiple mechanisms CD44-, folate- and caveolae-mediated [116].

5.2.5 Poly - ϵ -caprolactone

Poly- ϵ -caprolactone (PCL) is a synthetic biodegradable polymer widely used for drug delivery and tissue engineering [117].

Suksiriworapong and co-workers showed that PCL-PEG nanoparticles enter breast cancer cells by cholesterol dependent endocytosis [118]. Receptor-mediated endocytosis of lactobionic acid modified PCL nanoparticles was observed in HepG2 cells [119].

Ex vivo methods may also be used to inquire the endocytosis mechanism of nanoparticles. Hence Ravi *et al.* used the rat everted gut sac to study the endocytosis of PCL nanoparticles. Clathrin- and caveolae-mediated mechanisms are involved in the uptake of these carriers in the rat intestine [120].

5.2.6 Other polymers

Several other polymer nanoparticles endocytosis has been investigated. Some of these examples are reported in the table 2.

Interestingly Madlova *et al.* showed that a low % (0.5-1.3% of the particle dose) of PVA nanoparticles translocated Calu-3 monolayer after 14 hours, suggesting that nanoparticles, once sequestered, are retained inside the cell and do not translocate readily [121].

5.3 Lipid nanoparticles

Lipid-based nanoparticles are promising since they are biocompatible drug delivery systems. A broad variety of lipids is available on the market and the most used categories are linear fatty acids (e.g. oleic, stearic, palmitic acid and their derivatives), cholesterol and phospholipids. Several types of nanoparticles can be synthesized starting from lipids, such as liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), lipid nanocapsules, lipoplexes (complexes of DNA and lipids) as well as hybrid polymer/polysaccharide-lipid carriers.

5.3.1 Liposomes

Liposomes are spherical vesicles that can be formed by one or more phospholipid bilayers. Liposomes have two alternative uptake mechanisms: endocytosis or fusion with the cell membrane [122]. Conventional liposomes constituted by phospholipids (DOPC) and cholesterol enter epithelial cells (HeLa and HT-29) by CME. Un *et al.* found that, after endocytosis, DOPC is colocalized with the ER and Golgi apparatus, whereas cholesterol is found only in the Golgi network [123]. Li and co-workers showed that docetaxel-loaded cationic liposomes are endocytosed by a lipid-raft-mediated mechanism by epithelial cells (HepG2 and A345) [124].

Stealth nanoparticles have been prepared by PEGylation of liposomes. He *et al.* found that PEGylated liposomes were internalized by tumor cells through CME, while polycaprolactone modified PEGylated liposomes by a clathrin and caveolin mediated mechanism [125]. Other PEGylated liposomes functionalized with Ephrin A2 specific targeted peptide enters lung cancer cells through caveolae-mediated mechanism [126]. Moreover stealth liposomes can also transcytose epithelial cells monolayers [127].

5.3.2 SLN and NLC

Solid lipid nanoparticles (SLN) are carriers formed by a lipid that is solid at room and body temperature. These particles have been prepared to control and target the drug release as well as

improve the drug stability. However these systems showed some disadvantages related to their stability. Indeed these particles may crystallize and release the drug. Hence nanostructured lipid carriers (NLC) have been developed. NLC are composed by a blend of solid and liquid lipid in order to improve the loading capacity of the carrier and to reduce its release during the storage [128]. The solid lipids most used in the preparation of SLN and NLC are stearic and palmitic acid derivatives.

Clathrin-dependent endocytosis is involved in the uptake SLN by human epithelial cells (A549 and Hela cells) [129]. Solid lipid nanoparticles (SLN) transcytosis has been observed across MDCK cells monolayer. Approximately 2.5% of the total SLN crosses the monolayer and exocytosis from the apical side is also detected. Lysosomes are the main destinations of SLNs, and the inhibition of endosomal acidification increases their transcytosis [130].

Clathrin and caveolae mediated endocytosis of 180nm negatively charged SLN have been identified in Caco-2 cells [10]. Similarly positively charged SLN use the same mechanism than negative ones in rat everted gut sac model [131]. Additionally Chai *et al.* showed that 90nm negatively charged SLN are endocytosed by Caco-2 cells also through micropinocytosis [132].

In a like way NLC are endocytosed by epithelial cells through a clathrin and caveolin mediated mechanism [133-135].

5.3.3 Lipid nanocapsules

Lipid nanocapsules are formed by three principal components: an oily phase, made by tryglicerides, an aqueous phase, made of water and sodium chloride salt, and a non-ionic surfactant, derived from PEG. Soya bean lecithin is used to increase the nanocapsule stability [136].

Paillard *et al.* showed that the internalization of lipid nanocapsules in rat glioma cells is mediated mainly by a clathrin- and caveolae-independent mechanism [137]. The same laboratory previously found that the uptake of lipid nanocapsules is cholesterol dependent [138]. Contrarily Caco-2 cells endocytose lipid nanocapsules through a caveolae-mediated pathway [139]. Moreover Roger and co-workers showed that P-glycoprotein, ATP-binding cassette transporter [140], affects the lipid nanocapsules transport across Caco-2 monolayer [141].

5.3.4 Other lipids

Lipid-polymer hybrid nanoparticles have been developed by combining a wide variety of materials and chemical functions. However each particle investigated shows a specific endocytic pathway [142-145].

5.4 Inorganic nanoparticles

Inorganic materials have several applications in nanomedicine. These nanotechnologies have been applied in molecular imaging [146], photodynamic therapy [147], radiomedicine [148], theranostic [149], gene delivery [150] and as targeted delivery systems (e.g. magnetic nanoparticles) [151]. Hence the understanding of inorganic nanomaterials interaction with the biological substrate is essential.

5.4.1 Silica

Silica nanoparticles are a broad class of nanoparticles based on SiO₂. These carriers are further classified into non-porous and mesoporous silica nanoparticles. Mesoporous silica nanoparticles are characterized by homogeneous pores that can range between 2 and 50 nm [152]. Many mechanisms have been identified in the uptake of silica nanoparticles.

Nowak and co-workers showed that A549 uptake 20nm silica nanoparticles through a caveolae-independent and actin-dependent mechanism [153]. Silica nanoparticles were found co-located with flotillin in endocytic vesicles in epithelial cells; moreover flotillin-depleted epithelial cells showed a decreased uptake of the same nanoparticles [154]. The same authors showed that other particles (SicastarRed and AmOrSil) were incorporated in flotillin labeled vesicles, indicating the involvement of flotillin in trafficking or storage mechanism [155].

Walker *et al.* found that cholera toxin B modified mesoporous silica nanoparticles are endocytosed through a clathrin and caveolin dependent mechanism [156]. Soenen *et al.* showed that also 25 nm non-porous silica particles enter cells (vascular, neural and adrenal gland cells) by using clathrin, but 45 and 75nm particles use macropinocytosis [157]. Similarly silica-based nanoparticles modified with monoclonal antibodies against the $\alpha 2\beta 1$ integrin (130nm) enter osteosarcoma cells through a macropinocytic path [158].

Silica nanoparticles can also be exocytosed. Yanes *et al.* showed that phosphonate silica nanoparticles uses mainly lysosomal exocytosis to exit the cell [159]. In contrast metallic oxide nanoparticles have been found to transcytose the Calu-3 barrier, between 12.1-17.9% for SiO₂-NP after 24 hours [3].

5.4.2 Calcium phosphate

Calcium phosphate is a biocompatible and biodegradable inorganic material that has been applied in nanomedicine, especially in bone tissue engineering. Indeed several forms of calcium phosphate have been studied, such as hydroxyapatite $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ a compound chemically similar to the inorganic component of bones [160, 161].

Bawer and co-workers found that needle-shaped hydroxyapatite nanoparticles are endocytosed by hepatocarcinoma cells through a clathrin-mediated mechanism [162]. The coating of hydroxyapatite nanoparticles with magnetite induces a receptor-mediated uptake into osteoblast [163]. Similarly Kakizawa *et al.* observed receptor-mediated endocytosis of block-copolymer coated calcium phosphate nanoparticles into HeLa cells [164]. Sokolova and co-workers showed that negatively charged calcium phosphate nanoparticles enter HeLa cells through macropinocytosis in higher extent than the positively charged ones [165].

5.4.3 Gold

Gold nanoparticles are a useful model to understand the physicochemical properties that affect nanoparticles biodistribution. Thanks to its photothermal property, gold particles have been also applied to cancer diagnosis and therapy. Gold nanoparticles have a size ranging between 2-100nm and can be easily functionalized. These nanostructures can have spherical or rod-like shapes [166]. Pyshnaya and co-workers found that positively charged gold nanoparticles and nanorods modified with linear polyetylenimine enter melanoma B16, HeLa and kidney fibroblast in the same manner. These particles uptake is caveolin- and lipid raft-mediated [167]. Transferrin-modified gold nanoparticles are endocytosed by epithelial, glioblastoma cells and fibroblast through a clathrin-mediated mechanism thanks to the transferrin. Chithrani *et al.* showed that these carriers are also exocytosed in a size dependent manner: smaller nanoparticles are exocytosed faster than larger one [168].

5.4.4 Magnesium and Aluminium

Magnesium and aluminum are the principal components of double layered hydroxide, a class of anionic clay materials. These compounds have been used to prepare nanoparticles as vectors for drug delivery [169]. Double layered hydroxide have a particle size between 50-300 nm and are endocytosed through a clathrin-mediated mechanism by embryonic kidney cells, embryonic fibroblast, ovarian [169] and osteosarcoma cells [170, 171].

5.4.5 Other metals

Iron oxide nanoparticles are used for their superparamagnetic behavior. Hence they are mainly applied to tumor and central nervous system imaging. The safety of these particles has been discussed, however controversial and insufficient information are nowadays available [172]. Similarly to other particles iron oxide- polystyrene nanoparticles are endocytosed by HeLa cells through a macropinocytosis mechanism [173].

After intra-arterial injection, (anti-PECAM)-modified iron oxide NP were found to target and transcytose *in vitro* and *in vivo* the blood-brain-barrier (BBB) [174].

Silver is also used in nanotechnology. Recently Duran et al. reviewed silver nanoparticles interaction with cells. They discuss the relationship between the formation of protein coronas and their toxicity, however they do not specify the uptake pathway preferred by these particles [175]. For instance Ahlberg *et al.* found that polyvinylpyrrolidone (PVP)-capped silver nanoparticles enter mesenchymal stem cells through CME and macropinocytosis but not through caveolae-dependent mechanism [176].

Gadolinium based nanoparticles are used in theranostic and especially in magnetic resonance imaging (MRI). Wael and co-workers observed that 5 nm gadolinium nanoparticles are internalized by head and neck squamous cells through passive diffusion and macropinocytosis [177].

Quantum dots are small semiconductor nanocrystals having a size between 1-10nm broadly applied for optical imaging. Hild *et al.* reviewed the state-of-the-art concerning quantum dots cellular uptake. Thanks to their uniform and small size, quantum dots are useful for the investigation of nanoparticles endocytosis [178].

6. Discussion

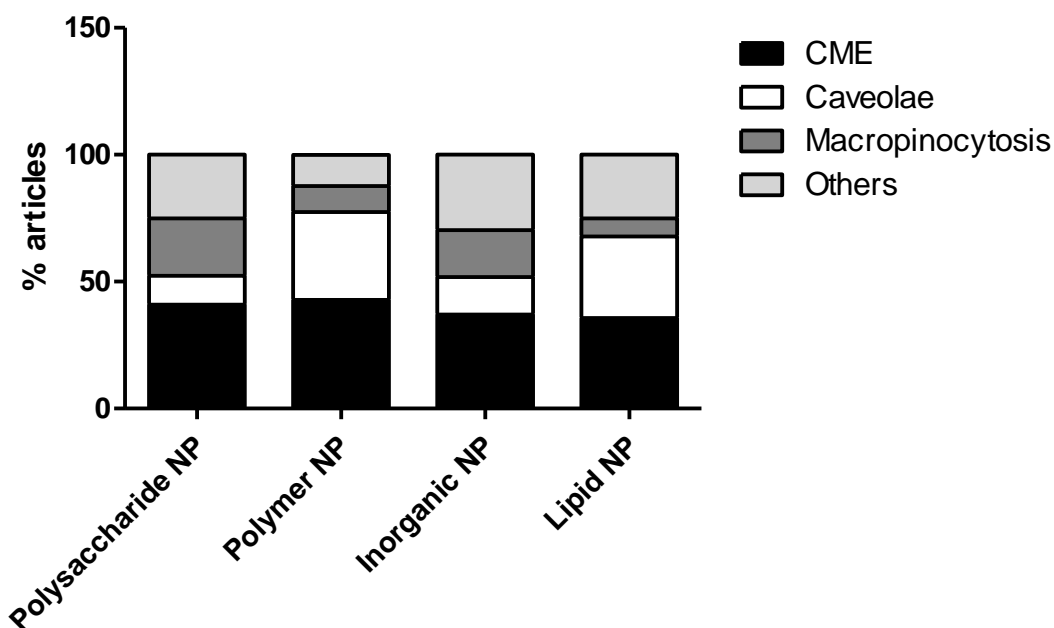


Figure 1 Occurrence of the endocytosis route used by nanoparticles made by different materials, considering the articles cited in this review.

Due to the huge variety of nanomaterials characteristics, paired with the large diversity of cellular models, it is difficult to draw a consistent conclusion that includes all the relevant elements. However some points are here discussed.

Unfortunately a lot of studies are performed without reliable positive controls. For instance transferrin is known to enter cells through the clathrin-pathway but it is not often used as control. Additionally the toxicity of the inhibitors may constitute a serious obstacle for the evaluation of the nanoparticles endocytosis pathway. The poor selectivity of some inhibitors should be taken into account. It has been evidenced that cholesterol is essential during the endocytosis process and its depletion perturbs particles internalization [179, 180]. Molecules that deplete cell's cholesterol are frequently used as inhibitors of caveolae-mediated endocytosis. However attention should be paid using these inhibitors since their caveolae-selectivity is uncertain. Nonetheless experimental artifacts can emerge using pharmacological inhibitors, due to the suppression of an entrance path and potential compensative mechanisms implemented by the cell.

Alternatively knocking down the clathrin- or caveolae-pathway of the cell is a complementary strategy used to understand particles uptake. This approach may be used in parallel with the use of inhibitors [94, 181].

Furthermore the cell phenotype influences the nanoparticles uptake. Endothelial and epithelial cells uptake is predominantly clathrin- and caveolae-mediated [182]. Macrophage polarization also affects the endocytosis. For instance Hoppstädter *et al.* showed that the M2-polarization of primary human monocyte-derived macrophages enhances the uptake of silica nanoparticles compared to M1 [183]. Macropinocytosis seems to be critical for the uptake in cancer cells [76]. Moreover defective endocytosis pathways have been observed in human cancer cells [184]. Kim *et al.* eventually highlights the dependence of the uptake on the cell cycle phase. Hence in G2/M-phase cells uptake more nanoparticles than in phase S, G0/G1 [185].

It is hard to define general rules for nanoparticles endocytosis since these mechanisms are dependent on a large number of factors. Other authors describe the influence of size, surface charge, shape, protein corona and cell division on nanoparticles endocytosis [55, 186].

The presence of numerous physicochemical characteristics of particles, which affect endocytosis, makes difficult to identify the parameter that most influences the uptake. Hence the definition of a hierarchy of parameters that we can modulate to induce a specific uptake of nanomaterials is ambitious and has not yet been achieved. However we propose here a comprehensive analysis of the state-of-the-art of nanoparticles endocytosis based on the material used to prepare nanostructured carriers. Thus we focus on the influence of the material nature on the uptake pathway. Nonetheless considering other physicochemical characteristics (e.g. size, surface charge, chemical modification, etc.) other conclusions may be drawn. It should be taken into account that all these variables interact with each other in the definition of the uptake pathway. Indeed many parameters should be considered during the rational design of nanoparticles and should be evaluated case by case.

Clathrin-mediated pathway is the endocytic mechanism involved in the endocytosis of the majority of nanoparticles. In figure 1 we reported the percentage of articles, cited in this review, and the incidence of the endocytosis path for the different nanoparticles categories. Our analysis shows that more than 40% of the polysaccharide and polymer nanoparticles here reported (40.9% and 42.8% articles respectively) use CME. This pathway is also preferred by inorganic and lipid nanoparticles since 37% and 35.7% of articles describe CME for these particles categories.

Regarding nanoparticles charge it has been noticed that positive polymers (e.g. PLA) and maltodextrin nanoparticles enter cells through a clathrin-mediated pathway preferentially [72, 187]. However also negatively charged chondroitin sulfate and heparosan showed to be endocytosed CME, but also by macropinocytosis [88]. Comparing different polysaccharides we observe that the charge seems not to be relevant in the choice of the endocytosis pathway.

Concerning the particle size smaller inorganic particles seems to enter cells preferentially by CME while larger through macropinocytosis [157]. The endocytosis of alginate nanoparticles was shown to be related to the size: 50-120nm nanoparticles are endocytosed by CME, whereas 420nm and 730nm particles by caveolae-mediated endocytosis and macropinocytosis respectively [92].

Studies show that when the uptake of nanoparticles occurs through a clathrin-mediated mechanism, caveolae-mediated endocytosis or macropinocytosis are also often involved [9, 10]. For instance polycaprolactone liposomes use both clathrin- and caveolin-mediated endocytosis [125].

Caveolae are involved mainly in the endocytosis of polymer and lipid nanoparticles, this path is described by 34.7% and 32.1% of articles respectively. Caveolae-mediated endocytosis is used also by inorganic and polysaccharide particles but in a much lower extent (14.8% and 11.4% of articles).

It seems that the size of the PLGA nanoparticles does not affect the endocytosis in MDCK cells, since 90nm and 274nm nanoparticles use both caveolae [101, 102]. However the coating of PLGA with a positively charged polysaccharide (i.e. chitosan) produced a switch in the uptake pathway to macropinocytosis. Hence the charge and the nature of the material play an important role in this case [102].

Polysaccharide and inorganic nanoparticles prefer macropinocytosis over caveolae-mediated endocytosis, since 22.7% and 18.5% of the analyzed articles report their macropinocytosis. However, only the 10.2% and 7.1% of articles about polymer and lipid nanoparticles respectively describe the macropinocytosis pathway. In case of inorganic nanoparticles (i.e. calcium phosphate) the charge plays a role in the uptake by HeLa cells. Indeed negatively charged particles are more endocytosed than positively charged one [165].

In the category “others” in Figure 1 we grouped all the articles that reported endocytosis mechanisms different from the main ones previously described. In this section the prevalent mechanisms were receptor-mediated endocytosis and lipid rafts.

7. Conclusion

In the last decades a lot of efforts have been done to better understand endocytosis mechanisms and intracellular trafficking. Although many pathways have been elucidated, not all processes have been clarified because of their complexity (e.g. clathrin- and caveolin- independent endocytosis). Much more data are available on clathrin-mediated pathway compared to the others, probably because it is the most known endocytic mechanism.

The introduction of nanoparticles as new technologies for intracellular drug delivery opened a new field of investigation. However it is still unclear how to modulate all the physico-chemical characteristics of nanoparticles to target a specific endocytosis pathway or intracellular organelles. Concerning transcytosis and exocytosis of nanoparticles and the opportunities to improve it, limited information are available. Due to the heterogeneity of nanoparticles composition, it is hard to find a general rule of nanoparticles interaction with the biological interface. Therefore it is necessary to evaluate case-by-case the impact of nanoparticles modification on the cell entry, exocytosis or eventual transcytosis.

Table 1 Polysaccharide nanoparticles endocytosis

Type of particle	Composition	Size (nm)	Z (mV)	Cells	Endocytosis mechanism	Ref
CS and TMC	CS/siRNA	200	-	MDCK	Megalín-dependent	[69]
	CS-ornithine conjugate	100-150	17-21	C6	CME, dynamin	[61]
	Chitosan polyplexes	76.7	17	Hela	Clathrin dependent and independent	[59]
	Glucmannosylated CS	100-200	6.30-12	Raw 264.7, Caco-2	CME, mannose and glucose-receptor mediated	[60]
	Cis-aconitate-modified CS-g-stearic acid	60	30.8	HEK-293	CME, Cav, Macropinocytosis	[63]
	(MTX+MMC)-PEG-CS	215	32.33	Hela	Folic acid receptor-mediated	[188]
	TMC Vit B12	321.4	26.2	Caco-2, HT29	CME, Cav	[189]
	Mannose-modified TMC-cysteine	150	20-30	Rat peritoneal exudate cells	lipid-raft and macropinocytosis	[190]
	Galactose-modified TMC-cysteine	52.4	27.2	QGY-7703	Gal receptor-mediated endocytosis, CME	[65]
	urocanic acid-modified galactosylated TMC	170	25	QGY-7703	Galactose-mediated, CME, endo/lysosomal escape	[66]
	Arginine, cysteine, and histidine modified TMC	120-150	22-31	HEK 293	CME	[62]
	TMC-PEI polyplexes	-	-	COS-7	CME	[64]
	Maltodextrin in	TMC CSK (CSKSSDYQC) peptide modified	198	19	Caco-2/HT29-MTX	CME, Cav, Macropinocytosis
Maltodextrin		63	25	16HBE	CME	[72]
				16HBE	Transcytosis	[74]
				BCEC	Transcytosis	[75]
PCL/maltodextrin	170	-8.3	LNCaP, DU145, PC3	CI, Macropinocytosis, CME, CI, Macropinocytosis	[76]	
Dextran	Dextran	-	-	Hela	CME	
				HUVEC	low cellular uptake	[91]
				HDF	low cellular uptake	
Chondroitin sulfate	ChS -CS	250	-	Caco-2	-	[192]
	ChS -CS	186.3	-38.7	COS7	Macropinocytosis	[86]
	Polyamidoamine Dendrimer-ChS	183.9	28.3	B16-F10	CME, Cav	[87]

	HA	90	-6.7	HCT-116	CD44-mediated, endosomal escape	[193]
	HA-CS	207	25.37	C6	Macropinocytosis, CME, Cav, CD44-mediated	[81]
	HA-coated CS/TPP	317	-52	RAW 264.7	CD44-mediated	[194]
	HA modified Mesoporous Silica	80	-27.9	HC- 116	CD44-mediated	[79]
Hyaluronic acid	HA–glycyrrhetic acid conjugate	190	-21.93	MDA-MB-231	CD44-mediated	[80]
	(SiNp)-curcumin complex HA conjugated	70	-26	Colo-205	CD44-mediated	[78]
	Hyaluronan-doxorubicin and cisplatin conjugates	-	-	MDA-1986	CD44-mediated	[195]
	HA with mono-functional fatty amines	90-1000	-20--8	A549, H69, MDA-MB468, Hep3B, B16F10	CD44-mediated	[196]
Folic acid	Folic acid-grafted CS dextran + succinic anhydride	150	-	KB	Folate receptor-mediated	[89]
	Folic acid Carboxymethyl-β-cyclodextrin	311	-7.53	SMMC-7721, Hela	Folate receptor-mediated	[90]
Cellulose	Ethyl cellulose and methyl cellulose	250	-11.7	Hela	CME, endolysosomal pathway	[83]
	Carboxymethylcellulose	120	-	EMT6, U937	CME	[84]
	Cellulose nanocrystals folic acid-conjugated	-	-	DBTRG-05MG, H4, C6	Folate receptor-mediated	[197]
Others	Heparosan	139.2	-17.4	Hela, A549	CME, Macropinocytosis	[88]
	Gracilaria lemaneiformis Polysaccharide selenium	50	-24	U87MG, C6	αvβ3 integrin-mediated endocytosis	[198]
	Oleoyl alginate ester	50-120	-31	Caco-2	CME	
		420		Caco-2	Cav	[92]
		730		Caco-2	Macropinocytosis	
	Cholesterol modified Pullulan	63	-	HepG2	CME, Macropinocytosis	[199]

Z potential (Z); chitosan (CS); Trimethyl chitosan (TMC); Polyethylenglycol (PEG); polyethilenimine (PEI); Polycaprolactone (PCL); Methotrexate (MTX); Mitomycin C(MMX); Vitamin B12 (Vit B12); Chondroitin sulfate (ChS); Hyaluronic acid (HA); tripoliphosphate (TPP); Clathrin-mediated endocytosis (CME); Caveolin-mediated endocytosis (Cav); Clathrin-independent endocytosis (CI)

Table 2 Polymer nanoparticles endocytosis

Type of particle	Composition	Size (nm)	Z (mV)	Cells	Endocytosis mechanism	Ref
Polystyrene	Polystyrene	50	-	AT1	Passive diffusion	[94]
	Polystyrene	100	-	AT1	CME, Cav	[94]
	Polystyrene	44	-	BOEC, HCF	non-endocytic transcellular flux	[200]
	Polystyrene	293	-13	Calu-3	Transcytosis	[121]
	Polystyrene	100	-	BMDM, 293T, L929	Macropinocytosis, phagocytosis, CME, Cav, CI, CavI	[95]
	Polystyrene	120-603	-	HeLa	Macropinocytosis (excavator shovel like mechanism)	[201]
	Polystyrene	40	-	A549	CME, Cav	[202]
	Polystyrene	40	-	J774A.1	Macropinocytosis, Phagocytosis, CME	[202]
	NH ₂ -labeled polystyrene	60	35	BEAS-2B	Cav	[180]
	PAEA-g-PEG-b-PS	14-24	8-20	MLE 12	CME	[203]
	PAEA-b-PS	14-25	8-21	MLE 12	CI	[204]
	Amidine polystyrene	20-100	-	AEC	CME, Transcytosis	[54]
	Carboxylate polystyrene	20-100	-	AEC	Paracellular passage	[54]
PLGA	PLGA	132	-8.9	Caco-2	CME	[97]
	PLGA	97	-	HAVSM	Exocytosis	[103]
	PLGA	-	-	HBE	Exocytosis	[104]
	PLGA	-	-	Caco-2	Exocytosis	[104]
	PLGA	-	-	renal proximal tube	Exocytosis	[104]
	PLGA	130-180	-5	Caco-2	Exocytosis	[98]
	PLGA	69	-12.5	HASMCs	CME-fluid phase endocytosis+endosomal escape	[99]
	PLGA	80-90	-25	MDCK	Cav, CME	[101]
	PLGA	274	-13.8	MDCK	Cav	[102]
	PCL-PEG/PLGA	133-210	0	HEI-OC1, SVK-1	CME	[100]
	CS-coated PLGA	300	17	MDCK	Macropinocytosis, CME	[102]
	mPEG-PLGA-PLL, (Gal)-mPEG-PLGA-PLL	198.8	-	HepG2	sialic acid receptor-mediated, CME	[205]
		-	-	Huh7	CME	[205]

		-	-	PLC	sialic acid receptor, CME	[205]
	Protamine-coated PLGA	140-173	-	U87MG	Transcytosis adsorptive-mediated	[206]
	TMC PLGA	247.2	45.2	Caco-2	CME	[97]
	Dol-PLA-PEG-FA	89-122	-15--20	MCF-7, CCL-110	Receptor-mediated	[207]
Polyacrylates	PEG-b-PCL-g-PDMAEMA	100-170	10-35	HepG2	Macropinocytosis, CME, Cav	[107]
	HPMA-oligolysine	rods 20/100	-	HeLa, CHO-K1	Cav	[208]
	Pluronic F127 PDMAEMA	80-180	5-20	HEK 293T	CME, Cav	[112]
	p(LAMA-r-AAPBA)	289-353	-25	Calu-3	CME, lipid raft/Cav	[106]
PEI	PEI and PAMAM polyplexes	90-150	-	HeLa	CME, Cav	[115]
	ChS-PEI	-	-	U87, A549	CME, folate, CD44	[116]
PCL	PCL-g-SS-LBA	85-140	-	HepG2	Receptor-mediated	[119]
	PCL PEG	20-235	-4--7	SKBR3	Cholesterol-dependent	[118]
	PCL	195	-19.7	rat everted gut sac	CME, Cav	[120]
	CD-PVM/MA	273.7	-9.82	everted intestinal ring model	CME, Cav	[209]
	PEG-PLA	125.93	-30.87	HUVEC	Cav, lipid raft	[210]
	2-deoxy-D-glucose PEG-co-PTMC	71	-	RG-2	Cav, CME, GLUT-mediated	[211]
	PEGylated polyester	104-118	-37--15	Caco-2	lipid raft, CavI	[212]
	PEGylated PAMAM	200-250	-	C2C12, HepG2	Cav	[213]
Others (PEO, PEG, PVA,...)	lysine-linked ditocopherol PEG 2000 succinate	20.5	0	Caco-2	Cav, CME, transcytosis CI CavI	[214]
	PPE	61	53	HeLa	Cav	[215]
	polyoxyethylene sorbitol oleate	-	-	RBL-2H3	Receptor-mediated endocytosis	[216]
	poly(β-amino ester) polyplexes	200	10-17	MDA-MB 231	Cav, CME	[217]
	graphene oxide PNVCL	438	-	KB	Energy-dependent endocytosis	[218]
	PAMAM cholesterol	80-160	50-60	MCF-7	Cholesterol-dependent endocytosis	[219]
	PVA	183-231	3-19	Calu-3	Transcytosis	[121]
	Alkyl-capped silicon nanocrystals	5	-	Hela, SW1353	Cholesterol-dependent	[220]

Poly (d,l-lactide-co-glycolic) acid(PLGA); Polyethylenglycol (PEG); Polyethilenimine (PEI); Poly- ϵ -caprolactone (PCL); poly(acrylamidoethylamine-graft-poly(ethylene glycol))-block-polystyrene (PAEA-g-PEG-b-PS); 4-O-beta-D-Galactopyranosyl-D-gluconic acid monomethoxy (polyethylene glycol)-poly (D,L-lactide-co-glycolide)-poly (L-lysine) ((Gal)-mPEG-PLGA-PLL); Dodecanol-poly(d,l-lactic acid)-b-poly (ethylene glycol)-folate (Dol-PLA-PEG-FA); poly(dimethylamino ethyl methacrylate) (PDMAEMA); N-(2-hydroxypropyl) methacrylamide (HPMA); glycopolymer poly(2-lactobionamidoethyl methacrylate-random-3-acrylamidophenylboronic acid) (p(LAMA-r-AAPBA); Polyamidoamine (PAMAM); Chondroitin sulfate (ChS); poly(ϵ -caprolactone)-graft-SS-lactobionic acid (PCL-g-SS-LBA); poly(methyl vinyl ether-co-maleic anhydride)-graft-hydroxypropyl- β -cyclodextrin amphiphilic copolymer (CD-PVM/MA); poly(trimethylene carbonate) (PTMC); poly(phenyleneethynylene) (PPE); poly(N-vinylcaprolactam) (PNVCL); Clathrin-mediated endocytosis(CME); Caveolin-mediated endocytosis (Cav); Clathrin-independent endocytosis (CI), Caveolin-independent endocytosis (CavI)

Table 3 Lipid nanoparticles endocytosis

Type of particle	Composition	Size (nm)	Z (mV)	Cells	Endocytosis mechanism	Ref
Liposomes	DOPC, Chol	110	0	HeLa, HT-29	CME	[123]
	PEGylated peptid-modified	157,3	-3.64	A549	Cav	[126]
	DDAB or DOTAP, DPPC or DOPE and Chol	121-195	34-70	HepG2 and A375	Lipid rafts, CavI	[124]
	(anti-EGFR mAbs) hybrid liposomal immunocerasomes	-	-	A431, DU145, HL-60	receptor-mediated endocytosis	[221]
	PEGylated liposomes DOPC and DOPE	125	-16	A549	macropinocytosis, membrane ruffling and blebbing,	[222]
	LPPs	105-121	-2	4T1	CME, Cav	[125]
	LDP2000	97.7-112	-17	4T1	CME	[125]
	PEGylated (PAMAM) dendrimers in liposomes	119	-	Caco-2	Transcytosis, P-gp efflux pump	[127]
SLN and NLC	Glyceryl tribehenate SLN	167	23	rat everted gut sac	CME, Cav	[131]
	SLN, cetyl palmitate,; NLC, cetyl palmitate, miglyol-812	180	-30	Caco-2	CME, Cav	[10]
	SLN stearic acid	250	-25	A549 and HeLa	CME	[129]
	TX-Lf-BCNU-SLNs	100-160	20-37	HBMECs	increased TEER	[223]
	SLN Octadecylamine, Glycerol monostearate	86.7-91.6	-28	Caco-2	Transcytosis, Macropinocytosis, CME, Cav	[132]
	SLN soya lecithin, stearic acid, Tween 80 adenosine modified	76-98	-19--24	MCF-7, DU-145	adenosine receptor-mediated	[224]
	SLN Monostearin and poloxamer 188	88,3	-28.78	MDCK	Cav, CME, Transcytosis	[130]
	SLN Compritol 888 ATO	161	-	Caco-2	CME	[225]
	SLNs stearic acid, salmon calcitonin, peptide ligand	240-409	-27--19	Caco-2/HT29-MTX	CME, Cav	[226]
	NLC	13,4	-9.5	A549	CME	[134]
NLC stearic acid DDAB18	49,2	29,8	RAW264.7	Cav, CavI	[133]	

	NLC chitosan hydrochloride	73-744	-7-+22	HLE	CME	[135]
Lipid nanocapsules	BNC HBsAg	105	-	SKBR3 and HeLa	HER2 receptor-mediated	[227]
	lipid nanocapsules	22-50-102	-7.2--1.9	F98	CI, CavI	[137]
	lipid nanocapsules	51.5-55.6	-	Caco-2	P-gp	[141]
	lipid nanocapsules	26-132	-	Caco-2	Cav	[139]
	lipid nanocapsules	50	-	9L, F98	Chol-dependent, lipid rafts	[138]
Others	FA-BSA-(DOPE)oleic acid	116,1	4,89	MCF-7, HepG2, MDA-MB-231	-	[142]
	PEGylated (DSPC, DSPE-PEG2000, Chol)-mesoporous silica	80-220	-	HT29, SKBR3	HER1 or HER2 receptor-mediated	[143]
	HDL-mimicking peptide phospholipid	18-23	-	CHO	Cav	[144]
	fatty glyceride-CS-enoxaparin	247-274	20	Caco-2	CME, Cav	[145]

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC); Cholesterol (Chol); Didecyldimethylammonium bromide (DDAB); 2-dioleoyl-3-trimethylammoniumpropane (DOTAP); 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); anti-epidermal growth factor receptor monoclonal antibodies (EGFR mAbs); soybean phosphatidylcholine, Chol modified with PEG-b-PCL copolymers (LPP); PEGylated liposomes consisting of DSPE-PEG2000 (LDP2000); Polyamidoamidine (PAMAM); Solid Lipid Nanoparticles (SLN); Nanostructures Lipid Carriers (NLC); tamoxifen lactoferrin Behenic acid (TX-Lf-BCNU); Bio-nanocapsules (BNC); Hepatitis B antigen (HBsAg); Folic acid (FA); 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPE); P-glycoprotein (P-gp); Human epidermal growth factor receptor (HER); transepithelial electrical resistance (TEER); Clathrin-mediated endocytosis(CME); Caveolin-mediated endocytosis (Cav); Clathrin-independent endocytosis (CI), Caveolin-independent endocytosis (CavI)

Table 4 Inorganic nanoparticles endocytosis

Type of particle	Composition	Size (nm)	Z (mV)	Cells	Endocytosis mechanism	Ref
Silica	lactosaminated mesoporous silica	100	-	HepG2, SMMC7721	CME	[228]
	Mesoporous silica arginine-HyA	20-50/50-200	-	CNE1-LMP1	energy-dependent	[229]
	silica	20	-	A549	CavI, actin-dependent	[153]
	CTB modified mesoporous silica	164	-19	HeLa	CME, Cav	[156]
	PLC-PLA, PLC silica	80-100	-27--19	Murine microglial	Macropinocytosis and phagocytosis	[230]
	non-porous silica	25, 45 and 75	-15	HUVEC, C17.2, PC12	CME, Macropinocytosis	[157]
Calcium Phosphate	amorphous CpP/retinol HyA coated magnetite (Fe3O4)	45	-	MC3T3	CME	[231]
	CP	120	-	HeLa	macropinocytosis	[165]
	HyA	100	-	HAP	CME	[162]
	Block copolymer-coated CP	170	-	HeLa	receptor-mediated	[164]
	HyA	-	-	osteoblast	receptor-mediated	[163]
Gold	Gold Nanorods PEI	37-49	53	BHK-21, B16, HeLa	Cav, lipid raft	[167]
	Gold PEI	65-75	34	BHK-21, B16, HeLa	Cav, lipid raft	[167]
	Tf-coated gold	14-50-74-100	-	HeLa, SNB19, STO	CME	[168]
	PEO nanogels inorganic gold np	132	-	HUVECs, hMSCs	CME	[232]
	dendrimer and gold dendrimers	-	-2,3	KB	folic acid receptor-mediated	[233]
	AuNRs (PEG-PCL-LA)	90	-	U87MG	receptor-mediated	[234]
Mg and Al	Mg and Al hydroxyde	100	40	HEK293T, NIH3T3, CHO-K1	CME	[169]
	Mg and Al hydroxyde	127-136	5-20	MNNG/HOS	CME	[170]
	Mg and Al hydroxyde	50-200	18-30	MNNG/HOS	CME	[171]
Others	PEG-folic acid iron oxide	67-74	-	HeLa	receptor mediated	[235]
	iron oxide polystyrene	126	-44	HeLa	Macropinocytosis	[173]
	PVP-capped silver	120	-20	hMSC	CME, Macropinocytosis	[176]
	folic acid selenium	180	-	HepG2	folic acid receptor mediated	[236]
	NaGdF4:Ce/TbCaP	120	-25,7	HEK, MCF-7, MDA, HeLa	receptor-mediated	[237]

graphene quantum dots	3-12	-	MDCK	lipid raft	[238]
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Hydroxyapatite (HyA); Cholera toxin B (CTB) ; Poly- ϵ -caprolactone (PLC); Polylactic acid (PLA); Calcium polyphosphate (CpP); Calcium phosphate (CP); Polyethyleneimine (PEI); Transferrin (Tf); poly(ethylene oxide) (PEO); Magnesium (Mg); Aluminium (Al); Polyethyleneglycol (PEG); Polyvynylpirrolidone (PVP); NaGdF₄:Ce/TbCaP; Clathrin-mediated endocytosis(CME); Caveolin-mediated endocytosis (Cav); Clathrin-independent endocytosis (CI), Caveolin-independent endocytosis (CavI).

Abbreviations (Cells)

16HBE, human bronchial epithelial cells	HAP, near-haploid human cells
4T1 cells, mammary gland tumor	HASMCs, Human arterial smooth muscle cells
9L, rat gliosarcoma cells	HAVSM, human arterial vascular smooth muscle cells
293T, human embryonic kidney	HBMECs, Human Brain Microvascular Endothelial cells
A375, Homo sapiens skin malignant melanoma	HCF, human colon fibroblasts
A549, lung epithelial cells	HCT -16, Cell Line human colon carcinoma
AEC, alveolar epithelial cells	HDF cells, normal human fibroblast cell line
AT1, immortalized alveolar type I epithelial cells	HEK 293 cells, human embryonic kidney cells
B16-F10, mouse melanoma cell line	HEI-OC1, cochlear cells, House Ear Institute-organ of Corti 1
BCEC, Brain capillary endothelial cells	Hela, human cervical carcinoma cells
BEAS-2B, human bronchial epithelium	Hep3B, liver cancer
BHK-21, hamster kidney	HepG2, hepatocellular carcinoma
BMDM, bone marrow-derived macrophage	HL-60, Human promyelocytic leukemia cells
BOEC, bovine oviductal epithelial cells	HLE, human lens epithelial cells
C17.2, murine neural progenitor cells	hMSCs, human mesenchymal stem cells
C6, glioma cell line	Huh7, hepatocyte derived cellular carcinoma cell line
C2C12, mouse myoblast cell line	HT-29MTX, human epithelial colon cells
Caco-2, human epithelial colorectal adenocarcinoma	HUVEC, human umbilical vascular endothelial cells
Calu-3, Homo sapiens lung adenocarcinoma cells	J774A.1, mouse monocytes, macrophages
CCL-110, normal fibroblast cells	KB, Homo sapiens HeLa contaminant Carcinoma
CHO-K1, chinese hamster ovary cells	LNCaP, prostate cancer cell line
CNE1-LMP1, nasopharyngeal carcinoma cell line	MC3T3, osteoblast precursor cell line
colo-205, human colon carcinoma cells	MCF-7, breast cancer cell lines
COS7, transformed African green monkey kidney fibroblasts	MDA-MB-231 cells, Homo sapiens mammary gland/breast
DBTRG-05MG, human brain tumor cells	MDA-MB468, breast cancer
DU145, Prostate cancer cell line	MDA-1986, head and neck squamous cell cancer cell line
EMT6, murine mammary carcinoma	MDCK, Madine Darby Canine Kidney
F98, rat glioblastoma cell	MLE 12, mouse cell line alveolar type II cells
H4, Homo sapiens brain neuroglioma	MNNG/HOS, osteosarcoma cells
H69, small cell lung cancer (SCLC)	NIH3T3, mouse embryo fibroblasts

PC3, Prostate cancer cell line

PC12, rat pheochromocytoma cells

QGY-7703, human hepato-carcinoma cells

RAW 264.7, murine macrophages

RBL-2H3, basophilic leukemia cell line

RG-2, rat glioma cells

SKBR3, breast cancer cells

SMMC-7721, Human hepatocellular carcinoma cell line

SNB19, human glioma cell line

STO, mouse embryonic fibroblasts

SVK-1, Stria vascularis K-1

SW1353, human bone chondrosarcoma, fibroblast-like cell line

U937 cells, Human leukemic monocyte lymphoma cell line

U87MG, Human glioblastoma cells

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1.1.1 Nanoparticles for vaccine and macromolecules delivery

In 1976 Birrenbach and Speiser published the first paper about nanoparticles in vaccinology and introduced the word nanoparts defined as hydrophilic micelles containing drug molecules, used as immunological adjuvants (Birrenbach *et al.*, 1976). In 1981 Kreuter reported the microencapsulation of influenza vaccine in polymethylmethacrylate particles (Kreuter *et al.*, 1981). From the nineties on, we have witnessed a high increase of publications on vaccine administration via nanoparticles (Figure 1), especially in the last two decades. This shows the growing interest of the scientific community in the combination of these two domains: nanoparticles and vaccines.

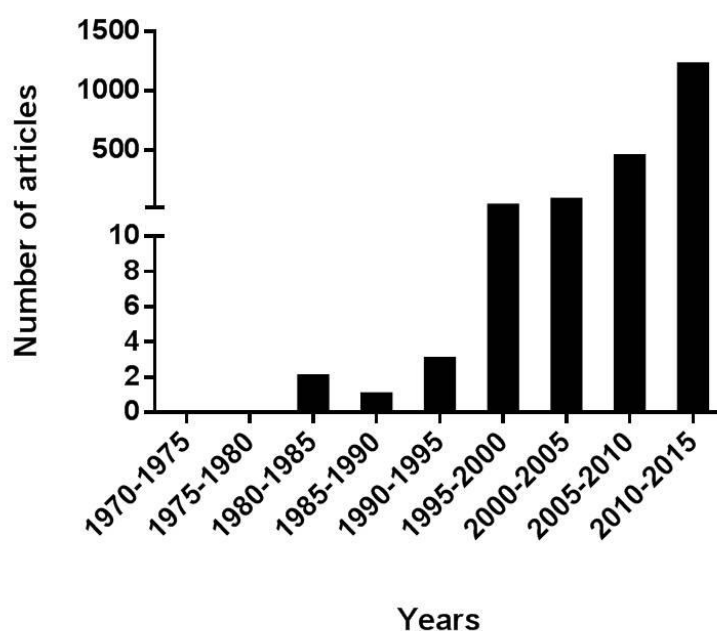


Figure 1 Publications about “vaccine nanoparticle”.

Histogram of summed publications on “vaccine nanoparticles” referenced in PubMed from 1970.

Among nanoparticles, particulate antigens are generally more immunogenic than soluble ones (Lycke, 2012). For instance, nasally administered N-trimethyl chitosan nanoparticles loaded with influenza antigens elicit higher immunogenicity compared to the solution prepared with the same components (Amidi *et al.*, 2007).

The rationale of nanoparticle application in vaccine development comes from the mimicry of the viral pathogens in terms of size and shape. Fife and co-workers observed that the immunogenicity depends on the carrier size and reported that the optimum size is within the viral range (40-50 nm) (Fife *et al.*, 2004).

Nanoparticles are generally efficient in the intracellular delivery improvement of drugs, therefore the administered dose can be reduced. This can also be used for vaccines, especially for the more expensive recombinant ones.

An additional advantage of the nanoparticles use are the protection against antigen degradation and the vaccine stabilization (Zhao *et al.*, 2014).

Nanoparticles as vaccine platform can target specific cells involved in the stimulation of the immune response. A way for nanoparticles to improve their interaction with immune cells is through targeting moieties, linked to the nanoparticle surface (Reddy *et al.*, 2007).

Nanoparticles in vaccinology may have multiple functions: as (i) adjuvant or (ii) immunomodulator (Zazo *et al.*, 2016; Zhao *et al.*, 2014). When nanoparticles behave as adjuvants, they improve the antigen immunogenicity, hence the vaccine potency, by acting locally and simultaneously with the antigen (e.g. depot effect) (Castignolles *et al.*, 1996; Vicente *et al.*, 2014). Most nanoparticles are not immunogenic *per se*, but allow the reduction of the antigen dose therefore acting as adjuvants (Brito *et al.*, 2014). For instance loading of *Toxoplasma gondii* extract in maltodextrin nanoparticles enhance the humoral and Th1/Th17 responses, while the plain nanoparticles do not stimulate the immune response (Dimier-Poisson *et al.*, 2015).

Additionally some nanoparticles are immunomodulators while systemically triggering the immune response (e.g. ISCOMs) (Coulter *et al.*, 2003; European Medicines Agency, 2006; Ilinskaya *et al.*, 2016).

Amongst several routes of vaccination, the mucosal one (e.g. nasal, oral) is advantageous as it potentially induces local and systemic protection against infections. Moreover it is convenient to mimic the natural route of pathogen entry in the body, such as the nose for the influenza virus (e.g. Flumist®) or the oral route for *Vibrio cholera* vaccine (e.g. Dukoral®). It is particularly convenient to induce an immune response as similar as possible to the natural one to promote local protection that blocks pathogen entry in the body.

This work will focus on the nasal administration of nanoparticles for vaccine delivery.

An ideal vaccine formulation administered through the nasal route should keep the antigen stable in the target region (e.g. nose-associated lymphoid tissue, NALT) for an adequate period of time, which is necessary for the antigen to interact with immune cells and provide their activation (Jabbal-Gill, 2010; Zaman *et al.*, 2013). Nasally administered solutions of vaccines remain in the nasal cavity for a period of time (e.g. 15 min) too short for effective antigen uptake (Illum *et al.*, 2001; Soane *et al.*, 1999). For this reason the use of mucoadhesive nanoparticles is advantageous to regulate the antigen nasal residence time. Chitosan, for example, is a mucoadhesive polymer known to reduce the mucociliary clearance and extend the permanence of the formulation in the mucosa (Aspden *et al.*, 1997; Illum *et al.*, 2001).

To achieve the required immunity, optimal antigen release kinetics has to be taken into account. For instance antigens released too slow, or too fast in high doses, may induce tolerance instead of the effective immune response (Woodrow *et al.*, 2012). With regards to nanoparticles carrying antigens and adjuvants, the antigen cross presentation (i.e. exogenous antigen presented on MHC class I) can be reduced by an inadequate timing in the adjuvant delivery. This means that if the adjuvant is released by the nanoparticles too early or too late after the antigen, the cross presentation may be compromised (Wilson *et al.*, 2006; Woodrow *et al.*, 2012).

In the review presented below, entitled “Nasal nanovaccines”, several classes of nanoparticles investigated for nasal vaccination are described and issues concerning experimental research on nanoparticles for vaccine delivery are discussed.

PUBLICATION 2: NASAL NANOVACCINES

Nasal nanovaccines

B. Bernocchi^{a,b,c}, D. Betbeder^{a,b,c,d}

^a Inserm, LIRIC—UMR 995, F-59000 Lille, France

^b Université de Lille, LIRIC—UMR 995, F-59000 Lille, France

^c CHRU de Lille, LIRIC—UMR 995, F-59000 Lille, France

^d University of Artois, 62000 Arras, France

Abstract

Nasal administration of vaccines is convenient for the potential stimulation of mucosal and systemic immune protection. Moreover the easy accessibility of the intranasal route renders it optimal for pandemic vaccination. Nanoparticles have been identified as ideal delivery systems and adjuvants for vaccine application. Heterogeneous protocols have been used for animal studies. This makes complicated the understanding of the formulation influence on the immune response and the comparison of the different nanoparticles approaches developed. Moreover anatomical and immunological differences between rodents and humans provide an additional hurdle in the rational development of nasal nanovaccines. This review will give a comprehensive expertise of the state of the art in nasal nanovaccines in animals and humans. Safety issues are also discussed due to the potential nose-brain passage of nanovaccines components.

Key words: nanoparticles, nasal vaccines, vaccine delivery

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1. Introduction

Several recent studies have focused on the use of nanoparticles for nasal vaccine delivery. Nasal administration is convenient to avoid the parenteral route and increase the patient compliance. Targeting the nose-associated lymphoid tissue (NALT) with nanoparticles and, as a consequence, stimulating the mucosal immune response via the production of a persistent immunological memory, has been investigated and seems to be successful [1-3]. Nevertheless, few such products have reached the market, even if they are safe, easy to produce and cost effective.

Preclinical studies are mostly performed in rodents. However problems concerning translational medicine highlight the limitations of available research, which are primarily anatomical and immunological differences between mice and humans that make it difficult to foresee the clinical efficacy and safety of nanovaccines. Furthermore the protocols used in term of number of vaccination doses, volumes, anesthesia and controls have a strong influence on the immunogenicity of the nanovaccines and it is difficult to compare the different nanosystems developed. Standardization of such experiments is necessary. The aim of this review is to give an overview of the state of the art of the use of nanoparticles for nasal vaccine application in animals and humans.

2. Nasal vaccination: why nanoparticles?

The nasal route is receiving growing interest and some low molecular weight drugs have already been approved and reached the market [4]. Examples of molecules delivered via the nasal route are butorphanol for pain relief (previously Stadol NS®, Bristol Myers, now sold as a generic), gonadotropin-releasing hormone (LHRH, Kryptocur®, Sanofi-Aventis) for cryptorchidism, LHRH agonists used in some fertility treatments (e.g. Buserelin, Supercur®, Sanofi-Aventis), and desmopressin for diabetes insipidus (Minirin® by Ferring or DDAVP® Nasal Spray by Sanofi-Aventis) [4-6].

However for larger molecules, such as proteins, the nasal uptake is very low and it is consequently necessary to develop strategies to improve drugs' absorption [7]. The mass cut-off for permeation of molecules in the nasal epithelium is approximately 1000 Da [4, 8] and absorption enhancers are required to ameliorate the mucosal delivery of larger molecules [9, 10]. Nanoparticles have been identified as successful adjuvants since they act as delivery systems and/or immune-modulators for vaccine applications [11-14]. The main rationale of using nanoparticles to deliver vaccines is their ability to protect antigens against proteolytic degradation and to improve cellular delivery of drugs [15, 16]. Interestingly, via the nasal route, nanoparticles are also able to by-pass the mucus and interact directly with mucosal cells, triggering the immune system [17, 18]. It is also possible to modify the physicochemical properties of particles (such as charge, shape and composition), thus increasing the choice for their use as potential protein carriers [19-21]. Thanks to their size, nanoparticles can also mimic viruses, given that the diameter of viruses is generally below 100 nm [22]. Like viruses, their nanometer size allows nanoparticles to by-pass mucus barrier therefore increasing nanoparticle-cell interaction [23].

Furthermore, nanoparticles may establish a sustained release of the antigen in the mucosa, in order to improve the chances of antigen uptake by the cells.

All these considerations make nanoparticles good candidates for mucosal route delivery systems for proteins. However, nasal administration may favour nose-brain passage of toxins, thus rendering this route of administration potentially highly toxic [24].

3. Nose features for vaccine delivery

3.1 Comparison of mice and humans NALT

It is perhaps instructive to compare key anatomical elements of rodent and human noses in order to understand how the immune system is triggered by this route.

In rodents, the lymphoid tissue is known as nose-associated lymphoid tissue (NALT) and it is concentrated at the bottom of the dorsal nose duct [25]. It is a paired, bell-shaped tissue that is characterized by an accumulation of lymphoid cells and its complete formation is observed around 5-8 weeks after birth [26].

Human adenoids and tonsils are the principal components of NALT and are an important feature of the human mucosal immune system [26]. A ring-shaped formation was recognized in 1884 by Waldeyer, and this structure is nowadays named “Waldeyer’s ring”. It is made of the adenoid, or nasopharyngeal tonsil, the paired tubal tonsils, the paired palatine tonsils and the lingual tonsil [27]. The tonsils are secondary lymphoid organs situated in the lamina propria of the pharyngeal wall. Macroscopically, the tonsillar surface is characterized by various narrow epithelial channels, called crypts, which penetrate deep into the underlying lymphoid tissue. These crypts considerably increase the tonsillar surface area and play an important role in the respiratory immune defense, since they are designed to trap foreign material [2, 28].

The nasal cavity differs both anatomically and histologically between mice and humans. Murine respiratory epithelium consists of a typical single-layer epithelium with columnar epithelial cells in the turbinate portion of the nasal cavity, whereas pseudostratified columnar epithelium covers the olfactory epithelium in mice [29]. In contrast, a single-layer epithelium is not observed in the human nasal cavity, and both the upper respiratory and olfactory surfaces are covered by a pseudostratified columnar epithelium [30, 31]. Notably, tight junction molecules (e.g. occludin, JAM-A, ZO-1, ZO-2, claudin) are expressed in the human upper airway and nasal epithelial cells [32]. These structures make the human nasal epithelium poorly permeable, while anatomical and histological differences, associated to differences in the immunological systems observed between rodents and humans [33], might explain the difficulties observed for translational studies on nasal vaccines [34].

3.2 Mucosal immune system activation by nanoparticles

The mucosal immune system can be anatomically and functionally divided into two main components: the inductive sites and the effector sites. The inductive site is composed of the organized mucosa-associated lymphoid tissues (MALTs) and regional mucosa-draining lymph nodes; here antigen-specific immune responses are initiated. The effector sites, such as the lamina propria, the stroma of exocrine glands and surface epithelia, are involved in antibody production and cell-mediated immune responses [26, 35].

It has been shown that the NALT is a mucosal inductive site for humoral and cellular immune response in the upper airways. After nasal viral infection we assist to germinal centers development, IgA⁺ and IgG2a⁺ B cell expansion and cytotoxic T cells (CTL) generation [36].

In the NALT all the immunocompetent cells required for the generation of an immune response are present. Indeed, antigen presenting cells (APC) like dendritic cells and macrophages are found there, as well as T and B cells, but also antigen-sampling M cells [26]. These inductive sites are connected through the common mucosal immune system to effector sites for the generation of antigen-specific, Th2-cell-dependent IgA responses, Th1-cell and cytotoxic T lymphocyte-dependent immune responses, which function as the first line of defense at mucosal surfaces [37, 38].

The presence of M cells has also been identified in the NALT [39, 40]. Their role is to perform a ‘sampling’ of luminal antigens so that cells of the immune system can come into contact with potential pathogens. The M cells thus combine two important functions: maintenance of the barrier and initiation of mucosal immune responses [40]. Their presence has been reported in the adenoidal epithelium [32], and it is thus widely accepted that NALT M cells are key players in the uptake of nanoparticles for the subsequent induction of antigen specific IgA immune responses [26].

In certain areas, beneath the epithelium, there are resident dendritic cells (DC) that express tight junction molecules, which in the gut allow them to sample the antigen, penetrating the epithelial monolayer as described *in vitro* by Rescigno *et al.* [41]. The mechanism of antigen sampling carried out by transepithelial dendrites of DC has also been observed in mouse NALT and in human adenoidal epithelium [32, 42, 43]. Thus, DC and macrophages might play an important role in nasal nanovaccines uptake [44].

The first barrier that nanoparticles face once nasally instilled is the mucus and epithelial cells. Once nanoparticles have crossed the mucus layer they come into contact with epithelial cells.

Epithelial cells do not only form a simple barrier to xenobiotics, they are also involved in innate immunity since they can orientate the immune response by cytokine secretion [45, 46] and have a role as APC [47]. Indeed, these cells express MHC class II, in addition to the MHC class I that is expressed by almost all nucleated cells. The presence of MHC class II, a feature typical of APC, has been reported at the level of nasal turbinates [48]. This observation suggests that epithelial cells could be implied in the uptake of nanovaccines, antigen presentation and immune response activation [49, 50].

The intranasal administration of antigens may stimulate the formation of germinal centers in NALT, leading to the clonal expansion of B cells and generation of antigen specific IgA in the respiratory tract that induces antigen specific immunity. However the nasal deposition of antigens has also been shown to be effective for the induction of systemic unresponsiveness - a form of mucosal induced tolerance [36, 51].

Different subsets of APC can induce an adaptive immune response locally by presenting the antigen to lymphocytes via the MHC. The T helper lymphocytes may also be directly primed by antigens in the NALT or draining lymphatics to stimulate an adaptive immune response [2]. Immature DCs migrate from the NALT to draining lymph nodes and stimulate a T helper or CTL response after maturation [52, 53]. Activated T cells migrate to the effector sites: T helper cells activate macrophages, NK cells and eosinophils, while cytotoxic T cells eventually lyse infected cells. Moreover, DCs activate B cells and induce surface IgA expression in activated plasma B cells [53]. At the level of the effector sites (upper airways and gut mucosa), polymeric IgA bind to the polymeric Ig receptor (pIgR). Dimeric IgA are exocytosed at the epithelial level and becomes secretory IgA (sIgA) [54]. The sIgA promote the clearance of antigens and pathogenic microorganisms by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by mucociliary clearance [55]. Thus, sIgA is a characteristic feature of mucosal immunity. However more research is needed to fully understand the cell mechanism(s) that are stimulated by nasal nanovaccines.

3.3 Immunological aspects of nano-antigen delivery

Once antigens loaded into nanoparticles are endocytosed by APC, many intracellular mechanisms can be activated. Depending on the pathway taken and antigen intracellular delivery, the epitopes of processed antigens can be presented as either major histocompatibility

complex (MHC) class I or MHC class II. Interestingly, after endocytosis nanoparticles may deliver the antigen to elicit MHC class I or class II presentation [56, 57].

Generally nanoparticulate formulations are endocytosed by cells and the intra-cellular traffic follows the endo-lysosomal pathway before the protein is delivered and degraded in the endosomes. Degraded peptides are associated with MHC class II and are presented on cell surface, where they can activate CD4⁺ T helper cells, therefore stimulating cytokine secretion and antibody responses.

However, nanoparticles may promote the endosomal escape of the protein. In this case the cytosolic delivery of the antigenic protein is possible and the antigen may undergo proteasome degradation. Peptides degraded by the proteasome are transported to the endoplasmic reticulum by transporters of antigen processing (TAP) and associate MHC class I. Cellular expression of peptide-associated MHC class I activates CD8⁺ T cells, and hence cell-mediated immunity [58-62].

Ideal vaccines should be able to activate both of these pathways, thereby inducing cross-presentation [59]. However, subunit vaccines are not effective in cytotoxic T cells activation and in this case vaccines are administered with adjuvants [63]. Interestingly the antigen encapsulation in nanoparticles may direct the antigen presentation towards a different or combined immune response. This orientation can be affected by multiple factors, such as the mechanism of uptake, and is dependent upon the nanoparticles physico-chemistry, such as the size and the surface charge.

Antigen endosomal escape has been observed for protein loaded in maltodextrin porous nanoparticles [64]. Polymeric nanoparticles made of propyl-acrylic acid have also been shown to promote cytosolic delivery of the antigen and MHC I presentation [22]. The release of lactic or glycolic acid from PLGA erosion may have a synergistic action on the endosomal acidification, improving the antigen degradation and presentation through MHC II [65, 66].

Antigen transcytosis through the nasal mucosa may also be advantageous. Indeed, some nanoparticles might promote the transcytosis of antigens or may be directly transcytosed through the mucosal barrier. It has been reported that the passage of immunogenic peptides across the epithelial barrier through M cells can stimulate the underlying immune cells [67].

4. Nanoparticles for nasal vaccine delivery

Different types of nanoparticles have been prepared to develop nasal vaccines. They can be grouped into four major categories: polysaccharides, polymers, lipids and protein nanoparticles. Complex systems, that mix different components, have also been developed (e.g. polysaccharide and lipid, synthetic polymer and polysaccharide,...) [3, 68].

It is complex to give a clear overview of the literature on nanovaccines. A lack of harmonization with respect to the immunization protocols (such as volume, dose, and concentration of the antigen, number of administrations, with or without anesthesia) are variously employed (Table). Furthermore, different experimental set-ups are used by different laboratories. All these parameters have the potential to greatly influence the immune response, and in addition many studies have used adjuvants, therefore it is impossible to clearly define the mechanisms that elicit a given immune response. Below, we report the main types of nanoparticles used and describe relevant studies concerning their potential as vaccine carriers and adjuvants.

4.1 Polysaccharide nanoparticles

Thanks to their biocompatibility, polysaccharides, such as chitosan and starch, have been widely used to prepare nanoparticulate delivery systems [69].

4.1.1 Chitosan nanoparticles

Chitosan is a co-polymer of glucosamine and *N*-acetylglucosamine, derived by the partial deacetylation of chitin, which is abundant in shellfish [70]. This mucoadhesive polysaccharide is soluble at acidic pH and has been applied as an absorption enhancer for small drugs [71]. Chitosan's degree of deacetylation and molecular weight influence the physicochemical characteristics of the polymer [72]. It is used as a vaccine adjuvant and it seems that its efficacy is dependent on its degree of deacetylation [73]. However in most of the published studies, the molecular weight, degree of deacetylation and purity of the polymer are not described [74]. Chitosan nanoparticles are mucoadhesive and thus prevent rapid nasal clearance, thereby improving the residence time of antigens in the nasal mucosa [71]. As mucociliary clearance is reduced this may extend the contact time between the NALT and the formulation [75, 76]. Soane *et al.* showed that the typical nasal residence time of administered solutions (15-20 min) can be quadrupled thanks to the application of mucoadhesive chitosan particles [77].

The adjuvant properties of chitosan nanoparticles are successful with various protein antigens such as recombinant anthrax [78, 79], hepatitis B (HBsAg) [80], influenza [81] and ovalbumin [82], but also with nucleotides like DNA [83].

Improved mucosal (IgA) and humoral (IgG) responses are generally observed in mice [78-81, 84] as well as in other animal models.

Chitosan nanoparticles loaded with tetanus toxoid are internalized by the rat nasal mucosa and trigger IgG and IgA production [85].

Dehghan *et al.* showed that dried chitosan nanospheres, carrying influenza whole virus, elicited strong humoral and cellular responses in rabbit [86].

Chitosan has also been used to prepare nanovaccine in one of its modified forms: N-trimethylchitosan (TMC) [87, 88]. Tafaghodi *et al.* showed that hepatitis B antigen formulated in TMC or chitosan nanoparticles elicited higher serum and nasal antibody titers after two intranasal immunizations in mice [89]. Recently, the ability of cationic chitosan to enhance Th1 and Th17 responses as well as DC maturation through type I interferon induction has been demonstrated [90, 91].

4.1.2 Association chitosan-polymers

Chitosan association with other polymers (e.g. alginate, poly-(ϵ -caprolactone)) gave either modest [82] or high [92] mucosal responses. Verheul and co-workers prepared TMC-hyaluronic acid nanoparticles and investigated the effect of PEGylation on nasal and intradermal vaccination. These nanoparticles elicited antigen-specific IgG titers but PEGylation cancelled this potential benefit of the nanoparticulate formulation [87].

Poly-(ϵ -caprolactone)-chitosan nanoparticles were used to improve HBsAg intranasal vaccination. Jesus S. *et al.* adsorbed different antigen amounts onto a fixed quantity of these nanoparticles and showed that the different doses elicited identical humoral and mucosal antibodies in mice nasal secretion [93].

Clinical trials

Chitosan application for nasal vaccination in the form of nanoparticles [94] or antigen-conjugates [95] has already reached clinical trials (phase I and II). In one study Norovirus virus-like-particles with chitosan as an adjuvant were intranasally administered twice to healthy volunteers, inducing specific IgA responses in 70% of vaccinated individuals. The vaccination

also reduced Norwalk virus symptoms and infection [94]. Huo *et al.* performed a clinical study mixing *Neisseria meningitidis* serogroup C polysaccharide (MCP) with (CRM)197, a non-toxic mutant of diphtheria toxin, and with chitosan. This preparation induced specific IgA in nasal washes and balanced IgG1/IgG2 responses following two intranasal vaccinations [95]. In a previous study, the same research group showed the ability of this formulation to elicit Th2 responses, studying (CRM)197-chitosan in combination with diphtheria toxoid [96]. Although these clinical studies gave promising results, no chitosan-based product for intranasal vaccination has yet reached the market.

4.1.3 Starch nanoparticles

Starch is a polysaccharide composed of amylose and amylopectin. This carbohydrate is abundantly found in plant amyloplasts, where it works as energy reserve [97]. Maltodextrins obtained by partial hydrolysis of starch are also used for the synthesis of nanoparticles [98, 99]. These biodegradable polysaccharides are widely used for nanovaccine applications.

Coucke *et al.* encapsulated influenza virus antigens within bioadhesive starch and propylacrylic acid mixtures. They reported that systemic antigen-specific IgG responses, but not mucosal IgA, were induced after intranasal delivery of the influenza vaccine in rabbit [100].

Positively charged maltodextrin nanoparticles surrounded by lipids are promising mucoadhesive polysaccharidic nanoparticles for intranasal vaccination. They were loaded with hepatitis B antigens and triggered greater cellular, humoral and mucosal immune responses than the free antigen [101].

These nanoparticles, loaded with inactivated influenza antigens, have been tested in a phase I clinical study. Significant mucosal IgA antibodies were produced in individuals who received two doses of the nasal influenza vaccine [102].

Dimier-Poisson and co-workers showed that lipid-maltodextrin nanoparticles can be loaded with high amounts of heterogeneous antigens, i.e. *Toxoplasma gondii* extract. These nanoparticles induced a strong humoral and cellular response, as well as a robust protection against acute and chronic disease in mice [3]. The mechanisms implied in this adjuvant effect are related to a TH1 and TH17 response, probably associated with an improved nasal residence of the antigen in the nasal mucosa [103].

4.2 Polymer nanoparticles

A co-polymer of lactic and glycolic acid, PLGA, is the synthetic polymer most employed for nanoparticle delivered vaccine development thanks to its biodegradability and biocompatibility [104]. Polylactide acid (PLA) was used in nanoparticle formulations to delay the delivery rate of low molecular weight drugs [105]. The PLA characteristics were then modulated by adding glycolic acid, hence developing PLGA. A wide variety of PLGA polymers is available on the market. These are made of different mole ratios of monomers (lactic and glycolic acid) and present either an ester or an acidic terminal group, which affect the hydrophobicity of the polymer. As a result of the variation of the ratio of the two acids in the co-polymer, the biodegradation rate is modified; an increase in the amount of lactic acid in the copolymer reducing the degradation rate [106].

PLGA nanoparticles may either encapsulate antigens in their matrix or adsorb proteins on their surface [22, 107, 108]. Encapsulating antigens in PLGA particles modulates the pharmacokinetics and allows sustained and controlled release of proteins [109]. Moreover, PEG coating of PLGA nanoparticles may favor the antigen passage across the mucosa [110, 111]. While PLGA nanoparticles are typically negatively charged, they can be made positively charged by adding cationic ligands (e.g. chitosan) [68].

PLGA nanoparticles can also be functionalized to enhance their permeability across the nasal mucosa. With this aim, Sundaram *et al.* developed transferrin-conjugated PLGA nanoparticles for gene-delivery across the nasal respiratory epithelium [112].

Primard and co-workers loaded PLGA nanoparticles with bovine serum albumin and a TLR7 agonist as adjuvant and this formulation induced effective mucosal and systemic humoral responses in mice [113].

PLGA have been combined with chitosan or glycol chitosan to encapsulate hepatitis B antigens and the greatest systemic and mucosal immune responses were observed with glycol chitosan PLGA. The authors proposed that this effect was related to its lower clearance and better uptake, compared to chitosan- or uncoated PLGA [114]. Particles made of PLGA have also received significant interest for veterinary vaccine development and other veterinary applications. Brandhonneur *et al.* showed that rE2 glycoprotein antigens loaded in PLGA microspheres induce a more intense and less variable response when administered nasally than orally in rabbit [115].

Kavanagh and co-workers nasally immunized calves with OVA PLGA in the presence of adjuvants (e.g. monophosphoril lipid A, cholera toxin), inducing specific IgA production [116, 117]. Greater IgA and IgG responses were observed in calves when Bovine parainfluenza 3 virus antigens encapsulated in nanoparticles. Cattle were vaccinated against foot-and-mouth disease with either chitosan-PLGA-DNA vaccine or chitosan-trehalose inactivated virus and the PLGA vaccine elicited higher levels of mucosal, systemic and cell-mediated immunity than the inactivated virus [68].

PLGA is not the only polymer employed for nasal nanovaccine development. Other studies have developed polymeric micelles made of amphiphilic polymers able to assemble antigens and micelles composed by antigen-grafted polymers. For example Noh *et al.* covalently bound poly(γ -glutamic acid) to cholesterol, aiming to deliver influenza antigens. The intranasal administration of these micelles produced high specific serum IgG titers and IgA in mice [17]. Recently, Li and co-workers showed the potential of intranasal vaccination with cyclodextrin-polyethylenimine 2k conjugate mRNA vaccine against HIV-1: the mRNA complex formulation elicited strong systemic, humoral and mucosal immune responses [118].

While PLGA-based vaccines have not yet reached the market, but have already been evaluated in clinical trials for example for the prevention of nicotine addiction and relapse [119]. So far, most of the studies on polymeric nanoparticles are still limited to the preclinical development.

4.3 Lipid nanoparticles

4.3.1 Liposomes

A liposome is a spherical vesicle with a liquid core surrounded by at least one phospholipid bilayer. By varying the lipid composition and the preparation method, different liposomes can be obtained (e.g. multilamellar vesicles (MLV), large unilamellar vesicles (LUV), small unilamellar vesicles (SUV)), of various sizes (20nm – 3 μ m). However their poor storage stability constitutes a considerable limitation for their application in vaccine delivery [120].

De Haan and co-workers first reported that free liposomes composed of phosphatidylcholine, cholesterol and dicetylphosphate had an adjuvant effect. This indicated that the immunomodulatory activity of these particles was inherent and unrelated to the vaccines they contained [121].

Tai and co-workers loaded liposomes with highly-conserved influenza-derived peptides, with monophosphoryl lipid A and trehalose-6,6'-dimycolate as an adjuvant. This formulation elicited potent innate and selective T cell-based adaptive immune responses, and induced protection against lethal challenge in mice [122]. Similarly, Ninomiya *et al.* have demonstrated that multilamellar vesicles (MLV) liposomes loaded with CD-40 antibody and influenza nucleoprotein peptide also trigger T-cell immune responses [123].

Most of the liposomes studied are negatively charged. However, positively charged nanoparticles interact better with the nasal mucosa and cationic liposomes have been developed as adjuvants for mucosal vaccines. Immunomodulatory lipids such as polycationic sphingolipids or cationic cholesterol derivatives have also been used to prepare liposomes that exhibit adjuvant activity upon mucosal delivery [22, 124, 125].

Recently, Tada and co-workers prepared 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl] (DC-chol) (DOTAP/DC-chol) liposomes as adjuvants for intranasal vaccination. The OVA DOTAP/DC-chol liposomes induced a significantly higher humoral and mucosal immune response compared to the free antigen in mice [126].

Liposomes have also been modified by chitosan-DNA complexes in order to efficiently trigger mucosal IgA production in a mouse model [127].

Proteoliposomes are liposomes carrying proteins, and they have been studied for influenza virus delivery through the intranasal route. These nanoparticles induced systemic and mucosal immune responses in mice as well as protection against virus challenge [128].

Liposomes were one of the first particles vaccine delivery system to reach clinical trials for intranasal vaccination. Childers *et al.* found that two intranasal administrations of liposomal formulations containing *Streptococcus mutans* antigen induced significantly higher IgA titers than the free antigen in healthy adults [129].

4.3.2 Virosomes

Virosomes are made of a phospholipid vesicle, as either a mono- or bi-layer, incorporating virus-derived proteins. NasalFlu, launched in Switzerland and developed by Berna biotech in 2001, is a trivalent virosomal inactivated influenza vaccine administered with heat labile toxin of E.Coli (LT) as an adjuvant. It was then withdrawn from the market owing to its link to Bell's Palsy disease in vaccinated individuals, reportedly due to the presence of LT [24].

Other licensed virosome vaccines are Inflexal®, a trivalent 'flu subunit, and Epaxal®, a hepatitis B vaccine by Crucell (Berna), but these are both injected intramuscularly or subcutaneously.

4.3.3 Lipid nanocapsules

Lipid nanocapsules have a hybrid structure between polymer nanocapsules and liposomes. Their inner core is made of medium chain triglycerides and is surrounded by lecithin and polyethylene glycol (PEG). The main advantage of lipid nanocapsules over liposomes is their relative stability and their solvent-free preparation [130]. These carriers have been prepared for mucosal vaccination by Li *et al.*, who combined toll-like receptor (TLR) agonists with antigen-carrying lipid nanocapsules. The pulmonary immunization of mice with this formulation elicited long-lived T cells in lungs and vaginal mucosa [131]. Even if lipid nanocapsules are promising tools for mucosal vaccination, currently there are no published studies concerning intranasal vaccination with these particles.

4.3.4 ISCOMs

Immunostimulating complexes (ISCOMs) are spherical, open cage structures of about 40 nm in size. They are made of cholesterol, phospholipids and Quil A extracted from the bark of a plant, the *Quillaja saponaria* [63]. *Quil A* is amphiphilic: the hydrophilic part is made of carbohydrates, while the hydrophobic portion is quillaic acid [132]. While ISCOMs are negatively charged, since they present glucuronic acid residues at their surface, cationic derivatives have been prepared to exploit the advantages of positive particles [133]. Cibulski *et al.* prepared ovalbumin-ISCOMs and found that they induced systemic and mucosal immune responses in mice after two intranasal immunizations under anesthesia [134].

Coulter *et al.* showed that ISCOMatrix™ with influenza antigens triggered higher antibody titers than the heat labile toxin [135]. Similar results were previously reported by De Haan and co-workers concerning liposomes [121]. Both research groups immunized mice twice under anesthesia, but instilled different volumes of vaccine: 12µl in the ISCOMatrix™ study [135] and 50µl in the liposome study [121].

Concerning larger animal models, mucosal IgA production was stimulated in Merino ewes after intranasal administration of ISCOMs [135]. Hägglund and co-workers intranasally vaccinated calves with ISCOMs, eliciting strong protection against bovine respiratory syncytial virus [136].

A virosomal H5N1 influenza vaccine with ISCOMatrix™ as an adjuvant reached phase I clinical trials, but while this vaccine is efficient in triggering specific-IgG, it is administered through intramuscular injection [137]. There are currently no examples of products on the market using ISCOMs for nasal vaccination.

4.4 Protein nanoparticles

Protein-based nanoparticulate vaccines are mainly proteosomes, composed of purified outer membrane proteins of *Neisseria meningitidis*. These are hydrophobic adjuvant/delivery systems that can be used for the mucosal administration of subunit vaccines [138].

Plante and co-workers intranasally vaccinated mice twice with proteosome-influenza vaccine under anesthesia. The subunit nanovaccine induced specific-serum IgG and mucosal IgA, as well as protection against virus challenge [139].

Influenza and shigella vaccines based on proteosomes have been tested in clinical trials. The intranasal administration of influenza proteosomes successfully induced nasal secretory mucosal antibodies (sIgA) and serum immune responses in healthy adults [140].

5. Nasal vaccines on the market

Few nasal vaccines for human use have been licensed and commercialized. The best known are FluMist® (Medimmune) and Nasovac™ (Serum Institute of India), quadrivalent and trivalent live attenuated influenza vaccines sold in the US and Asia, respectively [141].

There are more veterinary vaccines which use live attenuated viruses than humans one. Chickens are vaccinated against Newcastle disease and infectious bronchitis by a live spray vaccine marketed by Ceva under the trade name of Cevac® Vitabron L.

Merck have marketed various nasal veterinary vaccines for different species, including a nasally administered live vaccine to protect dogs against different diseases such as *Bordetella bronchiseptica*, canine parainfluenza virus and canine adenovirus type 2 (Nobivac®₃ ADT Intra-Trac®). Cattle are given nasal vaccines such as Once PMH IN® and Nasalgen® IP; the first contains an avirulent live culture of *Mannheimia Haemolytica* and *Pasteurella Multocida*, while the second the Bovine Rhinotracheitis-Parainfluenza3 modified live virus.

In the US FluAvert® (Merck), a modified-live equine influenza vaccine, and Pinnacle® I.N. (Zoetis Animal Health), a *Streptococcus equi* live vaccine, are available on the market for horse vaccination via the nasal route.

However, to our knowledge, no nasal vaccines employing nanoparticle technology have yet reached the market. Moreover, all these cited marketed vaccines involve the administration of live attenuated pathogens with the risk of reversion and toxicity due to infection, hence there is a need for the development of subunit and recombinant vaccines for nasal administration.

6. Conclusion

Nanoparticles have great potential both as delivery systems and as adjuvants for mucosal vaccines. Depending on the composition of the nanoparticles, it is possible to obtain a safe carrier able to associate and stabilize recombinant or subunit vaccines. Different particles have already been developed and seem promising for nasal vaccine delivery. In addition, the ease of administration via the nasal route makes it ideal for quick vaccination in case of pandemic emergency.

Attention should be paid to safety issues, with special regard to the *in vivo* fate of the nanoparticle delivery system and/or adjuvant. This aspect should be treated in order to prevent vaccine toxicity that may be particularly important in the case of nasal administration owing to the potential for passage of materials to the brain through the olfactory bulb.

Many studies have focused on the development of new nanovaccines and have been tested in animal models. However, results from clinical trials are still sparse, and the extrapolation of animal results to humans remains an issue.

Table Nanoparticles for nasal vaccination, immunization parameters and immune response evaluated.

Type of particle	Nanoparticles characteristics		Model	Immunization parameters			Imm. resp.	Ref.
	Size (nm)	Z (mV)		Admin.	Dose Ag (µg)	Anesthesia		
Chitosan	300-680	6-30	Mice	3* 15 µl	0.4 , 1 or 2.5	Yes	h, m	[78, 79, 83]
	143-200	26	Mice	2-3*20µl	10	No	h, m	[10, 80]
	80	14	Mice	2*10 µl	10	Yes	h, m	[89]
	210-310	23	Mice	5*20µl	100	Yes	h	[142]
	140	9.8	Mice	3*20µl	1	-	h, m	[81]
	207-603	17-26	Mice	3*25µl	2.5	-	h,c	[143]
	580	-	Rabbit	3*200µl	45	Yes	h, m	[86]
Chitosan or agarose	170-2000	-	Mice	3	10	Yes	m	[82]
TMC	250-400	7-21	Mice	2*10µl	10	-	h, m	[87, 88]
Chitosan or TMC	365-424	30-45	Mice	3	20	-	h, m	[84]
Chitosan and poly-(ε-caprolactone)	208	7-26	Mice	3*15µl	1.5, 5, 10	Yes	h, m	[93]
	12.6	23	Mice	3*10µl	15	No	h, m	[92]
Maltodextrin	70	38	Mice	3*12 ou 20µl	0.2, 1, 3 or 10	No	h,c,m	[3, 101]
PLGA, Glycol-CS PLGA, CS PLGA	200	-15-15	Mice	2	10	No	h,c,m	[114]
PLGA	290-430	-5.6-17	Mice	3*40 or 48µl	10 or 20	Yes	h,m	[113, 144]
	4000	-	Rabbit	2	10	-	-	[115]
	<2.5µm	-	Calves	2*2 ml	0,5, 1 or 5 mg	-	m	[116]
	<2.5µm	-	Calves	1*2 ml	-	-	m	[117]
	225	-	Calves	2*2 ml	-	No	m	[145]
Chitosan PLGA	500nm-2µm	-	Cattle	1/2/3	10-15	-	h,m	[68]

PVM/MA	148	-45	Mice	1	10-20	-	-	[146]
	200	-	Mice	3*6 μ l	3	-	h, m	[147]
PLA Microparticle	-	-	Mice	1*50 μ l	-	Yes	h	[148]
Cyclodextrin-polyethylenimine	117.3	26.4	Mice	2	10	Yes	h, c, m	[118]
Polystyrene	300-390	-	Mice	3*20 μ l	10	-	c	[149]
Cholesteryl Pullulan TNFα	27-42	-	Mice	3*30 μ l	-	Yes		[150]
Non-ionic surfactant vesicles and bilosomes	170	-37	Mice	4	50	-	h, m	[151]
Liposomes	30-100	-	Mice	2 or 3* 50 μ l	-	-	c	[122, 123]
	300	8	Mice	2* 50 μ l	25	Yes	m	[127]
ISCOM and ISCOMATRIX	40-50	-	Mice	2	2 μ g	Yes	h, m	[134]
	-	-	Mice	2* 12 μ l	-	Yes	h, m	[135]
	-	-	Sheep	2*500 μ l	-	No	m	[135]

Z potential (Z); (number of administrations \times volume instilled) (Admin.); antigen (Ag); immune response (Imm. resp.); the immune response is indicated as humoral (h), cellular (c) or mucosal (m); bibliographyreference (Ref.). Chitosan (CS), Trimethylchitosan (TMC), Polylactic-co-glycolic acid(PLGA), Poly methyl vinyl ether/maleic anhydride (PVM/MA), Polylactic acid (PLA), Tumor necrosis factor α (TNF α), Immunostimulating complex and Immunostimulating complex matrix (ISCOM and ISCOMATRIX)

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1.1.2 NPL positively charged porous nanoparticles with a lipid core

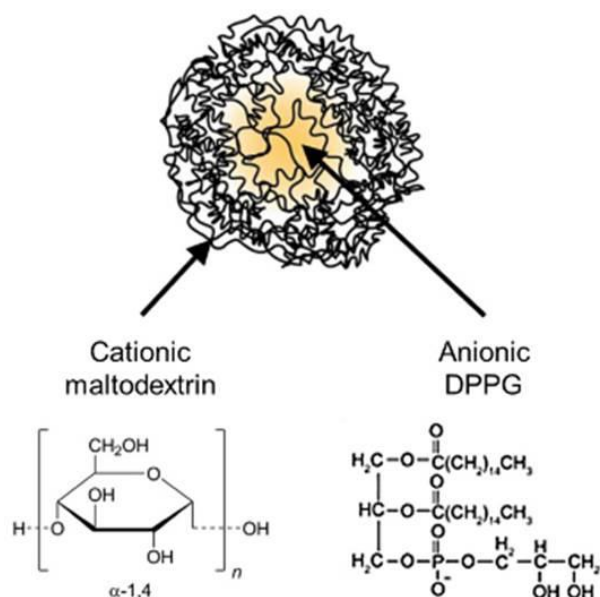


Figure 2 Representation of NPL.

The chemical formula of maltodextrin and dipalmitoylphosphatidylglycerol (DPPG) are reported. Image from (Dimier-Poisson *et al.*, 2015).

We used positively charged nanoparticles to intranasally deliver antigenic proteins. These nanoparticles (NPL) are made of a net of maltodextrin that traps proteins or nucleic acids and negative lipids, in our case the dipalmitoylphosphatidylglycerol (DPPG) (Paillard *et al.*, 2010). Maltodextrin used for NPL preparation is typically produced by starch hydrolysis through an enzymatic process. This polymer of D-glucose contains α -D-glucopyranosyl molecules linked through α -1,4 bounds (Chronakis, 1998). It is a polysaccharide generally recognized as safe (GRAS) by the Food and Drug Administration (FDA).

Maltodextrin nanoparticles (NP^+) are produced by chemical synthesis by grafting epichlorohydrin to reticulate the polymer and glycidyltrimethylammonium chloride (GTMA) to confer a positive charge to the particle (Betbeder *et al.*, 2002; Samain *et al.*, 1994).

The safety of the NP^+ has been previously investigated. NP^+ are not cytotoxic and genotoxic even at high concentration, therefore NP^+ are good and safe candidates for drug delivery (Merhi *et al.*, 2012). With regards to the mechanism NP^+ are endocytosed by airway epithelial cells

through the clathrin-pathway and exocytosed in a cholesterol dependent manner (C. Y. Dombu *et al.*, 2010).

The carrier behavior is modified by the introduction of a negative lipid inside the NP⁺ (NPL). Hence Dombu *et al.* showed that NPL deliver proteins in airway epithelial cells more efficiently than NP⁺. Moreover partial endo-lysosomal escape/cytosolic delivery of the protein is observed by ovalbumin loaded-NPL in the same cell model (C. Dombu *et al.*, 2012). This property of the NPL can be used to potentially induce MHC class I antigen presentation and consecutive cellular response in case of a vaccine formulation. NPL are highly stable carriers, able to associate a high amount of complex proteins (Dimier-Poisson *et al.*, 2015). They are effective as vaccine delivery carriers since they induce complete protection against parasitic challenge infection after nasal administration in mice (Betbeder *et al.*, 2014; Dimier-Poisson *et al.*, 2015). These carriers are also suitable to deliver lipophilic drugs, such as diminazene (Kroubi *et al.*, 2010).

1.2 Biological fate of nasally administered nanoparticles

1.2.1 Anatomy of the nose

The knowledge of carrier fate is crucial, especially for toxicity issues related to nanoparticles and adjuvants.

Once the nanoparticle formulation is instilled or sprayed in the nose, through either nostrils, it may reach different areas.

It first encounters the nasal vestibule that lies in the entrance of the nostrils (Figure 3) characterized by vibrissae or hairs and sebaceous glands where it can get trapped. The nasal septum, also called inner wall, divides the two nasal cavities. The outer wall is arranged in three or four turbinates, also called conchae that increase the surface of the nasal cavity increasing the chances for formulation-tissue contact. Turbinates irregularly divide the nasal cavity in three canals, each named *meatus*. Four different pairs of cavities, paranasal sinuses (frontal, ethmoid, maxillary and sphenoid) are observed on the lateral face of the nasal cavity. The sinuses are covered by a respiratory mucosa.

Arteries, veins and lymph vessels form the vascular net of the nasal tissue. Lymphatic vessels drain the absorbed material from the tissue to the lymph nodes. Lymph nodes localized at the submandibular level receive drained material from the anterior nasal cavity, whereas lateral,

pharyngeal, deep cervical and jugulofacial lymph nodes are connected to the middle and posterior side of the nose. M-cell aggregations or lymph corpuscles, adenoids and tonsils form the Waldeyer's ring around the nasal and buccal cavity. This formation is named nose-associated lymphoid tissue (NALT).

In the nasal cavity there are two types of epithelium: respiratory and olfactory. The main epithelium is a respiratory one, made of ciliated pseudostratified columnar cells. The olfactory region is located in the upper area of the nasal cavity where the olfactory epithelium lies. This is a direct pathway to the brain thanks to the presence of olfactory neurons inserted in the cribriform bone. M-cells within the lymphoid tissue are part of the adenoid tissue. Ciliated, columnar and goblet (mucus-producing) cells form the respiratory epithelium (Gizurarson, 2012).

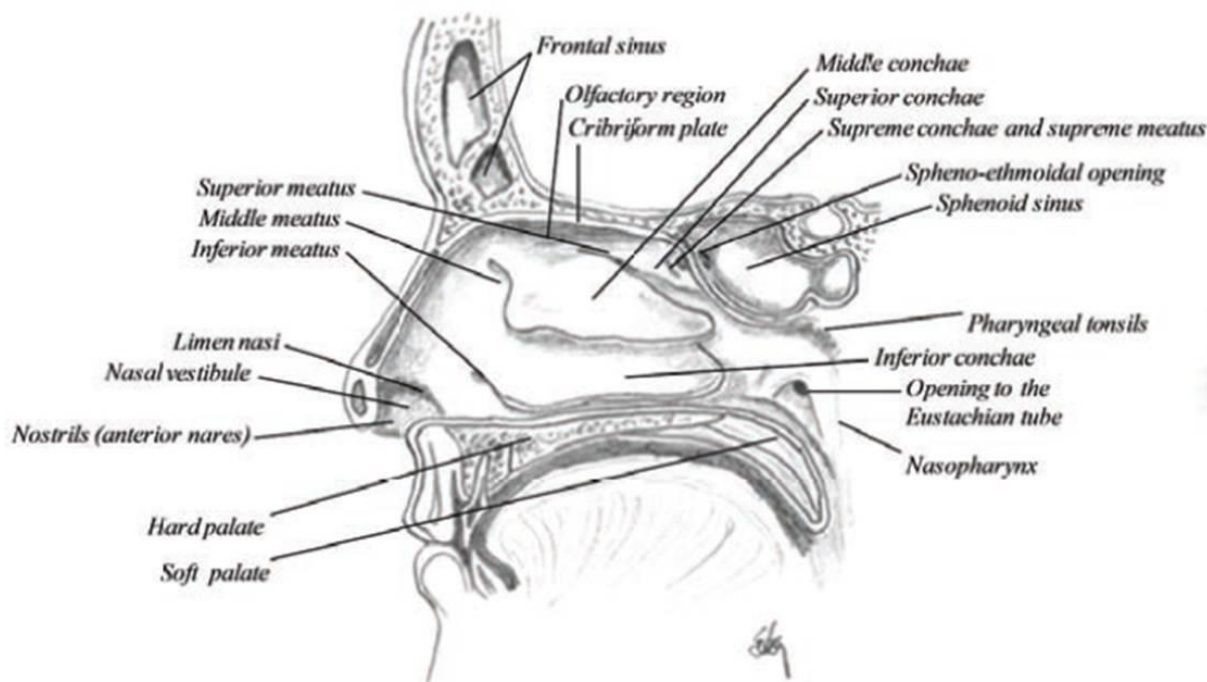


Figure 3 Sagittal section of the nasal cavity.

Image from (Gizurarson, 2012).

1.2.2 Nasal immune system: NALT

NALT is a secondary lymphoid organ, known as inductive site of the nasal mucosal immune system. It is indeed where the initiation of the immune response induced by the antigen administration takes place. The antigen sampling system (e.g. M cells) picks up the antigen in follicle-associated epithelium and transfers it to antigen presenting cells (APC), such as dendritic cells (DC). DCs prime naïve CD4⁺ and CD8⁺ T cells (Figure 4).

CD8⁺ T cells mature into cytotoxic T cells (CTL). CTL role is to kill infected cells and to fight viral infection.

The antigen presentation by DC to CD4⁺ T cells activates Th1, Th2 and Th17 responses as well as IgA class switching and B cells hypermutation in germinal centers. IgA⁺B cells migrate to the effector sites through cervical lymph nodes and peripheral blood. For nasally administered antigens, the effector sites are the lamina propria of the upper airways, the gut and the genital tract.

At the effector site, plasma B cells secrete IgA (sIgA). sIgA are transcytosed to the luminal side of the epithelium by the polymeric Ig receptor and block pathogen entry (Kiyono *et al.*, 2004; Lamichhane *et al.*, 2014; Lycke, 2012).

Multiple approaches are described in literature with regard to cell targeting. To improve vaccine efficacy, different strategies have focused on APC targeting, especially on dendritic cells. The nature of the target receptor, the type of APC, its activation state as well as the vaccine delivery system may affect the triggered immune response. Besides DC, macrophages, neutrophils and mastocytes can also present antigens to lymphocytes. Moreover, M cells targeting has been reported to improve immunity in mice (Alvarez *et al.*, 2013). M cells, known for antigen sampling function, have been identified in the nasal passage of mice and in NALT, to a much lesser extent (Kim *et al.*, 2011).

Epithelial cells represent the first physical barrier that separates underlying tissues from the external environment, thus protecting the body from pathogen's entry via the nose. Not only are these cells a mechanical barrier, they are also affect the immune response regulation (Pichavant *et al.*, 2003). Human bronchial epithelial cells may act as antigen presenting cells during viral infections. Papi A. *et al.* showed that MHC class I molecules, constitutively expressed on most of nucleated cells, are up-regulated subsequently to a rhinovirus infection in the respiratory

epithelium. However MHC class II, typically expressed by APC, is not up-regulated by the virus (Papi *et al.*, 2000).

In 1989, MHC II (HLA-DR) has been ubiquitously revealed in the lower respiratory tract and in the normal bronchial epithelium (Glanville *et al.*, 1989). MHC II molecules expression is necessary for the antigen presentation to T cells. The presence of these molecules in the airway epithelium suggested that this tissue contribute to immunoregulation by sampling antigens and directly interacting with T helper lymphocytes. MHC II was also observed in the nasal turbinates (Kalb *et al.*, 1991). Thus human bronchial epithelial cells have an accessory function in antigen presentation and may be an additional vaccine target (T. L. Li *et al.*, 2013; Salik *et al.*, 1999).

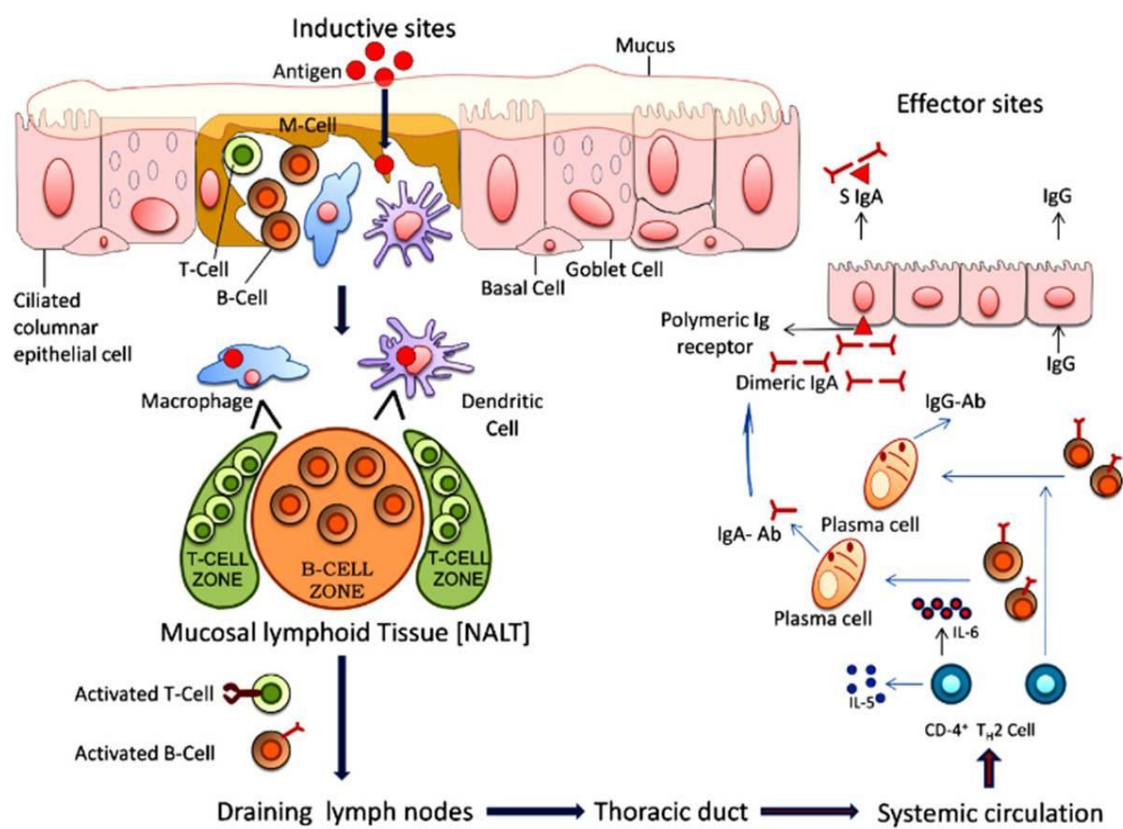


Figure 4 Mucosal immune response.

Schema reporting the activation of the immune response in the NALT inductive site, the migration of white blood cells and Ig production at the effector site (Gupta *et al.*, 2011).

1.2.3 Fate of nanoparticles after intranasal administration

Vaccines often activate a complex cascade of immunological response and the mechanisms of transport of nanoparticulate intranasal vaccines have been investigated nonetheless, these processes are not completely clear (Sharma *et al.*, 2009). The different compositions of nanoparticles, in terms of antigen, adjuvant and raw material, may differently trigger the immune response.

Nanoparticle biodistribution and uptake are affected by the size and zeta potential of the carrier (Kumari *et al.*, 2011). The use of cationic particles is advantageous thanks to the depot effect and their muco-adhesion. The electrostatic forces between the positive particle and anionic compounds on the cell surface, such as glycosaminoglycan and sialic acid, are accountable for this interaction. The increase of hydrophobicity can also favour the cell uptake thanks to the lipids interaction with the cell membrane (Zazo *et al.*, 2016).

Interestingly supplementing the particulate formulation with cationic adjuvants potentially produces a synergistic effect with the mucoadhesive polymer, prolonging the antigen persistence in the mucosa (Bento *et al.*, 2015).

To interact with the cells, nanoparticles should pass through the 10 μ m-thick mucus layer (Widdicombe, 2002). Once crossed, nanoparticles possibly gains contact with epithelial cells, M-cells or transepithelial dendrites of dendritic cells. Epithelial cells and M-cells may endocytose or transcytose the antigen-nanoparticles formulation, whereas dendritic cells preferentially phagocytes it. Differences concerning the immune response rise from various targeting possibilities at subcellular level (publication 1 Endocytosis of nanoparticles). For instance the antigen delivery into the cytosol induces MHC class I presentation, hence CD8+ T cell response, whereas protein endosomal degradation by APC provides MHC II presentation with subsequent CD4+ T cell activation (publication 2, Nasal nanovaccines).

Nanoparticles may be eroded by the action of enzymes or the matrix can be disaggregated by hydrolysis, this process might increase protein delivery in the cells.

Eventually the non-endocytosed nanoparticles or those who have been exocytosed after the uptake, are cleared by the nasal cavity. The nasal clearance rate of the formulation depends on the muco-adhesion of the formulation, therefore related to the material (e.g. alginate, chitosan) (Soane *et al.*, 1999; Tafaghodi *et al.*, 2004).

2. Influenza and vaccination

2.1 History of vaccination

It is popular knowledge that survivors of some diseases that caused epidemics, such as smallpox or plague, had become immune to the previously contracted infection. This observation gave birth and spread the practice of inoculation, also called variolation, as prevention from smallpox. Variolation consisted in the subcutaneous inoculation, *via* a lancet, of smallpox virus into healthy individuals. Despite 2-3% inoculated people died from the infection, this practice gave protection in most cases and diffused through Europe, Asia and America between 1700 and 1800.

The first vaccination occurred at the end of the XVIII century, when Eduard Jenner inoculated a patient with the cowpox virus collected by fresh lesion. Although the patient was inoculated against the smallpox virus, he did not become ill (Riedel, 2005).

The term “vaccination” derived from “*vacca*”, that means cow in Latin, was introduced over the following century.

Another milestone in vaccine discoveries was reached in the late XIX century by Louis Pasteur, who introduced the “Germ theory”. He investigated *Vibrio cholerae* infection, inoculating the fresh bacteria in chickens. However its experiment was failing for all chickens were dying after the inoculation. He once accidentally inoculated the attenuated bacteria and noticed protection from the subsequent infection with the active *Vibrio cholerae*. Several vaccines, wether live and killed vaccines, followed to fight against diseases like typhoid, plague, diphtheria and tuberculosis. Noteworthy is the finding of the *Mycobacterium bovis* by Albert Calmette and Camille Guerin in Lille in the beginning of the 19th century. The virulence of this strain was attenuated compared to the *Mycobacterium Tuberculosis*, previously discovered by Robert Koch, therefore it was suitable for tuberculosis vaccination.

Concerning influenza, the virus was first isolated by Richard E. Shope in 1931. Influenza vaccine was first licensed after the Second World War in the U.S.A. (Hajj Hussein *et al.*, 2015).

2.2 Influenza virus disease

Influenza is an acute viral infection of the respiratory tract which mainly affects the upper airways, sometimes extending to the lungs.

World Health Organization (WHO) estimated influenza annual attack rate at 5-10% in adults and 20-30% in children. This disease causes about 2-5 million cases of severe illnesses that yield 250 000-500 000 deaths (World Health Organization, 2014a).

After one or two days of virus incubation, the infected subject presents with fever, sore throat, myalgia, runny nose, coughing and headache, as a result of the cytokines produced by infected cells (Ge *et al.*, 2011). Complications lead to viral pneumonia and bacterial infections and may occur at any age. Children, elderly and immunocompromised patients feature a greater risk of complications compared to healthy adults (Halsey *et al.*, 2015; Rimmelzwaan *et al.*, 2001). Mortality is often related to infections of the lower respiratory tract, due to *Staphylococcus aureus* or Respiratory Syncytial Virus (RSV) infection (Nair *et al.*, 2010; O'Brien *et al.*, 2009). Patients with cardiovascular or metabolic diseases are also more likely to develop complications. Influenza viruses spread through the air. The virus transmission occurs *via* different modalities such as: (i) the contact with an infected surface or an infected individual; (ii) through airborne droplets. Respiratory droplets (size >5µm) or small aerosols (size <5µm) that remains suspended for longer time in the air, can be dispersed in the surroundings and inhaled by other people (Richard *et al.*, 2016). This enhances the viral dissemination.

The disease prevention through mass vaccination is considered the most cost-effective measure to limit the virus diffusion and protect against pandemic spread (Amorij *et al.*, 2010; Jang *et al.*, 2014; Rimmelzwaan *et al.*, 2001).

2.2.1 Influenza virus: classification and structure

Influenza viruses belong to the family of the *Orthomyxoviridae* and are characterized by eight negative senses - RNA (Bouvier *et al.*, 2008; Rossman *et al.*, 2012). Influenza viruses exist in three different *genera*: A, B and C, where the latter is the sole anti-genically stable. Influenza B and C viruses only infect humans, in contrast with Influenza A that also affects other mammalian and avian species.

The viral antigens used to classify A flu viruses are hemagglutinin (HA) and neuraminidase (NA). These antigenic glycoproteins may undergo modification causing the birth of new strains of influenza A (Couch, 1996).

Concerning influenza A, 18 different hemagglutinin (HA) and 11 different neuraminidase (NA) subtypes exist in nature; these have been originally identified in bats (Centers for Disease Control and Prevention, 2014).

In 1980, the WHO revised the nomenclature for influenza virus, firstly established already in 1971. The current nomenclature is displayed in figure 5.

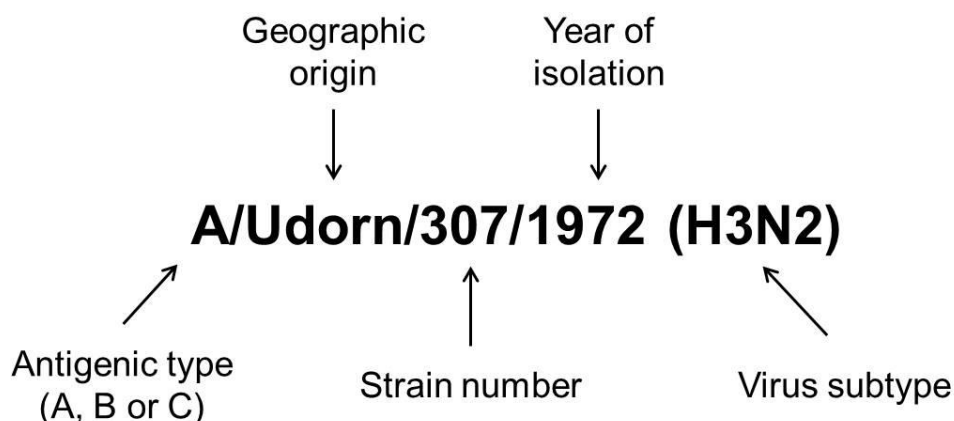


Figure 5 Influenza A virus nomenclature.

An index describing the subtype of HA or NA is present in case of influenza A (World Health Organization, 1980).

Influenza virus is pleomorphic: it exists in nature as spherical virions (100 nm of diameter) or as filamentous virions (100 nm-20 μ m of diameter) (Rossman *et al.*, 2012).

The virus is formed by a lipid envelope where HA, NA and proton ion channels M2 are inserted (Figure 6) (Bouvier *et al.*, 2008; Zebedee *et al.*, 1988). A middle layer of matrix protein 1 (M1) is found between the envelope and the nucleocapsid. In the viral core, the RNA, under the helical ribonucleoprotein form, is complexed with nucleoproteins. The RNA polymerase complex consists in PB1, PB2 and PA (Nayak *et al.*, 2009; Rossman *et al.*, 2012). Influenza B and C viruses differ from the A type for the presence of ion channels distinct from M2: BM2 and NB for B virus; CM2 for C virus (Bouvier *et al.*, 2008; Imai *et al.*, 2008; Liang *et al.*, 2010).

HA and NA surface antigens of seasonal influenza are capable to mutate and induce the antigenic drift of the virus. This enables the seasonal virus to escape from the host immune system.

Viral HA subtype defines the infection depth in the respiratory tract. Easily transmitted strains own an HA subtype that binds to the upper airways, as opposed to other strains (such as H5N1) which binds to receptor in a deeper part of the respiratory tract, i.e. the lungs (Ge *et al.*, 2011).

A pandemic disease occurs in case of a human infection from an unknown virus strain, derived from the animal reservoir (e.g. birds, pigs). This is called antigenic shift (J. C. De Jong *et al.*,

2000; J. K. Kelso *et al.*, 2013; Krammer *et al.*, 2015; World Health Organization, 2014b). Major alterations of the virus originate by reassortment of different subtypes of Influenza A: this may happen when the same animal (e.g. pig) is infected by an animal and a human strain at the same time and the virus undergoes modifications. As a result a new shifted virus capable to infect humans may generate and potentially cause pandemics (World health Organization, 2016). Pandemic influenza has already been reported as “Spanish Flu” H1N1 in 1918 and the “Swine flu” H1N1 in 2009.

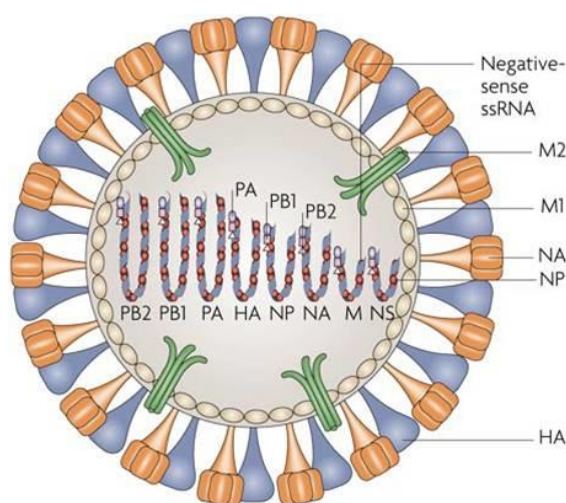


Figure 6 Schema of Influenza A virus.

Different proteins are labeled: hemagglutinin (HA), neuraminidase (NA), matrix protein 2 (M2), matrix protein 1 (M1), nucleoprotein (NP) and others ribonucleoproteins (PB1, PB2 and PA). Eight native-senses RNA lay in the capsid (Nelson *et al.*, 2007).

2.2.2 The infection mechanism

Viruses use the cell endocytic machinery in order to induce infection and replicate. Viruses can use multiple entry pathways depending on the host cell (Permanyer *et al.*, 2010; Piccini *et al.*, 2015; Schulz *et al.*, 2012). Viruses invade the host cell by mean of various endocytosis mechanisms, although one usually prevails on others. All these pathways can lead to successful infection (Lakadamyali *et al.*, 2004; Piccini *et al.*, 2015).

Influenza virus may enter cells *via* either a clathrin-mediated mechanism (CME) or a clathrin- and caveolin-independent pathway (Rust *et al.*, 2004; Sieczkarski *et al.*, 2002). These entrance paths are equally efficient for infection (Lakadamyali *et al.*, 2004).

Furthermore influenza A virus exists as spherical or filamentous virion (Rossman *et al.*, 2010). Spherical virions can invade cells by macropinocytosis as alternative pathway to CME, whereas the filamentous viruses mainly enter by macropinocytosis (Edinger *et al.*, 2014; Rossman *et al.*, 2012). The endocytosis of Influenza A virus is generally accepted as a receptor-mediated process; the primary receptor used by the virus is the sialic acid receptor (or N-acetylneuraminic acid receptor). This is bound by the viral hemagglutinin, a membrane protein highly expressed on influenza virions surface (Rust *et al.*, 2004). However, influenza virus infection can result from sialic acid independent-receptors (Edinger *et al.*, 2014; Stray *et al.*, 2000). A recent study shows that C-type lectin receptors, DC-SIGN (CD209) and L-SIGN (CD209L), are endocytic receptors and enhance influenza A infection, although sialic acid attachment can improve the viral entrance into the cell (Gillespie *et al.*, 2016). After internalization into cellular compartments, the virus localises in the early endosome and eventually reaches the late endosome. Because of the acidic pH, HA changes conformation and the viral envelope fuses with the endosomal membrane (Figure 7) (Edinger *et al.*, 2014; Lakadamyali *et al.*, 2004; Rust *et al.*, 2004). In the endosome the M2 ion channel let the protons flow into the virus. As a result of the acidification, M1 is disrupted and consequently ribonucleoproteins are delivered in the cytoplasm (Jing *et al.*, 2008). Influenza ribonucleoproteins use the nucleo-cytoplasmic traffic of the host to reach the nucleus, they enter the nucleus through the nuclear pore complex and replicate its genome (Eisfeld *et al.*, 2015; Resa-Infante *et al.*, 2011). New viral mRNA is transcript, transported to the cytoplasm and then translated into new viral proteins that are either imported in the nucleus or transported to the plasma membrane. The proteins PA, PB1, PB2 and NP are now required for genome replication and packaging into ribonucleoproteins (RNPs). Thanks to M1 and nuclear export protein the viral RNPs are transported to the cytoplasm and reach the plasma membrane. New viral particles containing HA, NA, M2 and M1 are assembled and released from the infected cell. M2 promotes the budding and NA cut sialic acid residues to induce efficient release, avoiding aggregation of the new virions (Air, 2012; Eisfeld *et al.*, 2015).

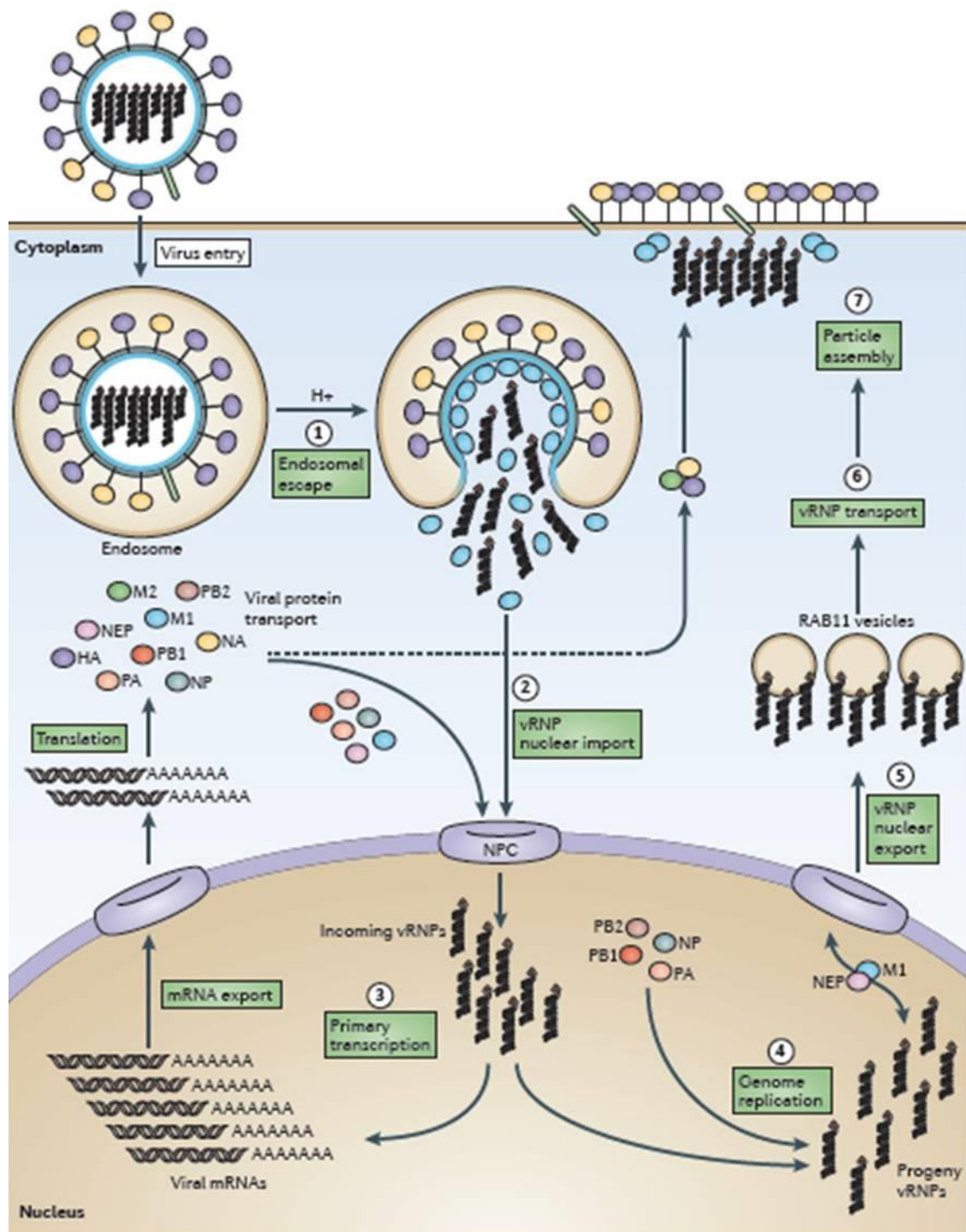


Figure 7 Influenza virus replication mechanism (Eisfeld *et al.*, 2015).

2.3 Influenza vaccines: inactivated (IIV) and live attenuated (LAIV) influenza vaccines

Currently licensed flu vaccines are mainly trivalent, consisting in two A virus strains (H1N1 and H3N2 subtype) and one B strain. Some formulations have been implemented with an additional B strain to generate quadrivalent vaccines (Gerdil, 2003). Most of current flu vaccines are inactivated (IIV) or live attenuated (LAIV) influenza vaccines. These are produced by virus inoculation in embryonated chicken eggs. The amplified viruses are collected by harvesting the allantoic fluid from the eggs and consequently purifying the extract by ultra-centrifugation (Eisfeld *et al.*, 2014; Gerdil, 2003).

Generally produced by cold-adaptation, LAIV are temperature-sensitive and they replicate slower than the untreated virus. In fact, these viruses grow at temperature under 25°C and they stop replicating at temperatures above 37.8°C (Esposito *et al.*, 2012). To disable virus ability to infect human cells, they undergo attenuation through chemical mutagenesis or multiple cell culture passages.

Alternatively virus inactivation can be performed by beta-propiolactone, formalin, heat or radiation treatment (S. D. Pawar *et al.*, 2015). The inactivated vaccine may consist of the whole virus, or it can be split by the use of a detergent or split and purified to obtain a subunit vaccine (Gerdil, 2003).

However IIV and LAIV vaccines present disadvantages related to the presence of ovalbumin that precludes administration to allergic people. LAIV vaccines also undergo age restrictions and are only to be administered to patients from 2 to 50 years old; unfortunately, the children and the elderly are an important target of the seasonal and pandemic vaccination (Centers for Disease Control and Prevention, 2015c; Esposito *et al.*, 2012).

Another disadvantage of LAIV vaccines is the variability of the immunogenicity between people. The possibility of reversion of the attenuated virus to the initial wild-type phenotype has not yet been demonstrated (Rimmelzwaan *et al.*, 2001).

Eventually subunit vaccines constitute an advantageous alternative to others IAV and to LAIV vaccines, since they do not contain the whole virus. Subunit vaccines are composed by proteins expressed in the virus (e.g. HA or M2 for influenza), which are extracted by chemical process and purified.

Vaccines can also be synthesized in culture cell; this process is much easier for the industrial scale up. For influenza vaccine synthesis, Madin-Darby canine kidney cells (MDCK), Per.C6 and Vero cells have been tested (Krammer *et al.*, 2015). To date, only MDCK cells found concrete application in the new influenza vaccine produced by Novartis, Flucelvax®, a trivalent inactivated influenza vaccine (Centers for Disease Control and Prevention, 2015a). This type of vaccine requires no embryonated eggs for viral replication therefore does not contain egg-derived contaminants (e.g. ovalbumin).

Alternatively a pure protein vaccine recently appeared on the market. Based on the HA, Flublok® has been the first recombinant influenza vaccine, FDA-approved in 2013. In order to produce this vaccine, HAs are expressed in insect cell line (*Spodoptera frugiperda*) using a baculovirus. The proteins (HA) are then extracted using a buffer and a detergent. The HAs are finally purified by chromatography (Cox *et al.*, 2015). Recombinant vaccines are convenient because they lack of infectious virus during production, do not require eggs, they are rapidly produced and their scale-up is accessible (Krammer *et al.*, 2015).

2.3.1 Administration routes of influenza vaccines

Influenza vaccines (IIV) are typically administered through the intramuscular route, as one single dose flu shot, to patients who had already contracted the virus or received the yearly vaccination (Rimmelzwaan *et al.*, 2001). Serum IgG are produced subsequently to intramuscular administration of flu vaccines. These antibodies are effective in the protection against the lower respiratory tract infection, but poor protection is conveyed in the upper airways, because of a lack of antibodies in the nasal mucosa. The use of a needle to administer these vaccines decreases the patient compliance (Amorij *et al.*, 2010).

For instance, Fluzone® is a quadrivalent intramuscular flu vaccine approved by FDA for children older than 6 months of age. For adults, FDA also licensed a quadrivalent intradermal influenza vaccine (Fluzone® Intradermal Quadrivalent) (Food and Drug Administration, 2016). Similarly, in Europe a split trivalent intradermal flu vaccine (Intanza®, by Sanofi Pasteur) administered *via* a microinjection system (e.g. Soluvia) has been licensed (Durando *et al.*, 2011). With a lower dose, intradermal vaccination yields comparable immunity to that of intramuscular injection (Frenck *et al.*, 2011; Saville *et al.*, 2008).

To circumvent the disadvantage of the needle used and to induce an effective mucosal immunity, nasal flu vaccines have been developed. The nasal route of administration has been exploited to mimic the natural route of infection of influenza virus. An efficient mucosal immune system stimulation does not only confer systemic protection but it also leads to local production of IgA, that protects the upper airways from viral infection, and serum IgG, for the lower airways protection (Holmgren *et al.*, 2012). Intranasal immunization has the advantageous possibility to induce antibody production in sites distant from the infection area e.g. the genital tract and/or intestine (Lycke, 2004b). Moreover the nasal instillation of LAIV promotes cytotoxic T cell (CTL) response and does not induce influenza-like symptoms (Belshe *et al.*, 2000; Rimmelzwaan *et al.*, 2001).

Nasal LAIV have been simultaneously developed in Asia and in USA (Krammer *et al.*, 2015). FluMist® was the first trivalent nasally administered LAIV vaccine approved in 2003 in the USA, similarly Russia had the NasoVac® (Holmgren *et al.*, 2012). The currently commercialised nasal flu vaccine is a quadrivalent LAIV (FluMist® Quadrivalent, Medimmune).

NasalFlu® was another trivalent one, but IIV, made of virosomes that reached the Swiss market in 2001, later withdrawn for the potential relation with Bell's palsy (seventh cranial nerve paralysis) as an adverse effect (Wong *et al.*, 2005). The mucosal adjuvant, *Escherichia Coli* heat labile enterotoxin, had been found to be accountable for inflammation (Halsey *et al.*, 2015; Wilschut, 2009). In fact, the nasal mucosa is a direct access to the brain thanks *via* the olfactory neurons. This pathway can be the cause of toxic events.

The oral route of vaccination is also a promising mucosal site able to induce effective protection after antigenic contact. This is supported by several oral vaccines on the market (like oral cholera vaccine (Lopez *et al.*, 2014), rotavirus vaccine (Greenberg *et al.*, 2009), poliovirus vaccine (Holmgren *et al.*, 2012). However, influenza oral vaccines are not yet available. Phase 1 studies have been performed using an adenoviral vector that could elicit influenza antibodies in 90% of individuals (Liebowitz *et al.*, 2015).

Another approach investigated for the oral flu vaccination is the use of a recombinant baculovirus expressing HA in his envelope. This induced broad mucosal, humoral and cell mediated immune responses in mice (Prabakaran *et al.*, 2014). Attenuated strain of Salmonella, expressing HA and NA of H5N1 strain, elicit complete protection against the lethal challenge with H5N1 and H1N1 in mice (Pei *et al.*, 2015). The high surface of the gastrointestinal tract is

an advantage for drug administration, but the harsh enzymatic conditions and the high dilution of the vaccine reflect the limits of this mucosal route. For these reasons, and because of the small doses of vaccine administered, the nasal administration is preferable for subunit and recombinant vaccines.

Sublingual administration has recently been investigated as mucosal route for flu vaccine and compared to the nasal route. Sublingual administration of adjuvanted IIV induced local and systemic specific immune response as well as CD4+ T cells and memory B cells. However, nasal administration induces higher immune response than the sublingual one (G. Pedersen *et al.*, 2012; G. K. Pedersen *et al.*, 2011).

Similarly, the eye mucosa has been suggested as an alternative route but only few studies concerning this route of vaccination are available (Hikono *et al.*, 2013; Seo *et al.*, 2010). To date, no sublingual or eye drop flu vaccines are commercialized.

2.3.2 Adjuvants

Adjuvants, from the latin *adjuvare*, to help, are used to support the antigenic immune response, especially for subunit vaccines. Accordingly to European Directive and the European Medicine Agency (EMA) adjuvants are defined as substances “*aimed at enhancing, accelerating and prolonging the specific immune response towards the desired response to vaccine antigens*” (European Medicines Agency, 2006).

Often subunit vaccines are not successful in cytotoxic T cell (CTL) activation because of their insufficient cytoplasmic delivery and MHC I presentation. In these cases the adjuvant supplement is especially needed.

Many adjuvants have been examined for influenza vaccination: oil-in-water emulsions, saponins and glycolipids, liposomes, bacterial toxins, cytokines, TLR agonist, polymers (Durando *et al.*, 2011).

Aluminum salts, e.g. Aluminium hydroxide, and its derivatives have been added to vaccines for decades. Aluminium acts by triggering the “inflammasome” in cells and generating proinflammatory response (Marrack *et al.*, 2009). Not all vaccines contain aluminium and, when present, it is added in small doses. Yet, safety concerns are often controversial.

MF59TM adjuvanted flu vaccines are a blend of influenza antigens and oil-in-water emulsion containing squalene. MF59TM was firstly licensed in 1997 as adjuvant for an influenza vaccine. Inactivated adjuvanted vaccine is commercialised under the name of FludTM (Novartis) (Durando *et al.*, 2011). The exact mechanism of adjuvanticity of MF59TM is unknown, but it can be partially related to its depot effect after intramuscular injection (Schultze *et al.*, 2008). Moreover, MF59TM induces recruitment of antigen presenting cells (APC) at the site of injection, contributing to the adjuvant effect (Dupuis *et al.*, 2001).

After nasal administration MF59TM-adjuvanted influenza vaccine did not show higher immunogenicity than the non-adjuvanted one (Boyce *et al.*, 2000).

In addition to MF59TM, aluminium hydroxide, AS04TM (aluminium hydroxide with monophosphoril lipid A), AS03TM (squalene and tocopherol emulsion stabilized by Tween[®] 80) and influenza virosomes are the only other adjuvants licensed in Europe. However, these adjuvants are not effective for intranasal vaccination (Amorij *et al.*, 2010; Fox, 2009). AS03TM has been licensed in a pandemic flu vaccine named Pandemrix[®] but it showed risk of narcolepsy as side effect (Centers for Disease Control and Prevention, 2015b).

Virosomes are reconstituted influenza liposomal vesicles, having HA and NA integrated in the phospholipid bilayer, with a diameter of 150 nm. These subunit injectable vaccines, commercialized under the name of Inflexal[®] V, consist in a blend of three types of virosomes, containing a specific strain of HA and NA (Herzog *et al.*, 2009; Mischler *et al.*, 2002).

The few above cited adjuvants are the only ones currently accepted therefore there is a great need of new safe adjuvants. The use of nanoparticles, as adjuvant and drug delivery systems like in the case of virosomes, is an advantageous approach.

Heat-labile toxin, from *Escherichia coli*, and cholera toxin, from *Vibrio cholerae* are the strongest known mucosal adjuvants, well-known for their toxicity and the high possibility to cause side effects. These toxins bind to the GM1 ganglioside receptor. The intranasal administration of radiolabeled cholera toxin showed that it can enter the olfactory nerves (van Ginkel *et al.*, 2000). Hence the access of bacterial toxins to the central nervous systems raises toxicity issues (Mutsch *et al.*, 2004). These molecules have been modified to reduce their toxicity while keeping the adjuvant propriety (Yamamoto *et al.*, 1997). An innovative approach has been investigated by fusing the enzymatically active subunit A1 of the cholera toxin (CTA1) with a B cell targeting moiety. Cholera toxin consists of five enzymatically inactive B-subunits

that forms a pentamer. The pentamer builds a ring around the subunit A, formed by a linker (A2) and an ADP-ribosyltransferase portion (A1) (Agren *et al.*, 1997). CTA1 acts on GTP binding proteins (like G α), as a result of this interaction an increase of intracellular cAMP is obtained (Lycke, 2004b). Lacking the B subunit, the cholera toxin does not bind to the GM1-ganglioside receptor that is present in almost all of mammalian cells (Lycke, 2004a, 2004b).

CTA1 has been linked to the c-terminal region of a dimer of the D-fragment of the protein A from *Staphylococcus aureus* (DD). DD binds the Ig-receptors present on B cells (Agren *et al.*, 1997; Eriksson *et al.*, 2004; Lycke, 2004a). CTA1-DD does not show any systemic toxicity and gave promising results in mouse model. This adjuvant shows strong class I and class II MHC restricted T cell immunity after intranasal or systemic administration (Eriksson *et al.*, 2004; Simmons *et al.*, 1999). Nasally administered CTA1-DD does not induce inflammatory events in the nasal mucosa and does not accumulate in the olfactory bulb, confirming the safety of this adjuvant (Eriksson *et al.*, 2004).

CTA1-DD has also been combined to another adjuvant ISCOMs (ImmunoStimulating COMplex). This couple is highly immunogenic and induces cell mediated, humoral and mucosal IgA responses after mucosal administration (Lycke, 2004b).

Immunostimulating complexes (ISCOMs) are 40nm micelles, having a cage-like structure, constituted by antigen, cholesterol, phospholipid and saponins, while ISCOMATRIX[®] have the same composition but do not contain the antigen. The saponins contained in ISCOMs are mostly *Quil A* or its purified compounds extracted from the bark of *Quillaja saponaria Molina* (Kersten *et al.*, 2003; Pham *et al.*, 2006; Sun *et al.*, 2009). In general, ISCOMs are prepared by centrifugation or dialysis. The former method involves three steps: at the beginning micelles of cholesterol and phospholipids are prepared using Triton X-100 as surfactant. The micelles are then centrifuged against a sucrose gradient including *Quil A*. A final dialysis stage is performed to eliminate the sucrose. The latter method, the dialysis, consists in the preparation of a micellar solution of all the ISCOMs components in a surfactant. The surfactant is consequently removed by dialysis. This method yields more homogeneous particle sizes (Sun *et al.*, 2009).

The ability to induce broad humoral and cellular immune response, such as CTL activation, is a great advantage of ISCOMATRIX (and also ISCOMs). This is linked to the antigen presentation by both MHC I and II receptors (Pearse *et al.*, 2005) allowing cross-presentation in APC. Indeed, ISCOMs are endocytosed by dendritic cells where they induce upregulation of both

MHC I and II (Villacres *et al.*, 1998). ISCOMs and ISCOMATRIX also induce upregulation of many cytokines (Sun *et al.*, 2009).

Yet, to obtain the approval of a new adjuvant for vaccine formulations, toxicity issues ought to be considered. For instance, three components of *Quil A*, QH-A, QH-B and QH-C have been separated and tested for toxic and adjuvant activity. For instance QH-B and QH-C have better adjuvant activity than QH-A. However, in terms of hemolytic activity and DL50, QH-B showed the most toxic profile, and it has been declared too toxic for clinical studies (Ronnberg *et al.*, 1995; Sun *et al.*, 2009). Noteworthy is the saponin QS21, an active fraction of the *Quil A*. Adjuvants containing QS21 are already tested in clinical trials (Zhu *et al.*, 2016)

ISCOMs have been combined with CTA1-DD to associate two different adjuvant mechanisms for a mucosal application (Mowat *et al.*, 2001). While APCs uptake ISCOMs, CTA1-DD binds to B cells, improving antigen presentation (Lycke, 2004b). The couple of adjuvants showed higher titer of IFN γ and T cells proliferation after nasal administration than nasal and subcutaneous (Mowat *et al.*, 2001). Intranasal administration of ISCOM carrying PR8, an influenza virus antigen, and CTA1-DD induced high enhancement of the immune response, e.g. serum antibodies, Th1 and Th2 responses and mucosal IgA (Helgeby *et al.*, 2006).

2.3.3 Looking for a Universal influenza vaccine

Influenza virus owns the ability to seasonally drift. Therefore, every year, vaccines should be adapted to the new emerging strains. The vaccine development and production, needed to adjust the vaccine to the mutated virus, takes at least 6-8 months. Moreover, seasonal vaccines cannot protect individuals from new hypothetical pandemic virus. Hence it raises the question of a universal flu vaccine: one that can ideally protect from the changes due to the drift and shift of influenza virus and also able to confer long-lasting protection (Krammer *et al.*, 2014). Different approaches aiming to develop a universal vaccine against influenza are currently under investigation, such as using HA, NA or M2 surface proteins as vaccine antigens. HA and NA are glycoproteins accessible on the virus surface and therefore strongly immunogenic, however they drift and shift, while M2 has remained almost constant since the Spanish flu (Fiers *et al.*, 2004). HA is composed by a globular highly-variable head and a stalk-conserved domain. Providing the vaccination with HA stalk-domain, antibodies neutralizing heterologous influenza virus can be produced, however weak protective efficiency is elicited since the stalk-domain is less immunogenic than the globular one (Jang *et al.*, 2014).

On the other hand, NA-specific antibodies avoid the release of the new virions from the infected cells preventing the viral spread and reducing the severity of the illness (Ebrahimi *et al.*, 2011; Wohlbold *et al.*, 2014). However, a NA influenza vaccine does not induce strong protection, which enables the use of this antigen together with more immunogenic proteins, like HA (Wohlbold *et al.*, 2014).

M2 extracellular domain (M2e) has been investigated as vaccine antigen but it retains a poor immunogenicity. This ion channel is highly represented on virus infected cells but scarcely on the virus itself and it is conserved in all human Influenza A strains (De Filette *et al.*, 2006; Fiers *et al.*, 2009). Moreover, due to its small size and its location in the viral membrane, M2e is not accessible to antibodies. To implement M2e potency, multiple approaches have been established (Ebrahimi *et al.*, 2011). A general strategy consists in binding M2e to a domain able to increase its immunogenicity: this can be achieved by obtaining particles as final reaction product. Therefore, M2e has been fused to GCN4, an eukaryotic transcriptional activator protein (De Filette *et al.*, 2008), or to the truncated heat shock protein (HSP70) molecule of *Mycobacterium tuberculosis* and *E. coli* (Ebrahimi & Tebianian, 2010; Ebrahimi, Tebianian, *et al.*, 2010). Linking the M2e peptide to the hepatitis B (HBc) virus core protein, Neiryneck *et al.* synthesized particles that could effectively protect mice against a lethal challenge after nasal or intraperitoneal administration of the particles (Neiryneck *et al.*, 1999). These particles were further optimized by linking multiple M2e sequences in tandem to the N-terminus of HBc. This induced greater protection than other constructs and higher anti-M2e antibodies than anti-HBc, supporting it as a good candidate for a universal flu vaccine. This candidate has also been implemented by co-administration of adjuvants, such as the non-toxic form of the heat labile toxin, LTR192G. This association enhanced M2e-specific antibodies and reduced morbidity conferring a complete protection against challenge (De Filette *et al.*, 2005). M2e-HBc was also nasally administered with the mucosal adjuvant CTA1-DD. This combination significantly increased protection, decreased morbidity and directed the immune response towards a balanced Th1/Th2 response, providing complete protection in mice (De Filette *et al.*, 2006). M2e-HBc particles were tested in Phase I clinical trials by Sanofi Pasteur. The particles were tested alone and in presence of two different adjuvants. This study concluded that the vaccine candidate ACAM-FLU-A was safe and induced immune response in most of the vaccinated individuals (Fiers *et al.*, 2009).

2.3.4 CTA1-3M2e-DD

CTA1-DD had been shown to be an optimal mucosal adjuvant candidate. This innovative adjuvant targeted approach has been conjugated with the idea of an M2e-based universal vaccine, since M2e vaccines are capable to induce intra- and heterosubtypic immune response against influenza (De Filette *et al.*, 2006; Neiryneck *et al.*, 1999). To overcome their weak immunogenicity, subunit vaccines often require the use of adjuvants. CTA1-DD has been therefore fused to M2e, to give CTA1-M2e-DD.

The production of CTA1-M2e-DD is achieved by expressing the protein vector in *E.coli* DH5 cells. After cell harvesting and centrifugation, the protein is collected as inclusion bodies. The inclusion bodies are washed and the protein extracted with 8M urea. The fusion protein is diluted and purified by ion exchange and size exclusion chromatography. The fusion protein containing a tandem of M2e, CTA1-3M2e-DD, is produced in the same way (Figure 8).

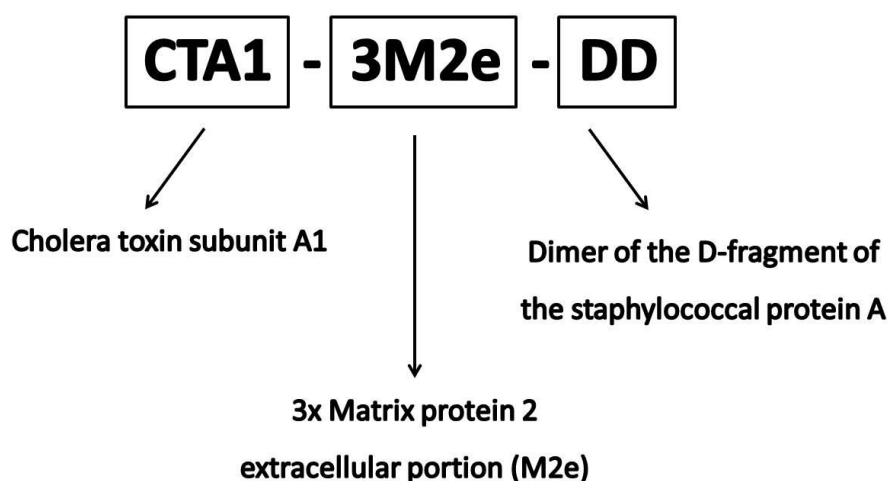


Figure 8 Adjuvanted antigen CTA1-3M2e-DD.

The fusion protein is composed by three parts: the adjuvant CTA1, subunit A1 of the cholera toxin; the antigen M2e, extracellular domain of the conserved porous channel of influenza A and a targeting portion DD, dimer of the D fragment of the staphylococcal protein A.

The B cell ability to recognize the M2e fused with the adjuvant has been questioned.

After intranasal administration of the new constructs, CTA1-M2e-DD induces high M2e-specific serum antibodies titer and M2e is still recognized by the B-cell receptor. In addition, to induce T cell proliferation, 1µg CTA1-3M2e-DD is as effective as 5µg of CTA1-M2e-DD. This supports

the synergistic effect induced by the tandem of M2e. Moreover, CTA1-3M2e-DD elicited protective immunity against lethal challenge in mice (Eliasson *et al.*, 2008).

CTA1-3M2e-DD has also been formulated with ISCOMs as particulate adjuvant/vector, since CTA1-DD and ISCOMs possess two complementary mechanisms of immune system stimulation. As a matter of fact, the former targets B cells whereas the latter dendritic cells (DCs) or more generally antigen presenting cells (APCs).

Immunogenicity of nasally administered CTA1-3M2e-DD/ISCOMs was assessed after prime and boost immunization in mice. Dose dependent M2e-specific B and T cell responses have been evidenced, reaching a plateau at the dose of 5 µg. In addition, IgA in BAL and serum antibodies (IgG1 and IgG2) were detected.

To exert its adjuvant property CTA1-3M2e-DD needs the ADP-ribosylating activity of CTA1 to be functional. That was proved by the immunization with an inactive mutated form of the fusion protein (CTA1(R7K)-3M2e-DD) with ISCOMs. The immunogenicity was drastically reduced by the inactive compound administration.

In addition, the stability studies of CTA1-3M2e-DD/ISCOMs showed that the vaccine is stable for 1 year at 4°C and that the lyophilized form can induce specific serum antibodies production after three intranasal administrations. Finally, this combination protected against lethal challenge with influenza virus (Eliasson *et al.*, 2011).

2.4 Influenza treatment: antiviral drugs

Whenever prophylaxis fails, treatment against serious influenza illness is needed. Antiviral drugs are therefore available as second line of defense. Antivirals act at different stages of the viral infection. In the case of Influenza three main proteins are traditionally targeted: neuraminidase (NA), M2 and RNA polymerase (T. C. Li *et al.*, 2015). Also RNA synthesis inhibitors, such as ribavirin, are also used (Air, 2012).

Neuraminidase inhibitors include Zanamivir (marketed as Relenza) and Oseltamivir (known as Tamiflu). These drugs mimic the natural substrate of NA, the sialic acid, showing high affinity for the viral target and blocking the release of the virions from the cells (Air, 2012; T. C. Li *et al.*, 2015).

Amantadine and its methyl derivative, named rimantadine, are the most popular M2 inhibitors. They are known to bind both the closed and open conformation of the proton pump of Influenza A virus (Hay *et al.*, 1985; Jing *et al.*, 2008). Adamantanes binding to M2 inhibit the uncoating of

the virus in the endosomes. However, these drugs are no longer extensively used for influenza infection treatment because of virus resistance (Air, 2012; T. C. Li *et al.*, 2015).

The favipiravir (or T-705), an RNA polymerase inhibitors, is active on influenza A, B and C as well as on other viruses.

In addition to the drugs inhibiting the classical targets, new molecules are under investigation, including the sialidase DAS181, an inhibitor of influenza virus attachment. DAS181 cleaves the sialic acid bond on human epithelial cells (Colombo *et al.*, 2016). Molecules suitable to bind sialic acid or blocking hemagglutinin maturation (like Nitazoxanide) are being investigated (Edinger *et al.*, 2014; T. C. Li *et al.*, 2015).

RESULTS

In this project we used cationic porous maltodextrin nanoparticles with a lipid core (NPL). NPL are an optimal tool for drug delivery, since they can improve the intracellular release of several molecules, in particular low molecular drugs and proteins (Dimier-Poisson *et al.*, 2015; C. Dombu *et al.*, 2012; Kroubi *et al.*, 2010; Loiseau *et al.*, 2002).

Firstly we investigated the loading of these nanoparticles with a model protein (ovalbumin, OVA) and their biodistribution after intranasal administration. These studies were required to further apply the NPL technology as adjuvant for a universal influenza vaccine in the UniVacFlu project.

Hence in the first part of this chapter we present the publication entitled “Mechanisms allowing protein delivery in nasal mucosa using NPL nanoparticles”, concerning the mechanistic study of protein-loaded NPL. Whereas, in the second part, we report results about formulation, stability and delivery of the universal influenza vaccine developed. These results indicate that NPL are an ideal platform for the mucosal delivery of proteins and vaccines.

Nanoparticles for protein delivery in the nasal mucosa

Presentation of the study

The study reported in this article assesses the interactions of porous cationic polysaccharide nanoparticles featuring a lipid core (NPL) with the nasal mucosa. The NPL were prepared, characterized and loaded with ovalbumin (OVA). We also prepared fluorescently labeled NPL and loaded them with a fluorescently labeled protein. The NPL and the protein were tracked *in vitro* and *in vivo*. These findings showed that NPL did not cross the epithelial barrier of the nose *in vitro* nor *in vivo*. NPL delivered the protein into the cell and prolonged the protein residence time in the nose.

**PUBLICATION 3:
MECHANISMS ALLOWING PROTEIN
DELIVERY IN NASAL MUCOSA USING NPL
NANOPARTICLES**



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Mechanisms allowing protein delivery in nasal mucosa using NPL nanoparticles



B. Bernocchi^{a,b,c}, R. Carpentier^{a,b,c,*}, I. Lantier^{e,f}, C. Ducournau^{f,e}, I. Dimier-Poisson^{f,e}, D. Betbeder^{a,b,c,d}

^a Inserm, LIRIC—UMR 995, F-59000 Lille, France

^b Université de Lille, LIRIC—UMR 995, F-59000 Lille, France

^c CHRU de Lille, LIRIC—UMR 995, F-59000 Lille, France

^d University of Artois, G2000 Arras, France

^e INRA, UMR1282 Infectiologie et Santé Publique, F-37380 Nouzilly, France. Laboratoire d'Expertise en Infection Animale

^f Université François-Rabelais de Tours, UMR1282 Infectiologie et Santé Publique, F-37000 Tours, France. Immunologie Parasitaire et Vaccinologie, Biothérapies Anti-Infectieuses

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ABSTRACT

The intranasal administration of proteins using nanoparticles is a promising approach for several applications, especially for mucosal vaccines. Delivery of protein within the epithelial barrier is a key point to elicit an immune response and nano-carrier has to show no toxicity. The aim of this work was to elucidate the interactions of cationic porous nanoparticles loaded with protein delivery for antigen delivery in the nose. We investigated the loading, the cellular delivery and the epithelial transcytosis of proteins associated to these nanoparticles containing an anionic lipid in their core (NPL). NPL were highly endocytosed by airway epithelial cells and significantly improved the protein delivery into the cell. *In vitro* transcytosis studies showed that NPL did not modify the *in vitro* epithelial permeability suggesting no toxicity of these carriers. Moreover protein and NPL did not translocate the epithelial barrier. *In vivo* studies demonstrated that NPL prolonged the nasal residence time of the protein and no NPL were found beyond the epithelial barrier *in vivo*, precluding a negative side effect. All together these results establish the NPL as a bio-eliminable and optimal vaccine carrier.

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1. Introduction

In recent years, nanoparticles have been increasingly studied for use in vaccine applications [1–3] owing to their potential adjuvant effect and ability to mimic viruses, characteristics related to their size, geometry and physical properties [4]. Furthermore, researchers have repeatedly reported that the use of particulate antigens is more successful than soluble ones in activating immune cells and affording longer term protection [5–7].

The mucosal route of administration is promising in order to trigger an effective immune response and protection against pathogens [8], and offers the possibility of needle-free vaccinations [9]. Notably, the nasal route is a convenient mucosal site for vaccine delivery since, besides being the natural route of infection of many pathogens, it is also non-invasive [10,11].

The translocation of nanoparticles has been extensively investigated at the blood–brain and the intestinal barriers [12–16]. However, little is known about transcytosis of nanoparticles in the airway mucosa. Scarce *in vitro* evaluations have been conducted in models of the airway epithelial barrier to improve understanding of the permeation mechanisms

governing drug and nanocarrier passage through the epithelium [17–19], but fewer *in vivo* studies have been published [20–23].

Paracellular transport is also considered as a potential pathway to overcome epithelial barriers [24] and to deliver drugs to immune cells found beneath the epithelium. The tight junction (TJ) opening can improve the drug permeation, although it could also be the result of a toxic effect [25]. Several *in vitro* models have been used to explore the interaction of xenobiotics with the airway epithelium. Human nasal septum epithelial cells RPM12650 ATCC® CCL-30™ were used however these cells pile and fail to reach a complete confluence [26,27]. The 16HBE140-human bronchial epithelial cell line are useful in the study of paracellular drug transport, since they form a pseudo-stratified monolayer and express TJ proteins [28–30] and have been established as a model system to investigate drug transport across the airway epithelium [31].

Porous nanoparticles [32] have recently been studied as vaccine delivery vectors [33]. It is possible to load these nanoparticles with proteins in order to deliver them into cells *via* endocytosis [34] and their use as nasal delivery systems for antigens has been shown to protect mice from acute and chronic infection against *Toxoplasma gondii* [33]. However, the mechanisms by which these nanoparticles deliver antigens within the airway mucosa and their ability to cross epithelial barriers have yet to be fully clarified.

* Corresponding author at: Inserm, LIRIC—UMR 995, F-59000 Lille, France.
E-mail address: rodolphe.carpentier@univ-lille2.fr (R. Carpentier).

In this work we aimed to better understand the interactions of these nanoparticles with the airway epithelial cells, their ability to cross this barrier, and to analyze the impact of the lipids within these nanoparticles on the delivery and the transcytosis of antigens in epithelial cells. *In vivo* studies were performed to track the antigen delivery in airway mucosa and its biodistribution after nasal administration. Toxicity issues are discussed.

2. Materials and methods

2.1. NP⁺ and NPL preparation

Polysaccharidic nanoparticles (NP⁺) and polysaccharidic lipidated nanoparticles (NPL) were prepared from maltodextrin (Roquette, France) as described previously by Paillard et al. [32]. Briefly, maltodextrin was dissolved in water by magnetic stirring at room temperature. A mixture of epichlorohydrin and glycidyltrimethylammonium chloride (GTMA, a cationic ligand; both from Sigma-Aldrich, France) in basic medium was added to the cationic polysaccharide leading to the formation of a gel. The gel was then neutralized with acetic acid and crushed with a high pressure homogenizer (Emulsiflex C3, Avestin, Germany). The nanoparticles (NP⁺) thus obtained were purified by tangential flow ultra-filtration (Centramate Minim II PALL, France) using a 1000 kDa membrane (PALL, France) to remove oligosaccharides, low-molecular weight reagents and salts. Purified NP⁺ were freeze dried. Lyophilized NP⁺ were resuspended in a 70% (w/w) aqueous solution of an anionic lipid (DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol from Lipoid, Germany) which was incorporated by the nanoparticles, thus obtaining NPL.

2.2. Labeling of the polysaccharide part of NPL

NP⁺ were covalently labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich, France) according to the following protocol: FITC was added to NP⁺ (NP⁺/FITC mass ratio of 10), solubilized in 0.1 M bicarbonate buffer (pH 9.5), and the solution was mixed for 6 h in the dark at room temperature. Afterwards the NP⁺-FITC were purified by tangential flow filtration and lyophilized. The NP⁺-FITC were then lipidated, as described above, in order to obtain NPL-FITC.

2.3. Labeling of the lipid part of NPL

Labeling of the phospholipids encapsulated in the NPL with DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine Perchlorate, ThermoFisher Scientific, France) was performed by mixing DiD (1 mg/ml in ethanol) for 30 min at room temperature with NPL with a final concentration of 0.7% (w/w of DPPG) obtaining NPL-DiD. After organic solvent evaporation the formulations were kept in the dark at 4 °C before use, while DiD loading was confirmed by gel permeation studies on a PD-10 Sephadex G25 desalting column (Sigma-Aldrich, France).

2.4. Protein labeling and loading into nanoparticles

The ovalbumin (OVA, Sigma-Aldrich, France) was labeled with FITC (Fluorescein-5-isothiocyanate, ThermoFisher Scientific), TRITC (tetramethylrhodamine-5-isothiocyanate, ThermoFisher Scientific, France) or CF750[®] Succinimidyl ester (Sigma-Aldrich, France) following the same protocol used for the NP⁺ labeling. The labeled protein was purified by gel filtration on a PD-10 Sephadex column, as above. The concentration of the labeled protein was then evaluated using the Micro BCA Protein Assay Kit (ThermoFisher Scientific, France) following the supplier's instructions. Labeled OVA (1 mg/ml) was post-loaded into pre-made sterile NPL (5 mg/ml) by mixing increasing amounts of OVA obtaining 1:0.5, 1:3 and 1:5 (w/w) OVA:NPL formulations.

2.5. Characterization of the NPL

The characterization of the size and zeta potential of the NPL were performed with a Zetasizer nanoZS (Malvern Instruments, France). For the size analysis, NPL (5 mg/ml) were directly measured. For the zeta potential analysis, 12 µl of NPL (5 mg/ml) were diluted to a final volume of 750 µl using distilled water and loaded into a disposable, folded capillary cell.

2.6. Characterization of OVA:NPL formulations

The size and the zeta potential of the OVA:NPL formulations were determined as described above. The analysis of the protein association to the NPL was performed by native polyacrylamide gel electrophoresis (PAGE), supplementing the formulations with electrophoresis buffer (Tris-HCl 125 Mm (pH 6.8), 10% glycerol, 0.06% bromophenol blue) and running the samples on a 10% acrylamide-bisacrylamide gel. A silver nitrate staining was subsequently performed to detect the unbound proteins and thus evaluate the amount of associated proteins.

2.7. NPL endocytosis and in vitro protein delivery

The 16HBE14o-(16HBE) human bronchial epithelial cell line obtained from Dr. Gruenert D. C. (Colchester, Vermont, USA), was maintained in Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific, France) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS, ThermoFisher Scientific, France), 100 U/ml Penicillin, 100 mg/ml streptomycin and 1% L-glutamine at 37 °C in a humidified 5% CO₂ atmosphere. The cells were plated at a density of 7.5 × 10⁵ cells/well in 6-well plates and used once they reached the confluence, after two days. The cells were treated for different times (0, 0.5, 1, 3, and 24 h) with NPL-FITC, NPL-DiD or with free OVA-FITC or OVA-FITC formulated in NPL. The cells were then analyzed with a BD Accuri™ C6 CFlow Sampler flow cytometer (BD Bioscience, USA).

2.8. In vitro transcytosis of NPL and ovalbumin across the airway epithelium

The *in vitro* transcytosis of NPL was investigated in a Transwell[®] model of the respiratory epithelial barrier. The 16HBE cells were seeded on Transwell[®] filters (3 µm porosity Transwell[®] filters, BD Bioscience, France) at a density of 1 × 10⁶ cells/Transwell[®] (0.9 cm²). The confluence was checked by transepithelial electrical resistance (TEER) measurement with an epithelial VoltOhmmeter (EVOM2, World Precision Instrument, USA) equipped with an STX2 electrode. The cells were pre-incubated for 30 min with Hank's Balanced Salt Solution (HBSS, Life Technologies, France) at 37 °C before measuring the permeability. A low molecular weight chitosan (Sigma-Aldrich, France) solution in HBSS at pH 6.5 (0.05% w/v) was used as positive control for the TJ opening and a 50 µg/ml solution of lucifer yellow (Sigma-Aldrich, France) as control for the paracellular and transcellular transport [35,36].

The cell monolayers were treated with 25 µg of NPL-FITC, NPL-DiD or with OVA-TRITC:NPL formulation, using HBSS as donor and acceptor medium. The TEER was checked after 30 min and every hour for 3 h moving the Transwell[®] in a plate with fresh acceptor medium before each measurement. The samples from the apical side and basolateral side were collected separately and the fluorescence was measured with a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Scientific, France).

2.9. Mice

Six-to-eight week-old Swiss OF1 mice were purchased from CER Janvier (France) and maintained under conventional conditions. Experiments were carried out in accordance with the guideline for

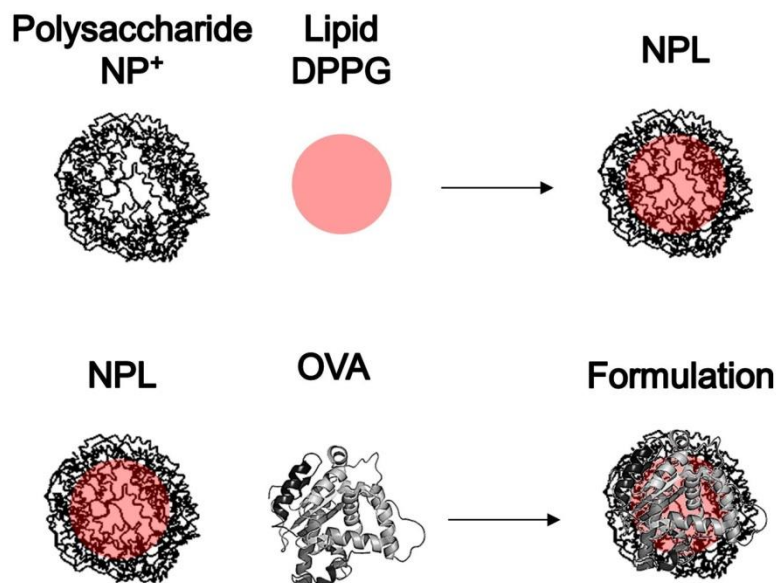


Fig. 1. Representation of NPL and ovalbumin (OVA) formulation. Premade polysaccharidic positive NP⁺ are post-loaded with an anionic lipid to obtain NPL. The antigenic protein is postloaded in NPL to prepare the formulation.

Table 1

Characterization of the formulations OVA:NPL. Size (Z-average), PDI (polydispersity index) and Zeta potential are measured in triplicate with a Zetasizer NanoZS.

	Z-average (nm)	PDI	Zeta-potential (mV)
NP ⁺	70.39	0.233	+45.9 ± 6.86
NPL	76.07	0.211	+44.2 ± 10.15
OVA	1.976	0.477	-7.77 ± 5.60
OVA:NPL 1:0.5 w/w	2782.67	1	+25.17 ± 6.937
OVA:NPL 1:3 w/w	76.71	0.261	+33.27 ± 9.173
OVA:NPL 1:5 w/w	62.39	0.236	+33.47 ± 8.417

animal experimentation (EU Directive 2010/63/EU) and the protocol was approved by the local ethics committee at Tours University (CEEA VdL).

To eliminate the background fluorescence caused by the grain-based diet, the animals were fed with the AIN-93M purified and dedicated diet for fluorescence optical imaging (TestDiet, United Kingdom) 7 days before instillation, and throughout the *in vivo* biodistribution study. To reduce background signal and light absorbance due to their fur, the animals were treated with depilatory cream one day before instillation and imaging process.

2.10. In vivo biodistribution studies of OVA alone or loaded in NPL after nasal administration

Three groups of non-anesthetized mice (3 mice/group) were designated as follows: control group (PBS), CF750[®] labeled ovalbumin (OVA-CF750[®]), formulation OVA-CF750[®]:NPL ratio 1:3 (w/w). Mice were administered with 10 µg of protein by nasal instillation, 5 µl in each nostril. The three groups of mice were maintained in separate boxes with water and diet *ad libitum*.

Longitudinal studies in individual animals were performed using the *In Vivo* Imaging System IVIS[®] Spectrum (PerkinElmer, Waltman, USA). Mice groups were successively imaged at 0.5, 1, 1.5, 2, 2.5, 3, 6, 24, 48 h following instillation, and one of the control mice was also imaged with each group at each time point. Acquisitions and analyses of images thus obtained were performed with the PerkinElmer Living Image software (version 4.2).

2.11. In vivo biodistribution studies of OVA and OVA:NPL in nostrils

Non-anesthetized mice were given 20 µl nasal instillations of solutions containing 8.3 µg of OVA-TRITC or the same amount of OVA-TRITC loaded in NPL-FITC at the ratio 1:3 (w/w). As a negative control,

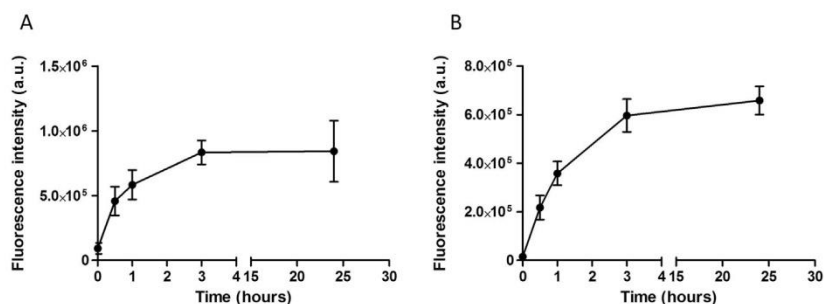


Fig. 2. Flow cytometry analysis of NPL endocytosis. 16HBE cells were treated for different times (0, 0.5, 1, 3 and 24 h) with NPL-FITC (A) or NPL-DiD (B). Results are expressed as mean ± SD of three independent experiments.

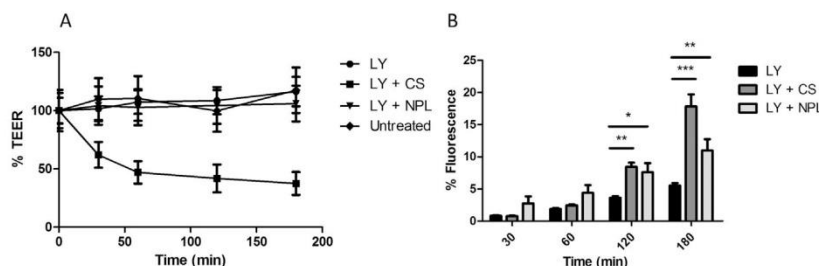


Fig. 3. A: Evaluation of tight junction opening, of 16HBE cells treated with either chitosan (CS) or NPL. Untreated cells were compared with cells treated with NPL, CS or lucifer yellow (LY). B: Evaluation of paracellular and transcellular permeability of LY. Cells monolayer with LY alone and in presence of CS or NPL. Results are expressed as the means \pm SD of triplicate measurements of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

mice received 20 μ l of PBS. The sacrifice was performed by cervical dislocation at different time points (1 and 3 h). The nasal cavities were then isolated and fixed in 4% paraformaldehyde (VWR International, France) for 24 h. Afterwards the nasal cavities were decalcified for 7 days in 10% EDTA (Sigma-Aldrich, France) and frozen in Tissue-Tek[®] OCT compound (Sakura[®] Finetek, USA), after which 10 μ m slices were obtained. The nuclei were stained with Hoechst 33342 (ThermoFisher Scientific, France). Then slices were mounted with Dako fluorescence mounting medium (Agilent Technologies, France) and imaged with a confocal microscope (LSM 710, Zeiss, France).

3.12. Statistical analysis

One-way ANOVA and Two-way ANOVA plus post-test were used to determine the significance of variations between groups using GraphPad[®] Prism software.

3. Results

3.1. Characterization of NPL

NP⁺ were made of porous cationic nanoparticles and lipids inserted into NP⁺ to produce NPL (Fig. 1). The mean diameters of NP⁺ and NPL were 70 and 76 nm and their polydispersity indexes were 0.23 and 0.21, respectively (Table 1). They were both highly cationic as their zeta potentials were +45.9 and +44.2 mV, respectively. Since the association of lipids with the NP⁺ did not significantly modify their size and zeta potential, we concluded that these lipids were inserted into the core of the nanoparticles.

3.2. NPL endocytosis

We evaluated NPL endocytosis by airway epithelial cells using flow cytometry. The polysaccharidic part of the NPL was covalently labeled with FITC (NPL-FITC) while the lipidic core was labeled with the lipidic dye DiD (NPL-DiD). Following either the polysaccharidic or the lipidic part, the NPL endocytosis into epithelial cells increases in a similar way, reaching a plateau after 3 h (Fig. 2A and B). The endocytosis rate

of NPL remained constant after 24 h. Moreover the kinetics profiles of endocytosis of the polysaccharidic or the lipidic parts of NPL showed the same trend for 24 h.

3.3. Evaluation of NPL transcytosis through the airway epithelial barrier

The 16HBE cells were cultured on Transwell[®] filters until the cells reached confluence (TEER value in the range of 250–750 $\Omega \times \text{cm}^2$). No decrease of TEER was observed after treatment with NPL indicating an absence of toxicity for these carriers. Chitosan (CS), that reportedly opens the TJ by an integrin mediated mechanism [37,38], was used as positive control (Fig. 3A).

To assess the permeability of the epithelial barrier, the transport of lucifer yellow (LY) across the airway mucosa was evaluated (Fig. 3). The paracellular permeation of LY reached 6% of the starting fluorescence after 3 h. A significantly higher permeation of LY (17.8% of initial fluorescence after 3 h) was observed when the tight junctions were opened using CS. The NPL induced a more rapid permeation of LY across the epithelial barrier (11% of initial fluorescence), but to a lesser extent than CS after 3 h.

Prior to the NPL transcytosis test, we first verified that NPL were able to cross the Transwell[®] filter (results not shown).

NPL-FITC or NPL-DiD were tested on the Transwell[®] model of the airway epithelial barrier in presence or absence of CS in order to evaluate NPL transcytosis. After 3 h incubation with NPL-FITC no significant transcytosis occurred. In addition, no fluorescence was detected in the basal compartment of the Transwell[®] treated with NPL-DiD (Fig. 4B). This confirmed that the NPL (both the polysaccharide and lipid parts) did not cross the epithelial barrier. Furthermore, even in conditions where tight junctions were opened, NPL transport was not increased (Fig. 4A and B).

3.4. Characterization of the OVA:NPL formulations

Different OVA:NPL formulations were prepared at different OVA:NPL (w/w) ratios.

Size analysis of the 1:0.5 OVA:NPL formulation by dynamic light scattering showed an aggregated formulation with a particle size of

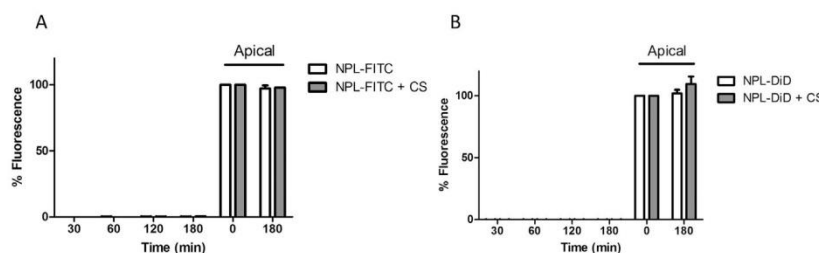


Fig. 4. Investigation of the *in vitro* translocation of NPL polysaccharide (A) and lipid part (B) across confluent 16HBE monolayers.

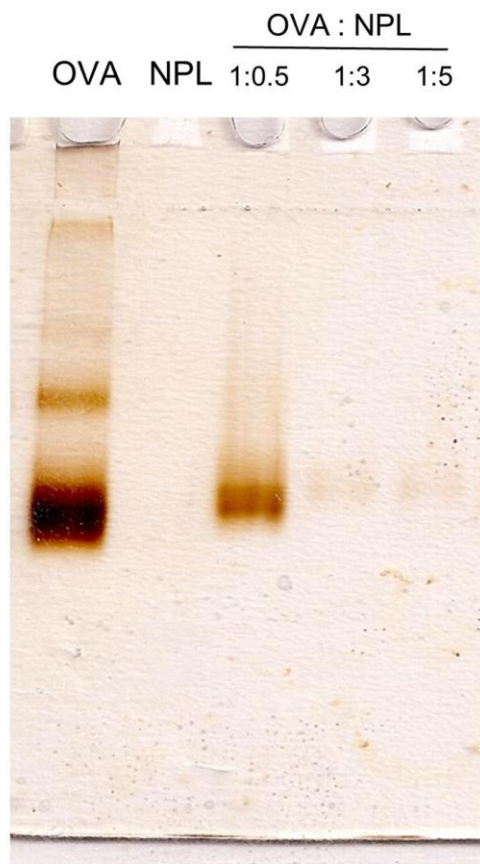


Fig. 5. Characterization of the ovalbumin (OVA) association to the NPL by native polyacrylamide gel (PAGE) electrophoresis using OVA:NPL at 1:0.5, 1:3 and 1:5 (w:w) ratios. Unbound proteins were revealed by silver nitrate staining.

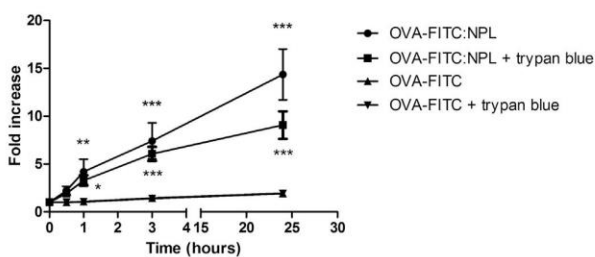


Fig. 6. Analysis of protein delivery by NPL into airway epithelial cells (16HBE) by flow cytometry. 16HBE were treated with free or formulated OVA-FITC for different time. Trypan blue was used to determine the % of protein bound on cell surface. Results are the mean of three independent experiments. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

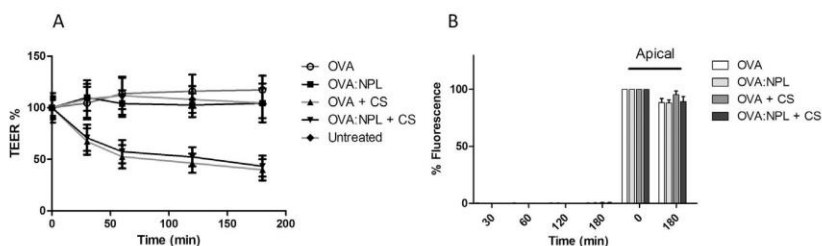


Fig. 7. *In vitro* passage of ovalbumin (OVA) free or formulated in NPL on 16HBE Transwell® model. Evaluation of the tight junction opening (A) and of the protein passage across the epithelial barrier (B).

2.7 μm and a high polydispersity index (Table 1). Moreover, compared to the NPL alone (+44.2 mV), a decreased zeta potential was observed for this formulation (from +44 mV to +25 mV). The OVA association to the NPL was assessed by polyacrylamide gel-electrophoresis (PAGE) in non-denaturing conditions revealing the presence of unbound proteins (Fig. 5).

Increasing the protein:NPL mass ratio to 1:3, we observed that the size and zeta potential of OVA:NPL was unchanged relatively to NPL alone (Table 1). Native-PAGE electrophoresis (Fig. 5) revealed that the formulation of OVA:NPL at mass ratios of 1:3 and 1:5 led to the complete association of the ovalbumin to the NPL. The 1:3 formulation fits the optimal criteria for the study of protein delivery (similar size and zeta potential than NPL alone, complete protein association) and was used in the following experiments.

3.5. Kinetics of protein delivery into airway epithelial cells by NPL

The OVA delivery by NPL into airway epithelial cells was evaluated by flow cytometry. The epithelial cells were treated for different times (0.5, 1, 3 and 24 h) with OVA-FITC either free or formulated in the NPL at a 1:3 mass ratio. Trypan blue was used to quench the extracellular fluorescence adsorbed on cell surface [39].

Compared to T_0 , a very low endocytosis was observed for OVA-FITC after 24 h (Fig. 6). Interestingly NPL highly increased OVA association with the cells (14.4 fold increase). Trypan blue experiment confirmed that OVA-FITC was endocytosed and that NPL highly increased its uptake.

3.6. *In vitro* transcytosis of free OVA or OVA:NPL

The transcytosis of free OVA or OVA:NPL was tested in the Transwell® model of the airway epithelial barrier, in the presence or absence of chitosan (CS). Neither free OVA nor OVA:NPL modified the TEER% in the absence of CS (Fig. 7A), while in the presence of CS the TEER% decreased to 45%, indicating the TJ opening.

The fluorescence of OVA-TRITC in the basal compartment of the Transwell® model was measured (Fig. 7B): after 3 h incubation, only 0.4% of the starting fluorescence of the OVA-TRITC was detected. No significant differences were observed in terms of OVA transcytosis between formulations even in the presence of CS. We concluded that NPL did not promote OVA transcytosis and that tight junction aperture by CS treatment was insufficient for the protein to cross the epithelial barrier.

3.7. *In vivo* biodistribution of OVA and OVA:NPL after nasal administration

We evaluated the *in vivo* biodistribution of free OVA versus NPL-loaded OVA for 2 days in mice using real time fluorescence optical imaging. Mice were nasally administered with free OVA-CF750® or OVA-CF750®:NPL; dorsal and ventral views of living animals were taken at defined time points (Fig. 8). As can be observed ventrally, a low fluorescence persistence of the unformulated protein is observed in the nose and totally disappeared after 1.5 h. Similar considerations

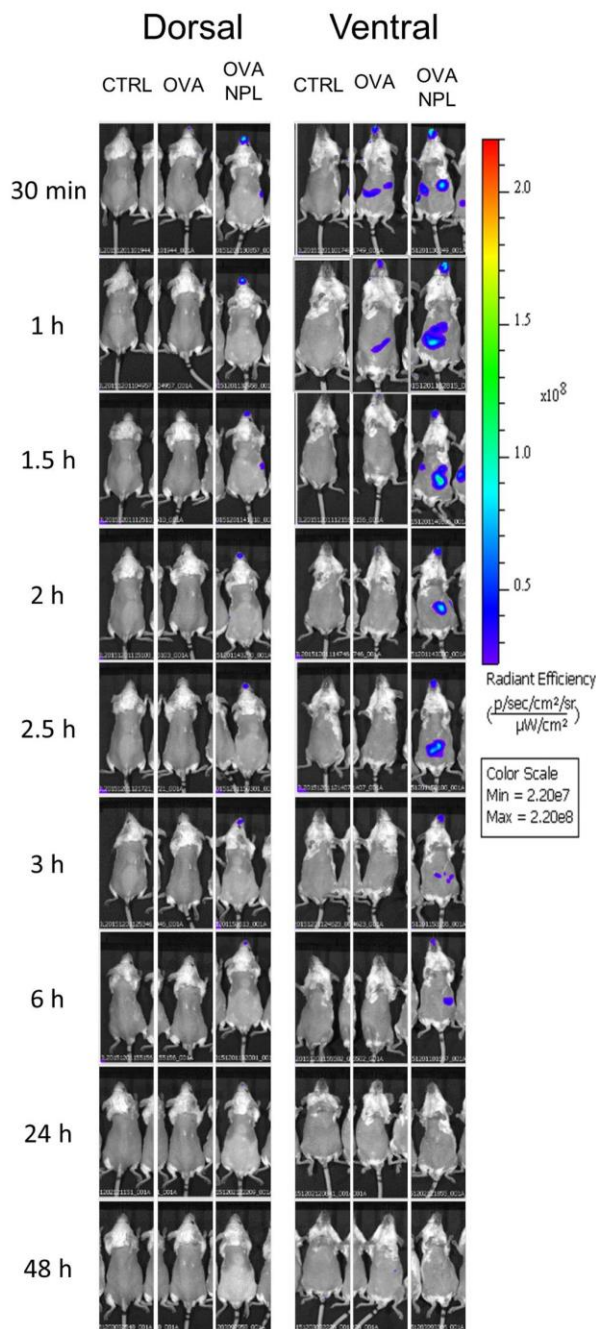


Fig. 8. Representative *in vivo* fluorescence images of OVA-CF750 biodistribution in mice. Mice were pre-treated with PBS (CTRL) or OVA-CF750, free and formulated in NPL. The ventral and the dorsal side of mice were imaged at different time points with *In Vivo* Imaging System IVIS[®] Spectrum (*n* = 3 per group).

have been brought dorsally. Moreover in the dorsal view we did not evidence fluorescence diffusion in the brain. Interestingly, NPL increased OVA residence time in the nose to at least 6 h (against 1.5 h for free OVA). Furthermore fluorescence related to labeled OVA was found in the feces (results not shown), suggesting its elimination.

3.8. Evaluation of NPL and OVA residence in the nose

Consistent with the *in vivo* distribution results of OVA showing a discriminative time point of 1.5 h, we wanted to determine the localization of NPL and OVA in airway mucosa 1 h and 3 h after administration.

Mice were nasally instilled with OVA-TRITC:NPL-FITC and nose tissue, sampled at three different depths (I, II and III; Fig. 9), was subsequently analyzed after nuclei staining. We observed the presence of NPL after 1 h, but only at the mucosal level. Additionally, after confocal analysis, NPL endocytosis in airway mucosa was observable 1 h after administration and became more pronounced after 3 h. The NPL were observed in all the different depths of the nasal tissue, and also at the surface of the nose-associated lymphoid tissue (NALT) at 1 and 3 h after the nasal instillation. However, no NPL was detectable on the basal side of the epithelium, underlining the inability of NPL to cross this cellular barrier *in vivo*. Contrary to what is observed in whole nose biodistribution studies (Fig. 8), OVA was not detectable in these sections (Fig. 9).

4. Discussion

The nasal route of administration has been investigated in recent years as an effective mucosal site suitable for non-invasive vaccine delivery, and able to induce both systemic and mucosal immunity. Many studies have shown that nasal administration of particulate antigens (Ag) is more immunogenic compared with soluble ones [8]. For instance, it has been demonstrated that administering the whole, inactivated influenza virus was more immunogenic than administration of split, subunit or virosome vaccines [5]. We obtained similar results more than a decade ago when we demonstrated that maltodextrin nanoparticles, covered by a lipid bi-layer and loaded with HBs Ag and beta-galactosidase, were able to induce strong mucosal as well as systemic antibody and cytotoxic T cell responses, while free Ag was poorly immunogenic [40]. Recently, we demonstrated that nanoparticles loaded with *Toxoplasma gondii* Ag after intranasal administration were able to induce strong TH1 and TH17 responses, and were able to protect mice against an orally administered lethal challenge with wild parasite [33]. Furthermore, we also demonstrated that these nanoparticles were highly endocytosed *via* the clathrin pathway and highly exocytosed *via* a cholesterol-dependent pathway, delivering Ag within the cytosol of airway epithelial cells [34,41]. These results might explain the increased immunogenicity observed [33].

In this study, we further investigated the role of these supramolecular nanoparticles, made of a polysaccharide matrix loaded with phospholipids in their core (NPL), as potential vaccine delivery systems in airway mucosa, and the different constituents of these NPL (Fig. 1) were tracked to assess their fate after endocytosis in the mucosa.

We first confirmed that the lipid loading into the NP⁺ did not vary the characteristics of size and zeta potential of the particles, suggesting the complete lipid incorporation into the maltodextrin structure (Table 1). As reported by Kroubi et al., the matrix saturation occurs at 70% of lipid loading (w/w); at higher percentages of lipid, irreversible aggregation of NPL was noted [42].

Due to the supramolecular structure of these nanoparticles made of polysaccharide and lipids we decided to follow the endocytosis of all their components in the cells in an effort to better define their role in protein delivery.

Interestingly, we observed similar uptake kinetics for both NPL components: polysaccharide and lipid (Fig. 2). This result suggests that the lipids are not released from the nanoparticles in the cells during their endocytosis. This is in contrast to liposomal preparations whose phospholipids were found to be converted to cellular phospholipid after lysosomal degradation [43], while our results indicate a high stability of the lipid (DPPG) inside the NPL.

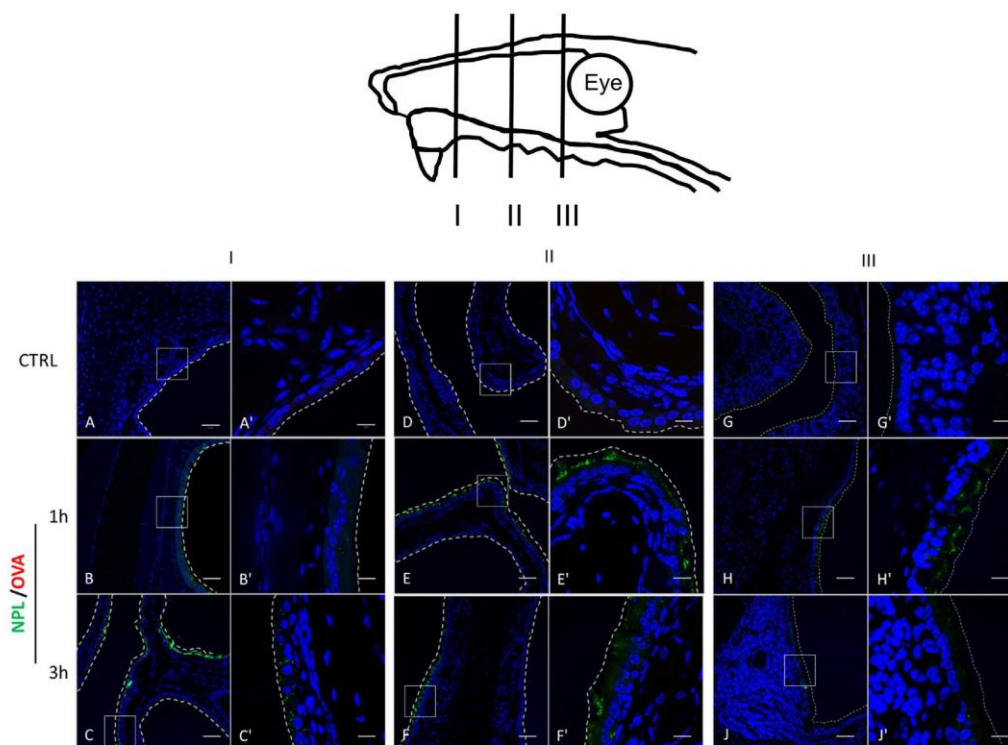


Fig. 9. Confocal microscopy analysis of frozen section of nasal mice tissue after different time points. Nasal biodistribution of the OVA-TRITC:NPL-FITC in three representative depth of the mice nose are reported, from the anterior segment (I) to the posterior segment (III). Enlargements of the regions in the white frames are reported. Nuclei were stained with Hoechst, NPL are labeled with FITC and OVA with TRITC. Scale bars 50 μm (A–J) and 10 μm (A'–J'). *In vivo* NPL endocytosis was confirmed and no NPL transcytosis was observed.

We thought it is essential to track the fate of these nanoparticles after nasal administration to fully understand how they deliver antigens in mucosa and potentially cross the mucosal barriers, both to learn their mechanism of action and investigate potential toxicity issues. We observed that NPL do not open the TJ and do not cross the airway epithelial barrier *in vitro* or *in vivo* (Figs. 4 and 9). Interestingly, in contrast to what is described for other particles [44], we were able to discount any nose-to-brain delivery of these nanoparticles as they were found not to cross the epithelial cells *in vitro* or *in vivo*. Nose-brain passage of nanoparticles and their potential toxicity would prevent further studies for vaccine applications [45].

However, in some circumstances, the epithelial barrier could become damaged leading to TJ opening [46,47]. Chitosan, a cationic polysaccharide polymer, opens TJ *in vitro* and *in vivo*, thus enhancing the passage of drugs unable to traverse the epithelial barrier by transcytosis by instead favoring paracellular passage [48]. We demonstrated that when the TJ were opened by the action of CS (Figs. 4 and 7), neither OVA (MW = 43,000 Da) nor NPL crossed the cells monolayer suggesting that opening the TJ aperture facilitates paracellular passage only for low molecular weight drugs. Furthermore cytotoxicity and genotoxicity studies were also performed on these cells and it was shown that even at high doses these NPL were not toxic [49].

The NPL can be loaded with a large amount of different proteins and the formulation is effective to induce humoral, cellular and mucosal responses when administered *via* the nasal route [33]. However, the underlying mechanisms of antigen delivery or antigen translocation by the NPL are not fully understood. Ovalbumin is a well-known vaccine model antigen, currently used as a template for nanoparticulate vaccines [3,50]. The formulated protein is efficiently delivered into airway epithelial cells (Fig. 6). Notably, we found that the formulation of OVA in NPL increased the protein delivery into cells 14 fold. We then studied

the protein fate and its possibility to traverse the airway epithelial barrier. *In vitro* we did not observe any transcytosis of the free or formulated OVA (Fig. 7). We thus concluded that protein (in this case ovalbumin) is not transported across the epithelial barrier by transcytosis and that NPL do not facilitate nor induce this mechanism.

To study the permeability of the *in vitro* epithelial barrier model, since NPL and OVA cannot cross it, we used lucifer yellow as a low molecular weight molecule. Lucifer yellow crossed the epithelial barrier *in vitro*, without modifying the TEER%, *via* paracellular transport and pinocytosis allowing potential transcytosis [35,36,51], and its passage was increased by the TJ opening (Fig. 3). Its transfer across the epithelium was also enhanced by the NPL. We suggest that this increase could be directly linked to the increased intracellular traffic due to NPL endocytosis in a non-specific manner (Fig. 3) as no interaction between LY and NPL was observed (data not shown).

Finally, *in vivo* studies were necessary to understand the real fate of the NPL and the encapsulated protein within nasal mucosa.

Biodistribution studies performed by *in vivo* imaging allowed us to follow the protein distribution. We observed that after 1.5 h the protein administered alone had totally disappeared from the nasal area (Fig. 8), while nanoparticle formulated protein was still present after 6 h. This result suggests that NPL stay in the nose and potentially protect protein from degradation as had already been observed *in vitro* [34]. At this stage we considered two complementary possibilities emanating from this result: either the longer residence time of NPL/protein was due to adsorption at the surface of the mucosa, or OVA delivery within cells was more efficient when formulated in NPL. Confocal microscopy studies clearly showed that NPL were found in mucosa cells of the nose (Fig. 9). Interestingly, we still observed after 3 h the NPL in airway mucosa cells and no NPL was observed in the tissues underneath confirming the *in vitro* results. Free OVA, due to its fast degradation,

was observed only in minute amounts and poorly detectable on the nose tissue slices.

5. Conclusion

We studied the dynamics of nanoparticle and protein interaction with the nasal mucosa. Taken together, our results provide a framework of the whole mechanism for the bio-distribution of nanoparticle protein formulations. After nasal administration the NPL are endocytosed by airway epithelial cells and deliver the protein into the cells. We suggest that the *in vivo* increased residence time of OVA in airway cells might be due to an increased cellular protein uptake from OVA:NPL compared to free OVA, due at least in part to the partial protection of the protein from degradation afforded by its encapsulation within the NPL. The NPL are probably subsequently exocytosed and, following the mucociliary movement, are endocytosed by other/deeper cells in the nasal epithelium. During these processes, the NPL continue to deliver part of the encapsulated protein but do not cross the epithelial barrier. Contrary to what has been observed with other nanoparticles, NPL did not cross the mucosal barrier. Considering the previously demonstrated ability of NPL formulations to stimulate the immune system, and our findings that they do not cross airway epithelial barriers, these carriers are thus extremely interesting candidates for nasal vaccine delivery.

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Supplementary data:

NPL endocytosis by THP-1 derived macrophages

The NPL endocytosis by macrophages was evaluated by flow cytometry. THP1-derived macrophages were treated for different times with the same amount of nanoparticles. In order to follow the polysaccharide part we used NPL FITC, while NPL DiD were used in order to follow the lipid loaded into the NPL.

The kinetics of NPL endocytosis in THP-1 derived macrophages assumed a different profile compared to the epithelial cells previously observed (Figure 2, Bernocchi *et al.*). In macrophages a continuous improvement of the fluorescence associated to the cells between 3 and 24 hours is observed (Figure 9). Both types of NPL labeling in epithelial cells or macrophages did not evidence any difference in kinetics profile. This suggests that DiD was not released from NPL neither in epithelial cells nor in macrophages. The release of DiD should lead to the cellular accumulation of the fluorophore and to a different kinetics profile when compared to NPL FITC.

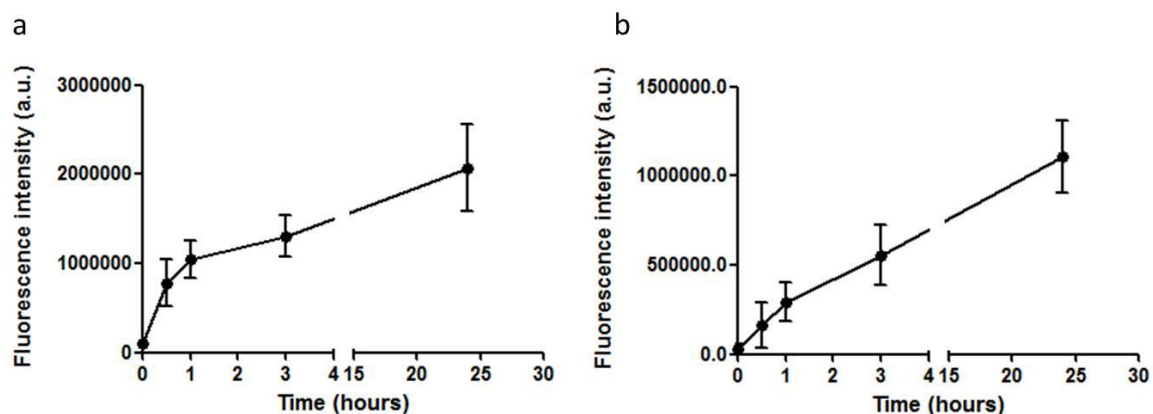


Figure 9 NPL endocytosis in THP1-derived macrophages.

The polysaccharide was covalently labeled with FITC (a); the uncovalent lipid interaction was simulated with DiD (b).

Conclusion

This study elucidated the mechanism of NPL interaction with the nasal mucosa. The understanding of the formulation biodistribution after intranasal administration is a fundamental requirement in order to prevent toxic events that might be given by the undesired brain drug delivery. Contrary to what has been observed for lactoferrin-polycaprolactone nanoparticles by Liu *et al.* (Liu *et al.*, 2013), NPL do not cross the nasal mucosal barrier, results by (Ducournau *et al.*, 2016). Moreover in this paper we found that the protein is delivered in the nasal mucosa. In the Figure 10 we propose the comprehensive mechanism of NPL interaction with the nasal mucosa and bio-elimination.

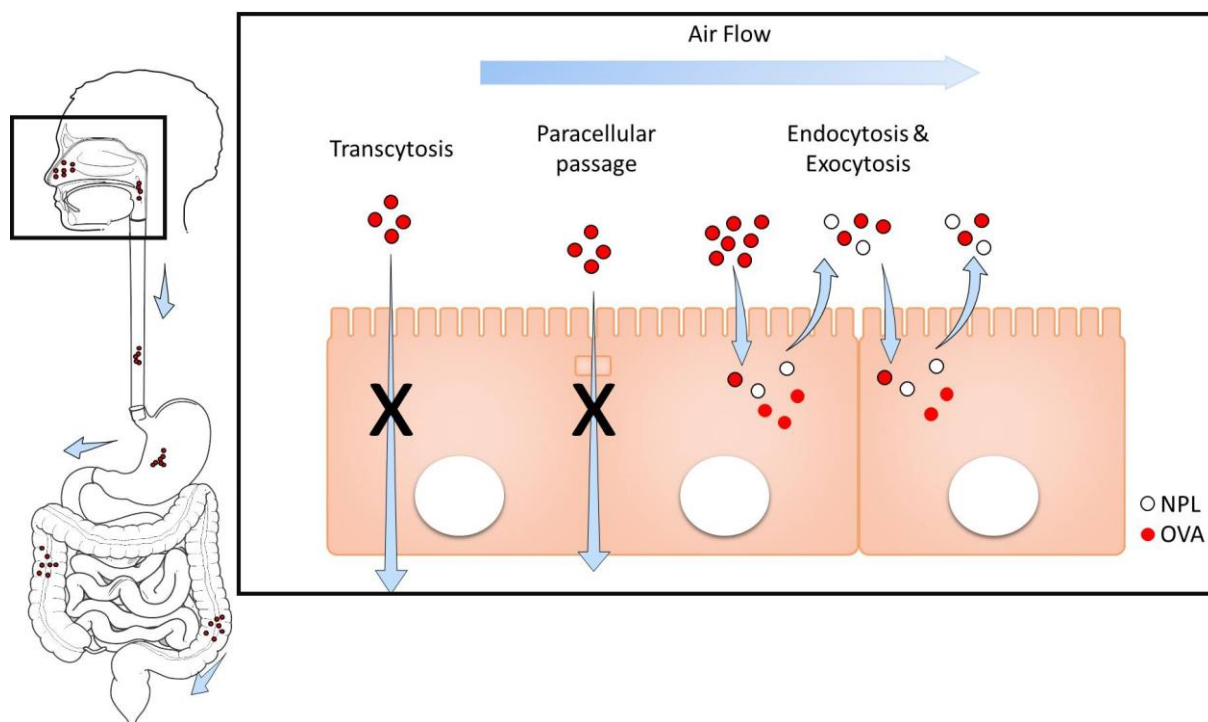


Figure 10 Mechanism of NPL interaction with the nasal mucosa.

Choice of the cell model: In vitro models of airway barriers

The vaccine formulation may have different ways to overcome the airway epithelial barrier. The epithelium filters the entry of ions and xenobiotics into the body. This process is under strict control thanks to the tight junctions. The tight junctions (TJ) are relatively narrow as the free diffusion cut-off ranges between 1-40Å (Plopper G., 2016). TJ opening may be induced by some molecules (e.g. chitosan). This may favour the passage of molecules or antigens present in the nasal cavity (De Magistris, 2006). The variation in the TJ opening may be evaluated *in vitro* via transepithelial electrical resistance measurement (TEER).

Alternatively the transcytosis pathway may be advantageous to cross the epithelial barrier. In this case the vaccine formulation is endocytosed by the cell at the apical level and exocytosed in the basolateral compartment (publication 1, Endocytosis of nanoparticles). According to which airway depth is to be investigated, different *in vitro* models are available: 16HBE14o-, Calu-3 and A549 are respectively bronchial, lower airways and alveolar cell lines. These cell lines form epithelia with distinct characteristic, (pseudostratified, columnar or alveolar) and have been used for drug transport investigations in the airways (Figure 11). Also primary cells like human alveolar-type I cells have been used for similar purpose (B. Forbes *et al.*, 2005).

In this study we chose 16HBE14o- cells because these cells have characteristics similar to the one found in nasal mucosal cells, such as shape, apical villi and tight junctions (Cozens *et al.*, 1994; Ehrhardt *et al.*, 2002; I. I. Forbes, 2000).

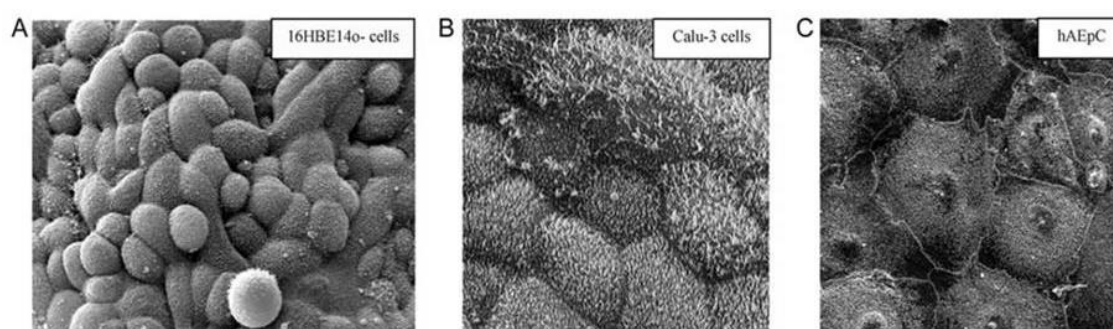


Figure 11 Scanning electron microscopy of airway epithelial cells.

(A) 16HBE14o- cell layer, (B) Calu-3 cell, (C) type I alveolar epithelial cells (hAEpC). (B. Forbes *et al.*, 2005)

Ovalbumin as a model of antigen for vaccine delivery

Ovalbumin (OVA) is the dominant protein found in avian egg-white. This glycoprotein of 45kDa belongs to the serpin (serine-protease inhibitor) superfamily although it does not show any protease inhibition activity, as opposed to other serpins. Hen egg-white OVA accounts for 386 aminoacids, one single disulfide bond and the N-terminus is acetylated. OVA exists in a native form and a more stable, compact and hydrophobic S-form. Native OVA has a three-turn alpha-helical loop and four beta-sheets. In contrast to other serpins, the cleavage of the loop does not lead to its insertion in the beta-sheets. This explains the lack of protease activity. Ovalbumin function is unknown but is supposed to be involved in chicken embryo development (Huntington *et al.*, 2001).

Despite its unidentified function, OVA is a well-characterized protein used as antigenic model thanks to its interaction with the immune system. OVA plays a role in asthma, oral tolerance and allergies. In fact, egg-based vaccines are not to be administered to allergic individuals for OVA presence as contaminant.

To investigate antigen delivery properties and the immune system stimulation, OVA has been incorporated in several potential vaccine carriers and has therefore been associated to numerous nanoparticles such as liposomes (Nakanishi *et al.*, 1997), virosomes (Bungener *et al.*, 2002), ISCOMs (Mowat *et al.*, 1991), PLGA nanoparticles (Schiller *et al.*, 2015) to improve the immunogenicity.

Indeed intranasal administration of chitosan particles loaded with OVA (0.4-1 μm) induces significantly higher IgG and IgA antibodies compared to the free protein in rats after 3 immunizations (Nagamoto *et al.*, 2004). On the other hand OVA-poly(γ -glutamic acid) nanoparticles induce CD8+T cells to produce IFN- γ , TNF- α and IL-2 but no antigen-specific IgG were detected (Uto *et al.*, 2009). Slütter *et al.* compared the immunogenicity of OVA-loaded PLGA, TMC and PLGA/TMC nanoparticles. They showed that a greater induction of serum antibodies and IgA is obtained after two nasal administrations of the positive TMC nanoparticles, whereas PLGA and PLGA/TMC nanoparticles induce negligible IgG titer. Although TMC nanoparticles failed in prolonging the nasal residence time of OVA and promoting DC uptake, they could deliver the antigen to B-cells (Slütter *et al.*, 2010).

In absence of adjuvants OVA and OVA-loaded liposomes induce poor mucosal and systemic response in mice (Vadolas *et al.*, 1995). Indeed OVA has been extensively used as vaccine model antigen for the development of new nanoparticles.

Nanoparticles for universal influenza vaccine delivery

Presentation of the study

This study is part of the program UniVacFlu, part of the European Union Seventh Framework Program FP7 that aims to develop a universal mucosal influenza vaccine. In this international project our partner synthesizes recombinant adjuvanted proteins and our role is to provide these constructs formulated with nanoparticles to test the protection from viral challenge and transmission. To design a new vectored influenza vaccine we prepared and characterized the formulations, investigated their stability and the antigen delivery in the airway epithelium. Studies investigating the mucosal and systemic immunity are currently on going in collaboration with the partners of the European consortium UniVacFlu.

This work will be included in original research articles in collaboration with the UniVacFlu partners.

POROUS NANOPARTICLES FOR THE MUCOSAL DELIVERY OF AN ADJUVANTED UNIVERSAL INFLUENZA VACCINE

1. Introduction

Nanoparticles increasingly play a substantial role in vaccine design, thanks to their ability to improve antigens stability and to enhance its delivery (Csaba *et al.*, 2009).

A nanoparticle formulation mimics the natural route of entry of influenza virus in the body and can potentially stimulate mucosal and systemic immunity (Woodrow *et al.*, 2012). Moreover, the mucosal immunization confers better protection from pathogen infection than the traditional injectable route. Protein-based vaccines are often not immunogenic enough to provide a sufficient immune response. To be effective, these vaccines need a delivery system and an adjuvant (Skwarczynski *et al.*, 2014).

Adjuvanted antigens, developed in order to circumvent the toxicity of the bacterial holotoxin cholera toxin, are fusion proteins based on the CTA1-DD adjuvant (Eliasson *et al.*, 2008; Eliasson *et al.*, 2011). These proteins contain a specific domain for B cells targeting, and possibly other antigen presenting cells, which strongly binds the Fc and Fb of immunoglobulins. DD is the dimer of the D-fragment, the synthetic analogue of *Staphylococcus aureus* protein A (L. C. Agren *et al.*, 1999). The adjuvant moiety is represented by the CTA1 domain of the fusion protein. This protein is the subunit A1 of the hexameric Cholera Toxin, known for its ADP ribosylating activity and its strong adjuvant function (L. Agren *et al.*, 1999). Nanoparticles may improve the delivery of the antigens in the mucosa, so we used porous cationic nanoparticles as drug delivery system of adjuvant and antigens for nasal application.

The nanoparticles used in this work are nanocarriers made of reticulated maltodextrin, positively charged (NP⁺) (Paillard *et al.*, 2010) and loaded with an anionic lipid (DPPG) (NPL). Hence these systems display a negative hydrophobic core surrounded by a positive polysaccharide shell. The formulation consists of three main entities: the maltodextrin, the lipid and the protein, associated by non-covalent interactions (Van der Waals forces and electrostatic interactions).

Stability is a fundamental prerequisite for licensing a vaccine. The currently marketed nasal influenza vaccine (FluMist®, Medimmune) has a shelf life of only 18 weeks and requires the cold chain (Kumru *et al.*, 2014). Hence there is a large margin of improvement with regard to vaccine stability. The use of nanoparticles to increase drug stability has already been recorded (Kumari *et al.*, 2010).

The aim of this work is to improve the immunogenicity of influenza virus antigens administered through the nasal route using nanoparticles as delivery system and to investigate the possibility to build a universal flu vaccine, able to induce cross-protection. Therefore the antigens CTA1-3M2e-DD, its inactive mutant CTA1(R9K)-3M2e-DD and hemagglutinin (HA) have been formulated with nanoparticles.

These constructs have been prepared by the conjugation of two approaches to develop an adjuvanted recombinant influenza vaccine. The mucosal adjuvant CTA1-DD conceived by Pr Lycke, from the University of Göteborg, have been fused with the M2e ectodomain, conserved epitope of influenza (Agren *et al.*, 1997; Eliasson *et al.*, 2008). Pr Fiers, from the University of Ghent, and then Pr Xaelens firstly developed the idea of universal influenza vaccines based on the tetrameric Matrix Protein 2 (Neiryneck *et al.*, 1999).

We characterized the loading of the antigens in NPL and assessed the stability for 3 and 12 months. We also investigated the ability of the NPL to deliver antigens into the airway epithelial cells and macrophages *in vitro* and we addressed the question of antigen passage across the epithelial barrier through transcytosis and paracellular pathways.

2. Materials and methods

2.1 Nanoparticles preparation

Nanoparticles (NP⁺) were produced as described by Paillard *et al.* Briefly maltodextrin (Roquette, France) was dissolved in 2N sodium hydroxide by magnetic stirring at room temperature. A mixture of epichlorohydrin and glycidyltrimethylammonium chloride (GTMA, a cationic ligand; both from Sigma-Aldrich, France) was added to the polysaccharide leading to the formation of a gel. After neutralization by means of acetic acid, the gel was crushed with a high pressure homogenizer (Emulsiflex C3, Avestin, Germany). The newly obtained nanoparticles (NP⁺) were purified by tangential flow ultra-filtration (Centramate Minim II PALL, France) using a 300 kDa membrane (PALL, France) to remove oligosaccharides, low-molecular weight reagents and salts. Purified NP⁺ were freeze dried. Lyophilized NP⁺ were

dissolved in water and a lipid (DPPG: 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol from Lipoid, Germany) was loaded into NP+ at a temperature above the gel to liquid phase transition temperature of the lipid, thus obtaining NPL.

2.2 CTA1-3M2e-DD and HA loading into NPL

The fusion proteins CTA1-3M2e-DD and CTA1(R9K)-3M2e-DD were kindly given by Mucosal Immunobiology and Vaccine Center (MIVAC) Development (University of Gothenburg). This adjuvanted antigen was loaded into premade NPL, by mixing the protein (1.66 mg/ml) with NPL (5 mg/ml) (1:3 mass ratio protein: NPL) and incubating for 30 minutes at room temperature. Different mass ratios of protein and nanoparticles (NPL) (1:0.5; 1:3; 1:5 or 1:10 protein:NPL) were prepared.

Lyophilized hemagglutinin (Recombinant Influenza A Virus H1N1 (A/Puerto Rico/8/34) Hemagglutinin/HA, Sino Biological Inc., China) was resuspended in Empigen® BB (N,N-Dimethyl-N-dodecylglycine betaine, Sigma-Aldrich, France) 1.98% obtaining a protein concentration of 1mg/ml. At room temperature hemagglutinin was incubated with either NPL or CTA1-3M2e-DD:NPL to obtain a formulation 1:5 (mass ratio) antigen:NPL.

2.3 Size and zeta potential measurement of Antigen-loaded NPL

The size and the zeta potential of antigens (CTA1-3M2e-DD, CTA1(R9K)-3M2e-DD and HA), NPL and antigen-loaded NPL were characterized by dynamic light scattering and laser doppler velocimetry with a Zetasizer nanoZS (Malvern Instruments, France). Antigen, NPL or antigen-loaded NPL were charged into the low volume quartz batch cuvette (ZEN2112) for particle size purposes. For the zeta potential analysis, samples were diluted in a final volume of 750µl and loaded into a disposable folded capillary cell.

2.4 Analysis of antigens association to NPL

The analysis of antigens association to NPL was performed by native polyacrylamide gel electrophoresis (PAGE). Antigens, NPL and antigen-loaded NPL were supplemented with the electrophoresis buffer (Tris-HCL 125Mm (pH 6.8), 10% glycerol, 0.06% bromophenol blue) and run on a 10% acrylamide-bisacrylamide gel. The gel was stained by the silver nitrate method to detect the unbound proteins.

2.5 Stability studies of CTA1-3M2e-DD-loaded NPL

The stability of the antigen CTA1-3M2e-DD, the NPL, the formulations CTA1-3M2e-DD:NPL 1:0.5 and 1:5 by mass ratio was evaluated over 3 months, in accelerated (40°C) and standard (4°C) conditions, and over 12 months at 4°C in sterile setting. The stability of the size and the zeta potential was measured by dynamic light scattering and laser doppler velocimetry, as previously described.

The stability of the antigen association to the NPL was evaluated by native PAGE as described above. The antigen degradation was assessed by SDS-PAGE, supplementing the samples with a denaturing buffer (Tris-HCL 125 mm (pH 6.8), 20% glycerol, 10% SDS, 2.5% β -mercaptoethanol and 0.06% bromophenol blue). The gels were stained by silver nitrate method.

2.6 CTA1-3M2e-DD labeling and loading into NPL

The antigen CTA1-3M2e-DD was labeled with FITC (Fluorescein-5-isothiocyanate, ThermoFisher Scientific) according to the following protocol: FITC (1 mg) was added to 10 mg of antigen (mass ratio of 10) solubilized in 0.1 M bicarbonate buffer (pH 9.5), and the solution was mixed for 6 hours protected from light at room temperature. The labeled protein was filtered by gel filtration chromatography on a desalting column (PD-10 Sephadex, Sigma-Aldrich). The concentration of the protein was then evaluated using the Micro BCA Protein Assay Kit (ThermoFisher Scientific, France) following the supplier's guide. The labeled antigen was loaded into pre-made NPL as previously described.

2.7 CTA1-3M2e-DD delivery in airway epithelial cells and macrophages

The 16HBE14o⁻ cells (16HBE), epithelial cell line, were maintained in DMEM (ThermoFisher Scientific, France) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS, ThermoFisher Scientific, France), 100 U/ml Penicillin, 100 mg/ml streptomycin and 1% L-glutamine at 37°C in a humidified 5% CO₂ atmosphere. The cells were plated at the density of 7.5×10^5 cells/well in 6-well plate and used after two days.

The THP-1 monocytes were maintained in RPMI 1640 (ThermoFisher Scientific, France) supplemented with 10% heat-inactivated FCS, 100 U/ml Penicillin, 100 mg/ml streptomycin and 1% L-glutamine, 0.05 mM β -mercaptoethanol (Sigma-Aldrich, France) at 37°C in a humidified 5% CO₂ atmosphere.

Monocytes were plated in a 12-well plate at the density of 8×10^5 cells/well in RPMI 1640 supplemented with 1% FCS and without β -mercaptoethanol. Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, France) was then added at the concentration of 20ng/ml in order to induce the monocytes differentiation into macrophages. After 72 hours the THP1-derived macrophages were washed twice with PBS and treated as explained below.

The cells were treated at different times (0, 0.5, 1, 3, and 24 hours) with CTA1-3M2e-DD-FITC or CTA1-3M2e-DD-FITC:NPL. The cells were analyzed with BD Accuri™ C6 CFlow Sampler flow cytometer (BD Bioscience, USA).

2.8 *In vitro* transcytosis of CTA1-3M2e-DD-loaded NPL through the airway epithelium

The *in vitro* transcytosis of CTA1-3M2e-DD free or loaded into NPL was studied in a Transwell® model of the respiratory epithelial barrier. 16HBE cells were seeded at a density of 1×10^5 cells/transwell (0.9 cm^2) on $3 \mu\text{m}$ porosity filters (Transwell®, BD Bioscience, France). The cell's confluence was verified by transepithelial electrical resistance (TEER) measurement with a Voltohmmeter (EVOM2, World Precision Instrument, USA) equipped with an STX2 electrode.

The cell's monolayers were treated with $8.3 \mu\text{g}$ of antigen associated or not to NPL (1:0.5 or 1:5 antigen:NPL mass ratio), using Hank's Balanced Salt Solution (HBSS, Life Technologies, France) as donor and acceptor medium. A low molecular weight chitosan (Sigma-Aldrich, France) solution in HBSS pH 6.5 (0.05% w/v) was used as positive control for the tight junction opening (TEER decreasing). The TEER was checked after 30min and every hour for 3 hours moving the Transwell® in a plate with fresh acceptor medium before each measurement. The samples from the apical side and basolateral side were collected and the fluorescence was measured with a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Scientific, France).

3. Results and discussion

3.1 Characterization of the formulations CTA1-3M2e-DD:NPL

The formulations of the antigen CTA1-3M2e-DD with NPL were prepared at different mass ratios and size and zeta potential were characterized using NanoZS from Malvern. CTA1-3M2e-DD had an average size of 160 nm, while the size of NPL was 70 nm. The formulations were performed at different protein:NPL mass ratios (1:0.5; 1:3; 1:5; 1:10). The dynamic light scattering analysis showed the presence of aggregates ($4 \mu\text{m}$) in the formulation antigen:NPL

(Ag:NPL) 1:0.5 by mass ratio, that were also macroscopically visible. The increase in Ag:NPL mass ratio led to the decrease in the particle size of the formulation (Figure 12a). The zeta potential analysis displayed a highly positive charge ($+45.63 \pm 1.65$ mV) for the NPL and a negative charge for the antigen (-19.47 ± 0.85 mV) (Figure 12b). All the formulations had a comparable surface charge except the formulation 1:0.5. The zeta potential of the formulation 1:0.5 Ag:NPL was $+11.47 \pm 1.68$ mV, while the other formulations displayed a value of +30mV. This supported the idea that part of the antigen was adsorbed onto the surface of the formulation 1:0.5. Increasing the NPL mass a smaller size of the formulation was obtained, implying that the protein was progressively disaggregated and encapsulated in the NPL (1:5 mass ratio). In order to assess if some antigen was free, we analyzed the formulation by native electrophoresis. The complete association of the antigen to the NPL was evidenced from the mass ratio 1:3 (Figure 12c). For a mass ratio 1:0.5, the antigen association to the NPL is partial, consistent with the results obtained by dynamic light scattering and the zeta potential analysis.

The same observations done for CTA1-3M2e-DD can be pointed out for CTA1(R9K)-3M2e-DD, the mutant protein lacking ADP-rybosilating activity (Figure 13). The free protein CTA1(R9K)-3M2e-DD is highly aggregated, since it shows a particle size of 332.4 ± 22.18 nm (Figure 13a). Moreover the average size of the formulation 1:0.5 is greater than $8\mu\text{m}$ and exhibit aggregates. Similarly to the case of the active protein, while increasing the Ag:NPL mass ratio a lower particle size is detected. The formulations of the inactive protein displayed zeta potential values similar to the one obtained for the active antigen.

This suggested that the modification in CTA1 primary structure did not significantly affect the protein-NPL interaction and the association is quantitative after the 1:3 ratio. We concluded that both proteins, active and inactive, associate to the NPL in a similar way.

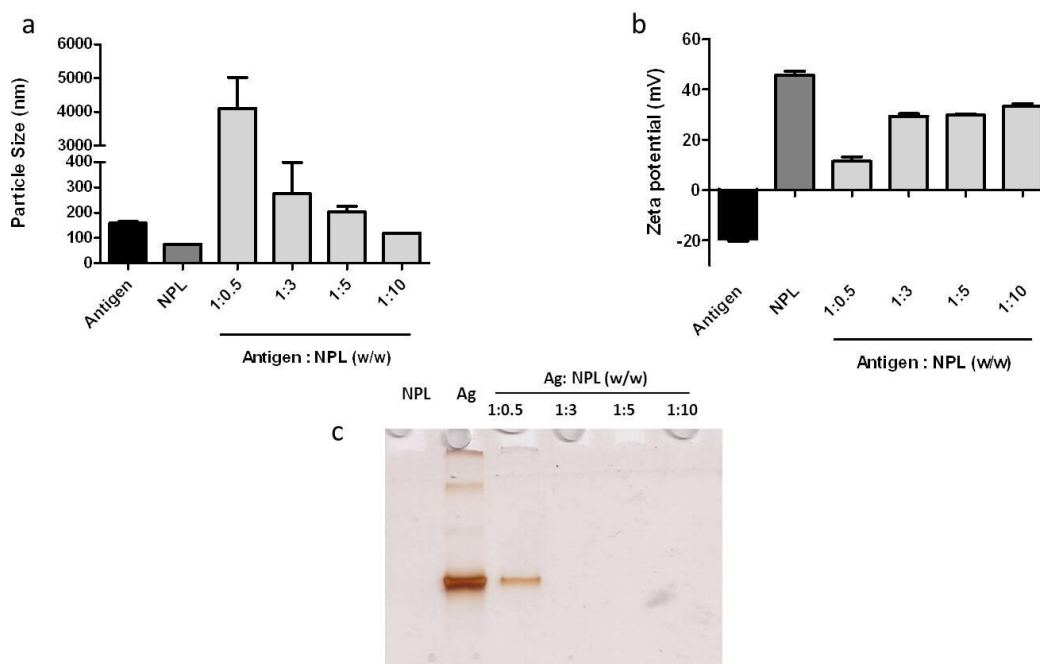


Figure 12 Characterization of CTA1-3M2e-DD NPL.

(a) Particle size analysis (b) Zeta potential (c) Native-PAGE electrophoresis.

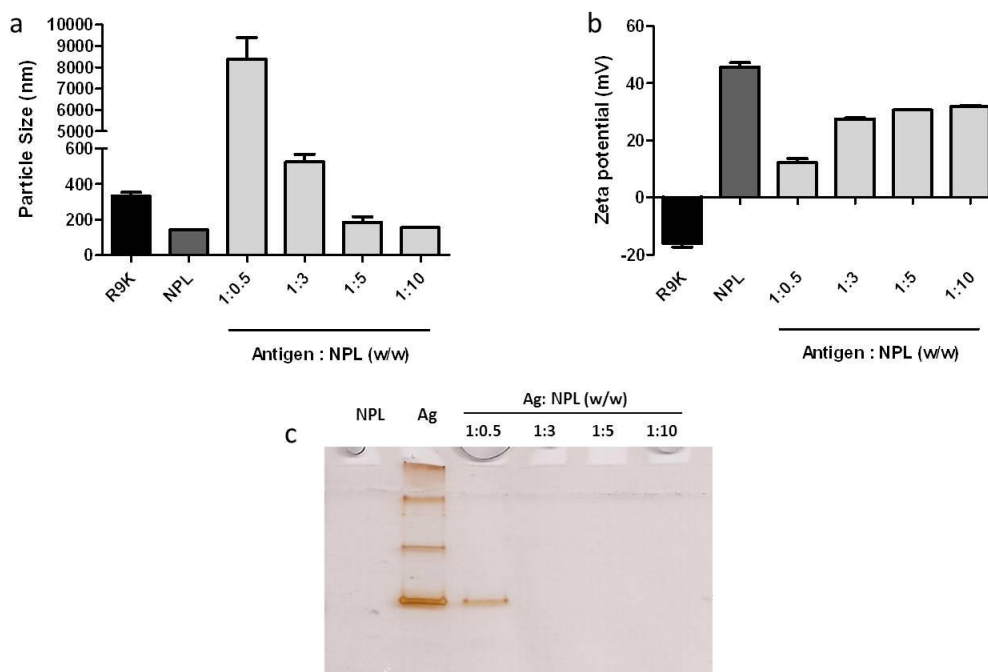


Figure 13 Characterization of CTA1(R9K)-3M2e-DD NPL.

(a) Particle size analysis (b) Zeta potential (c) Native-PAGE electrophoresis.

3.2 Characterization of the formulations HA:NPL and HA/CTA1-3M2e-DD:NPL

Hemagglutinin was associated to NPL or to CTA1-3M2e-DD -loaded NPL. Dynamic light scattering analysis showed that the free protein in presence of Empigen®BB has a particle size around 50nm (Figure 14a). The charge of the protein was negative (-10 mV). HA was loaded into NPL and the resulting formulation was about 100 nm. Hemagglutinin was also loaded into CTA1-3M2e-DD: NPL and CTA1(R9K)-3M2e-DD: NPL formulations, obtaining a particle size of 130 nm and 90 nm respectively. All the formulations containing HA had a comparable zeta potential of about +27 mV (Figure 14b). The antigen association was confirmed by native-PAGE, where a small fraction of HA was revealed unbound in all the formulations.

The structure of the HA could be accountable for this. Hemagglutinin has a stem portion and a globular moiety. We supposed that the former might more likely interact with the maltodextrin network than the latter because of its shape. Additionally, the globular portion has a considerable steric hindrance that may further obstruct protein-NPL interaction.

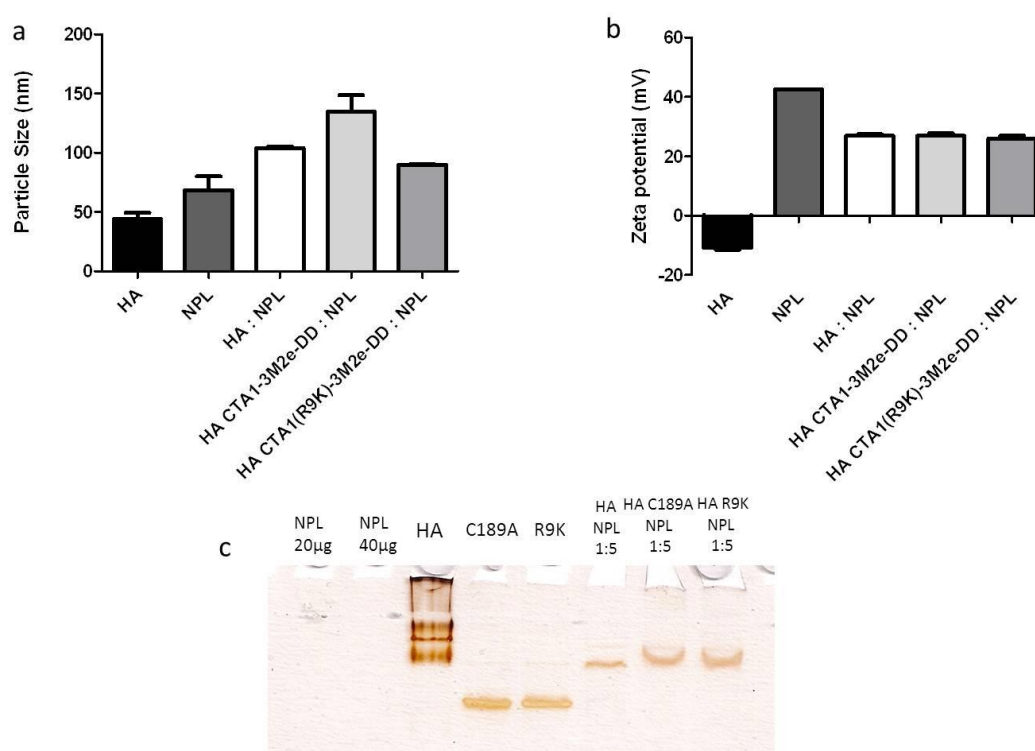


Figure 14 Characterization of HA CTA1- 3M2e-DD NPL.

(a) Particle size analysis (b) Zeta potential (c) Native-PAGE electrophoresis.

3.3 Stability of the formulations CTA1-3M2e-DD NPL

3.3.1 Stability of the antigen association to the NPL

The stability of the antigen association to the NPL was evaluated by native polyacrylamide gel electrophoresis (PAGE). In these conditions, only free antigens were able to migrate into the gel. The formulations 1:0.5 and 1:5 Ag:NPL by mass:ratio were considered for this study, according to the *in vivo* tests performed for the characterization of the immune response and protection against viral challenge (data not shown, manuscript in preparation by Lycke *et al.*).

The association of the antigen to the NPL was stable for both formulations at 4°C (Figure 15 and 16). A constant association of the antigen to the NPL (mass ratio 1:0.5) was observed for 6 months at 4°C, while a complete association was observed over 12 months (Figure 16). On the other hand the antigen was steadily associated to the NPL in the formulation 1:5 by mass ratio for one year, meaning that there is no release of the protein by the NPL at 4°C.

However the stability test in accelerated conditions (40°C) showed the disappearance of the band related to the unassociated protein in the formulation 1:0.5 during the time. We suggest that at 40°C the protein better associates to the NPL or that the formulation aggregates, preventing the protein entry into the gel. Concerning the formulation 1:5, the antigen association to the NPL was stable and complete at 4°C over one year and 40°C over 3 months, as no differences were revealed by native-PAGE.

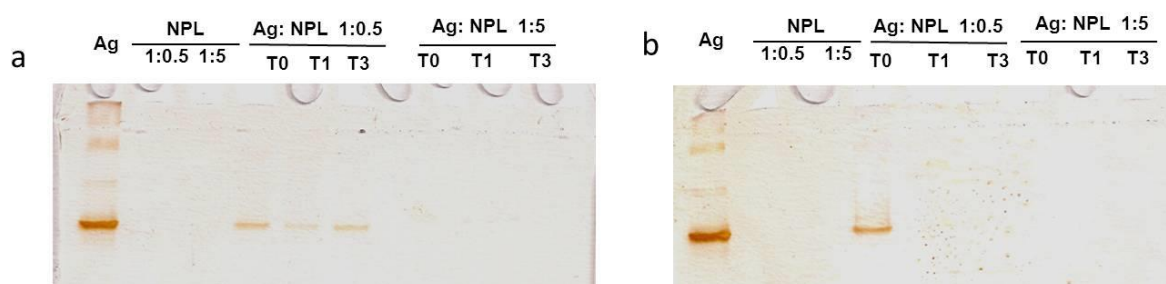


Figure 15 Characterization of the stability of CTA1- 3M2e-DD NPL: native PAGE.

a) 4°C, b) 40°C.

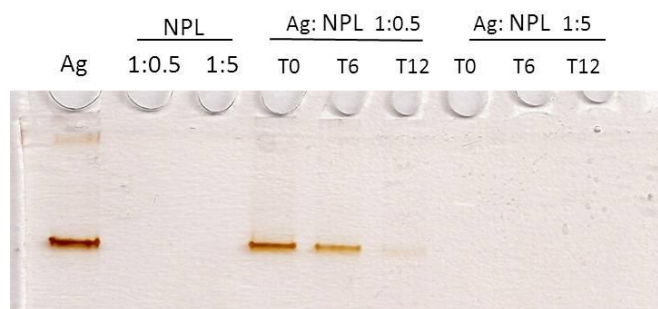


Figure 16 Characterization of the stability of CTA1- 3M2e-DD NPL 12 months.

3.3.2 Stability of the antigen free and loaded in NPL

In SDS-PAGE (Figure 17 and 18) the degradation and the aggregation of the protein free or formulated with NPL has been evaluated. The formulation 1:0.5 was stable over 12 months at 4°C since no band changes were observed in denaturing conditions. A slight degradation of the protein was noticed for the formulation 1:5 after 12 months as suggested by band appearance in the gel (MW<35kDa). However the band corresponding to the antigen (~45kDa) was the major one observed. A band at about 100kDa was noticed, highlighting the presence of protein aggregates.

The stability test in accelerated conditions (40°C) showed the degradation of the protein even if associated to the NPL for the mass ratio 1:5 antigen:NPL (Figure 17b) suggesting the needs of a preservative for the long term storage of the formulation, in order to block the protease activity. The technique used was unfortunately inadequate for the analysis of the formulations at 40°C, since the strong binding of the proteins to the NPL did not let the totality of the protein entering the gel. However some degradation fragments (size lower than 40 kDa) are observed.

The stability of the antigen alone has been assessed, as reported in figure 19. The antigen was stable at 4°C for three months but, as expected, it degraded at 40°C. Moreover we observed that the plain antigen is stable at 4°C for one year (Figure 19b).

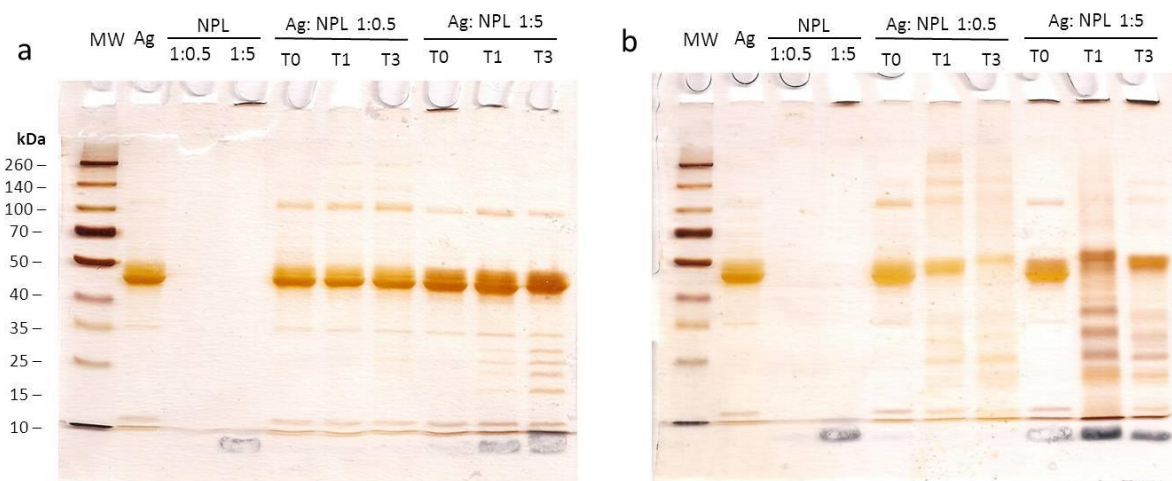


Figure 17 Characterization of the stability of CTA1- 3M2e-DD NPL: SDS PAGE.

a) 4°C, b) 40°C

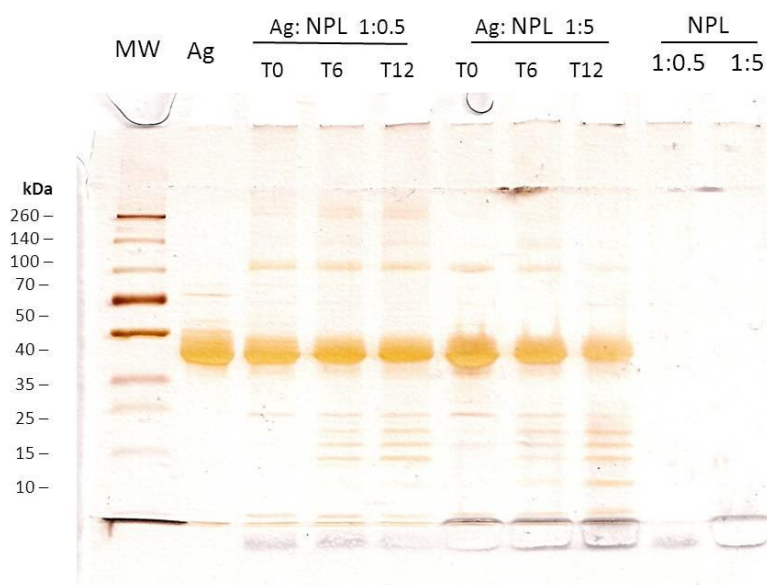


Figure 18 Characterization of the stability of CTA1- 3M2e-DD NPL 12 months.

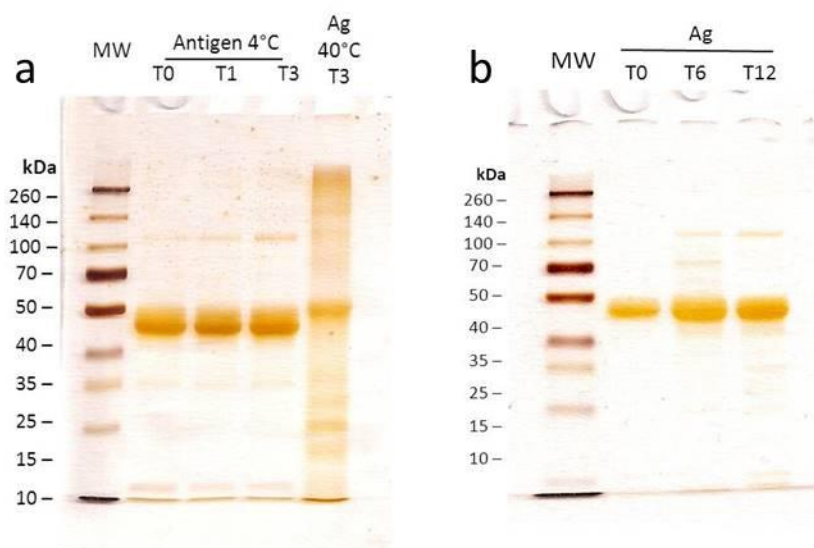


Figure 19 Characterization of the stability of CTA1- 3M2e-DD. Stability was evaluated by SDS-PAGE during 3 (a) and 12 months (b).

3.3.3 Colloidal stability: stability of the size and the zeta-potential

The dynamic light scattering analysis displayed the lack of size stability of the formulation 1:0.5, a very high polydispersity index (close to 1) and a decreased Z-potential. These results suggested that the formulation 1:0.5 had undergone a continuous rearrangement of the protein and NPL complex. This displacement appeared limited at 4°C, as the lower temperature reduced the fall of zeta potential (Figure 20). However the Z-average varied over 6 and 12 months and the broad polydispersity index (PDI) observed for the formulation 1:0.5 increased with the time. The zeta potential of the formulation 1:0.5 (Figure 21c) reached negative values indicating that the protein was on the surface of the formulation and masks the positive charge of the NPL.

In contrast, the size and charge of the formulation 1:5 were highly stable, since this formulation kept its size at ~200nm for one year. The PDI of the formulation 1:5 increased after 6 months but decreased again after 12 months (Figure 21), whereas at 40°C increased polydispersion was observed just after 3 months (Figure 20). A constant zeta potential was measured for the 1:5 formulation.

Macroscopically, a precipitate had already appeared in the formulation 1:0.5 after few hours from the preparation. This phenomenon was not observed for the formulation 1:5 and confirms the colloidal stability of this second preparation.

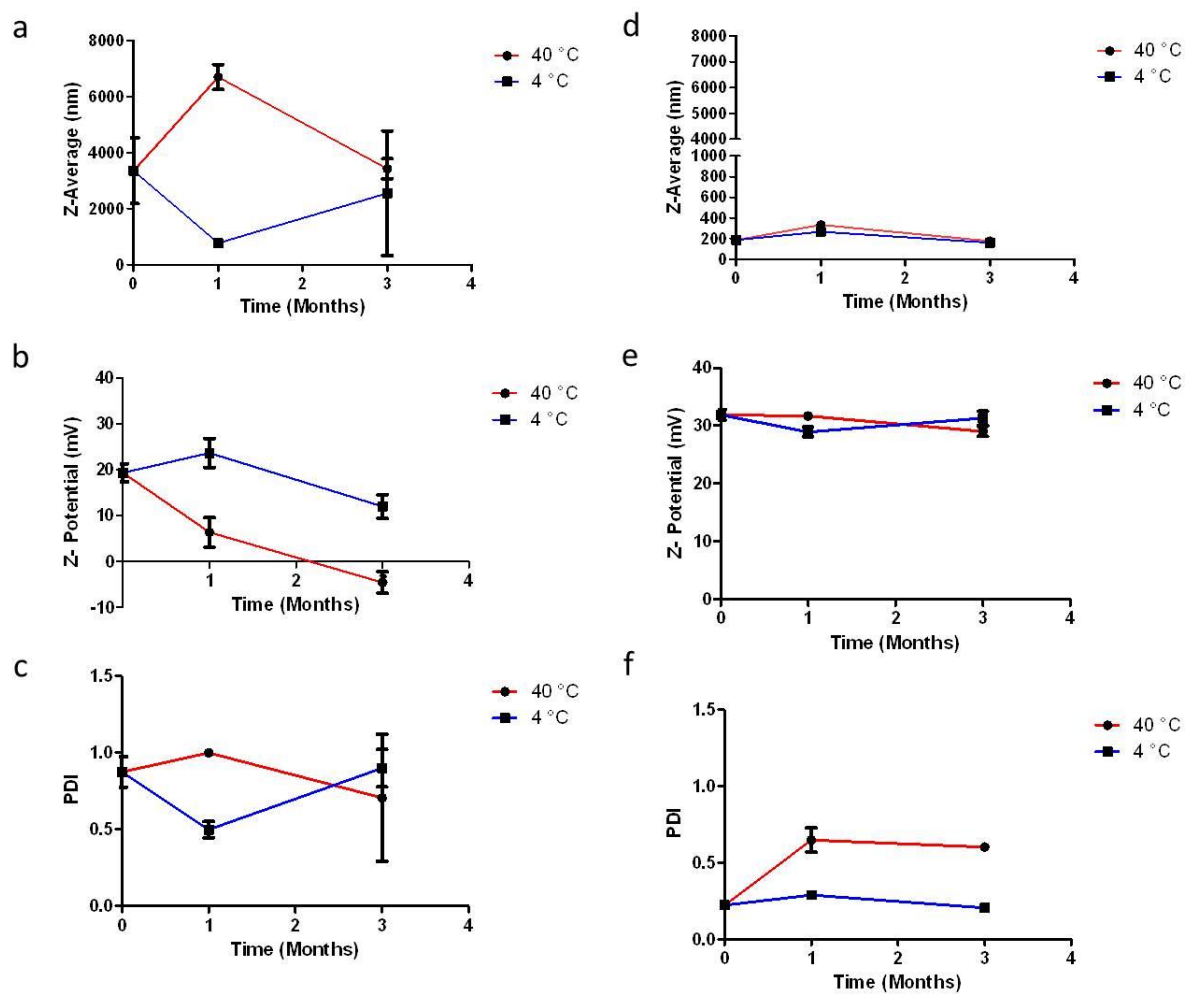


Figure 20 Characterization of the size and charge stability of CTA1- 3M2e-DD: NPL. The stability was evaluated at 4°C and 40°C. Ag:NPL 1:0.5 (a,b,c), Ag:NPL 1:5 (d,e,f).

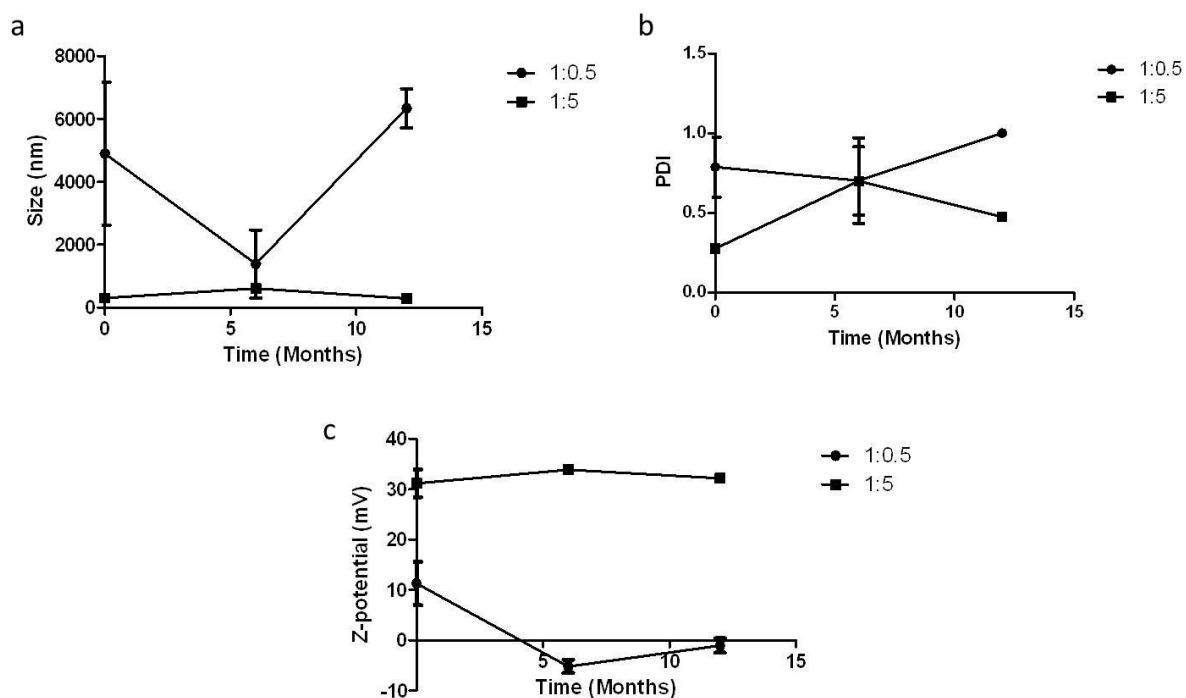


Figure 21 Characterization of the colloidal stability of CTA1- 3M2e-DD NPL12 months.

The stability of the NPL alone had been investigated at two different concentrations: the one used in the formulation 1:0.5 (0.308 mg/ml) and the one of the formulation 1:5 (3.08 mg/ml) (Figure 22). At 4°C a very high stability of size, charge and polydispersity of this carrier was observed independently on the concentration of the NPL. At 40°C a lower colloidal stability was observed, especially for the polydispersity index that increases significantly for the more diluted carrier. These results evidence the high stability of the NPL at 4°C (Figure 22). We also verified the stability of the NPL for one year at 4°C, these were found overall stable (results not shown).

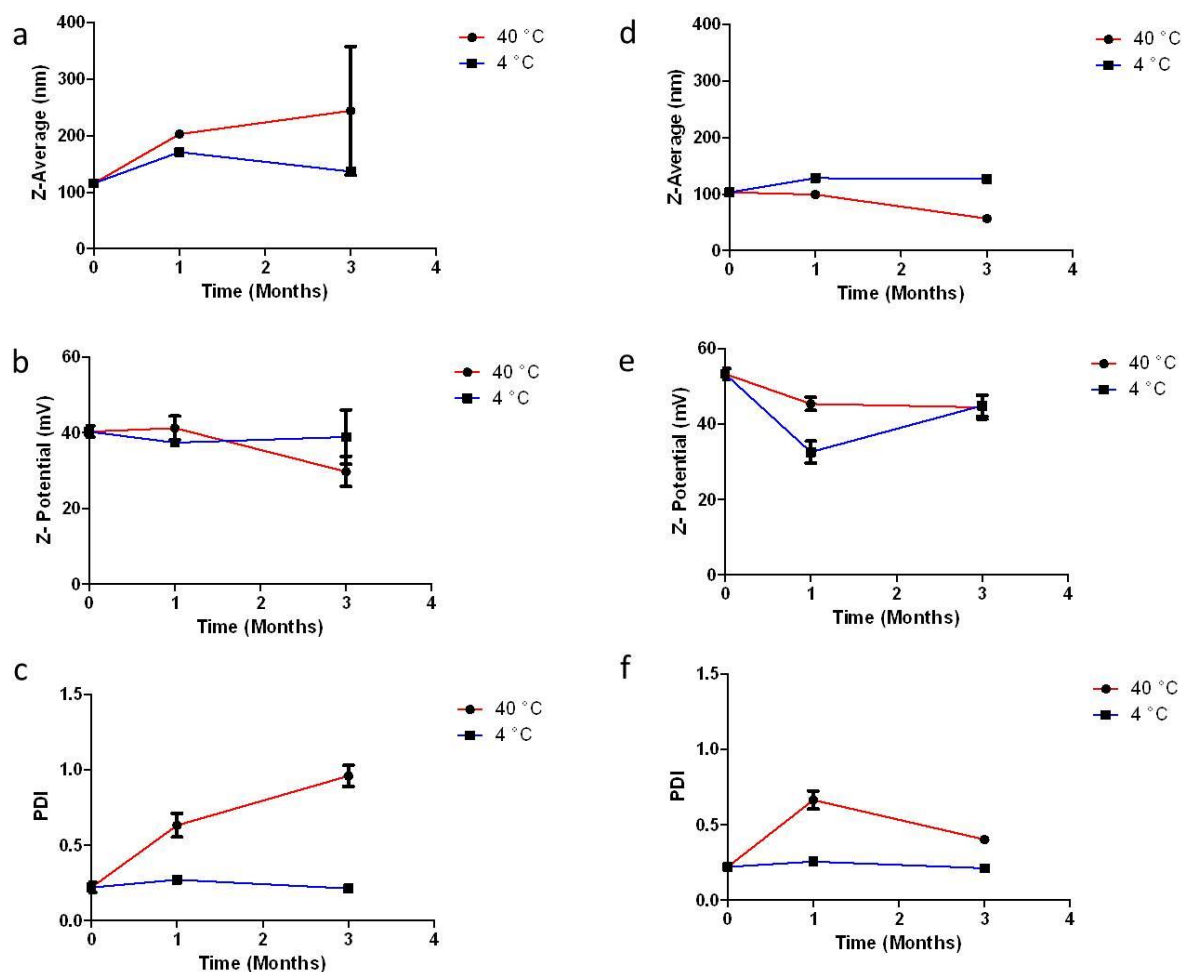


Figure 22 Characterization of the NPL size and charge stability.

The stability was evaluated at 4°C and 40°C.

The colloidal stability of the free antigen was evaluated (Figure 23). The protein had a z-average about 250 nm and a broad polydispersity (PDI=0.5), suggesting the presence of aggregates in the solution. Despite the stability of zeta-potential, the size detected varied during the time reaching a value of approximately 70 nm. The PDI increased after 3 months at 40°C but decreased at 4°C. The increased PDI at 40°C might be attributed to the antigen degradation observed by SDS-PAGE (Figure 17). The free antigen underwent important size and charge variation during one year of storage at 4°C (results not shown).

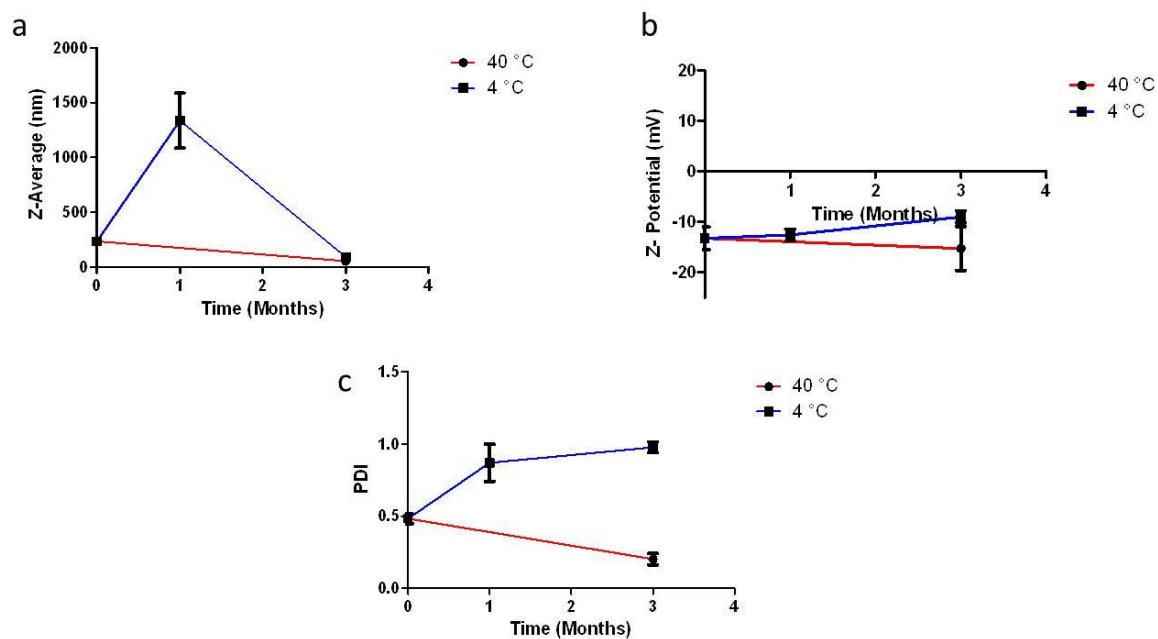


Figure 23 Characterization of the stability of CTA1- 3M2e-DD.

We conclude that the antigen and its association to the formulation 1:0.5 was stable for 6 months, whereas the size and zeta-potential of this formulation were unstable. On the other hand the formulation 1:5 was highly stable for 12 months.

3.4 CTA1-3M2e-DD delivery in epithelial cells and macrophages

Epithelial cells (16HBE) and macrophages were treated at different incubation times with the FITC-labeled antigen CTA1- 3M2e-DD free or formulated with the NPL to evaluate the protein delivery.

NPL increased the antigen delivery in airway epithelial cells up to 12 fold compared to the unformulated antigen, after 24 hours (Figure 24a).

The kinetics profile suggested that the antigenic protein was step-by-step delivered in epithelial cells. The same result was observed in macrophages, where an increase of 9 fold was obtained after 24 hours using the NPL (Figure 24b). In THP-1 derived macrophages the kinetics profile of antigen delivery reached a plateau after 3 hours, underlining the phagocytic role of these cells.

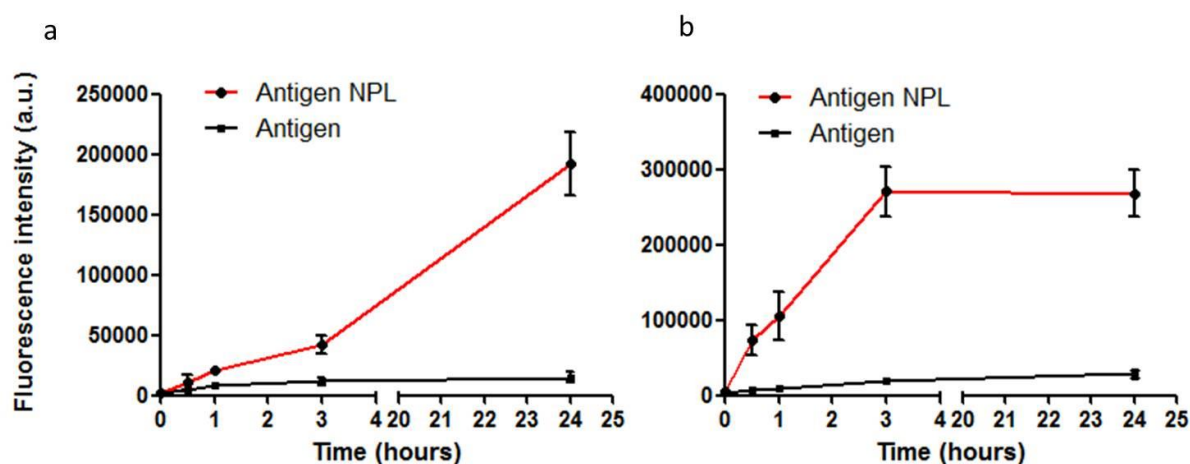


Figure 24 CTA1-3M2e-DD (Antigen) delivery in airway epithelial cells (a) and macrophages (b).

3.5 *In vitro* transcytosis of antigen-loaded NPL

The protein and NPL transcytosis had been investigated in an *in vitro* model of the airway epithelial barrier, in order to understand whether the NPL cross the epithelium to deliver the antigen to the immune cells found beneath or if the antigenic protein could cross the epithelial barrier under NPL assistance. Airway epithelial cells monolayers were treated with CTA1-3M2e-DD-FITC free or formulated with NPL in presence or absence of chitosan (CS). The CS is a chitin-derived polysaccharide able to open the tight junction (TJ) of epithelial cells through an integrin mediated mechanism (Hsu *et al.*, 2013).

In figure 25 is reported the TEER%. The TEER% was constant after treatment with CTA1-3M2e-DD-FITC free or formulated with NPL in contrast, in presence of CS, the TEER% decreased to 45%, indicating TJ opening. We concluded that the NPL and the CTA1-3M2e-DD-FITC did not open the TJ. In agreement with our findings Markov *et al.* observed that cholera toxin did not open the tight junction in rat colon epithelium (Markov *et al.*, 2014). Concerning cholera toxin effect on tight junctions there are conflicting results. Indeed these results are in contrast with the observation that increased cAMP induces TEER increase and reduced paracellular permeability (Deli, 2009). However Guichard *et al.* observed epithelial tight junction disruption after treatment with the A subunit of the cholera toxin (Guichard *et al.*, 2013).

The fluorescence of CTA1-3M2e-DD-FITC that permeated in the basal compartment was measured: after three hours, 0.4% of the starting fluorescence of the CTA1-3M2e-DD-FITC was detected in the basal chamber. No significant differences were revealed in terms of fluorescence passage between the protein and the formulation even in presence of CS. We conclude that the CS-dependent opening of the TJ was not sufficient for the CTA1-3M2e-DD-FITC to cross the epithelial barrier. These results suggest the lack of paracellular passage or transcytosis, while formulated antigens and NPL entered the cells.

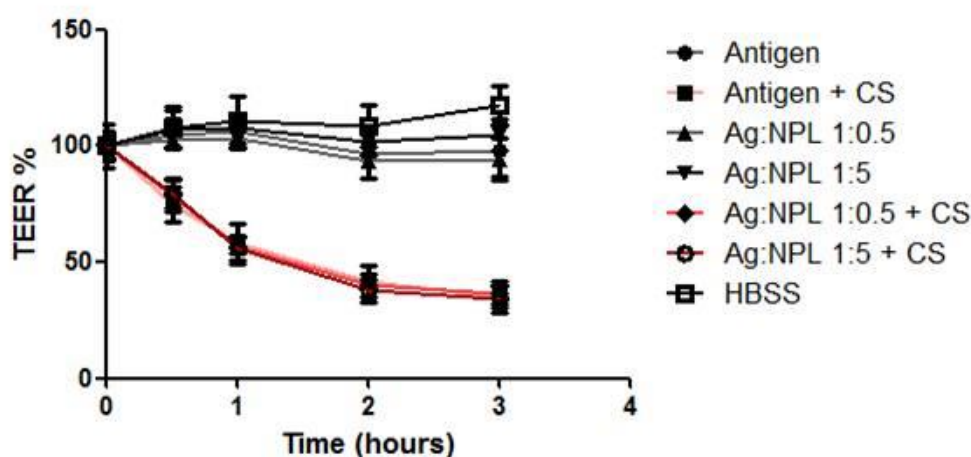


Figure 25 Tight junctions opening during the antigen treatment.

4. Conclusion

Antigen association to the NPL and the stability of the formulations were investigated. We observed a high stability of the formulation 1:5 at 4°C and we showed the complete antigen association to the NPL. However in presence of free antigen (formulation 1:0.5 w/w) the size and charge of the formulation were instable, confirming the stabilizing effect of the NPL on the antigen. NPL efficiently delivered the antigen in airway epithelial cells and macrophages. We also observed that the formulation do not cross the airway epithelial barrier and the adjuvanted antigen do not open the tight junction.

In vivo protection from viral challenge and transmission are currently under investigation by the Consortium partners using these formulations.

DISCUSSION

Nanoparticles for nasal vaccine delivery: mechanism and biodistribution

Drugs are generally administered as pharmaceutical formulation, hence in combination with excipients. Nanoparticles used in nanomedicine are to carry the drug to the target organ or tissue to ensure an efficient delivery. However, drug activity may be weakened or lost, in particular in case of fragile drugs, such as proteins and peptides. As a consequence the features of a formulation include the ability to stabilize and keep the active pharmaceutical ingredient (API) structure and activity. To maximize the surface area, necessary to improve drug release, the size of the formulation can be reduced to the nanodimension.

That is the reason why nanoparticles have an application in drug delivery. Nanoparticles can associate proteins in several ways, thus influencing the drug delivery. In fact, drugs can be either encapsulated within the particles or adsorbed on the surface, thus influencing the drug biodistribution and delivery. The immune response can be oriented to humoral or cellular responses according to how whether the antigen is encapsulated, adsorbed on the surface or mixed to the nanoparticles. Zhang and co-workers have recently shown that a formulation composed by the antigen encapsulated and mixed to the nanoparticles elicited a stronger immune response compared to that of the antigen being only encapsulated by the nanoparticles (W. Zhang *et al.*, 2014).

In this thesis antigens were associated to the NPL by post-loading, hence we studied their association to the NPL. The NPL loading is influenced by the antigen quality, such as the structure and the molecular weight, the protein aggregation and the Ag:NPL mass ratio. The different proteins studied are OVA, CTA1-3M2e-DD and HA. However the association conditions of these proteins to the NPL were not the same since they were dissolved in different aqueous media. OVA has been dissolved in ultrapure water, HA in Empigen® BB 1.98% solution and CTA1-3M2e-DD has been supplied in buffer (NaP 10nM, NaCl 0.16M pH=7.4).

We analysed the formulations prepared by mixing several protein: NPL mass ratios using different proteins: OVA and CTA1-3M2e-DD. We observed a different particle size of the formulation 1:3 relative to different proteins. Hence OVA: NPL has a particle size of 76 nm and a Z-potential of +33 mV, whereas CTA1-3M2e-DD: NPL has a size of 250 nm and a Z-potential of +30 mV. We concluded that OVA is loaded into the NPL, whereas in the case of CTA1-3M2e-DD formulation, NPL are probably surrounding the antigen (formulation 1:3). The result obtained for CTA1-3M2e-DD is in contrast to what observed for the total extract of *Toxoplasma gondii*, which is completely loaded inside the NPL, even if the extract alone showed a high

particle size (482.4 nm) (Dimier-Poisson *et al.*, 2015). This discrepancy may result from the aggregation of CTA1-3M2e-DD.

The antigen association to the NPL was analyzed by native-PAGE. In the formulation 1:0.5 by mass ratio part of the protein is still unbound. Conversely in the formulations 1:3 by mass ratio both OVA and CTA1-3M2e-DD are completely associated to the NPL. In case of HA a fraction of free antigen is revealed by native-PAGE in presence of a high amount of NPL (mass ratio 1:5). Nonetheless, this fraction of free HA detected by electrophoresis may be attributed to several factors: (i) the presence of trimers of HA, (ii) the use of detergents (Empigen® BB) or (iii) the strength of the electrophoresis-induced electric field. Indeed HA exists in trimers that may be too bulky to associate to the NPL. Moreover, we used a detergent to solubilize HA, this can form micelles that prevent the HA association to the NPL. The electric current applied by native-PAGE can be sufficiently strong to separate poorly bounded HA by the NPL or dissociate the micelles that associate part of the protein. However, most of the HA was bound to the NPL. Taken together these results overall suggest a different protein-NPL interaction, probably dependent mainly on proteins structure, presence in solution of dimers or trimers and use of detergents.

Additionally, this behavior may be attributed to the Z-potential of the proteins: in case of the *Toxoplasma gondii* extract the global negative charge observed (-33.6mV) differs from the NPL charge much more than the charge observed for the proteins used in this work (e.g. -10mV for the HA).

Most nanoparticles are not stable in solution or in presence of serum proteins. Our results showed that both the NPL and the formulation 1:5 CTA1-3M2e-DD: NPL are highly stable (at least one year at 4°C). We reported stability studies performed in absence of antibacterial agents commonly used in influenza vaccines (e.g. thiomersal). Even in absence of preservatives the antigen CTA1-3M2e-DD was not degraded.

The use of nanoparticles for intracellular drug delivery leads to a reduction of the administered dose (Korsmeyer, 2016). We demonstrated *in vitro* an increase of intracellular antigen delivery when using the antigen-loaded NPL compared to the free antigen (Figure 6 publication 3, Bernocchi *et al.* and figure 24 Part II: *Results*). This opens opportunities for dose-sparing strategies.

Accordingly, nanoparticles are particularly suitable for the administration of biological drugs, such as recombinant vaccines. Additional reasons for nanoparticles application in vaccinology

are found in their ability to act as adjuvants or immunomodulators (Ilinskaya *et al.*, 2016). The improvement of the nasal residence time of antigens is a considerable advantage of the nanovaccine formulation. In this way the possibility of local antigen uptake by M-cells, DC and epithelial cells may be maximized to obtain a relevant immune response. These cells are the main targets of a mucosal vaccine.

The nasal clearance of the nanoparticle should be therefore minimized. One strategy to achieve a prolonged antigen residence in the nasal cavity is the use of biocompatible mucoadhesive material to prepare the formulation.

Therefore polymers (e.g. chitosan and TMC) have been reported to extend the nasal residence of antigens. Even the simple conjugation of OVA with chitosan can slow the protein clearance in the nasal cavity, in contrast to the unconjugated antigen that is almost completely cleared from the nose within 2 hours (Slutter *et al.*, 2010). Hence the use of nanoparticles to attain a sustained antigen release is reasonable. Moreover the mucoadhesion of NPL have been previously reported following sublingual administration (Razafindratsita *et al.*, 2007).

In a previous work Dimier-Poisson *et al.* investigated the efficacy of NPL loaded with the total extract of *Toxoplasma Gondii* by nasal administration. This vaccine triggered humoral and specific Th1/Th17 cellular responses and protection against the oral parasite challenge (Dimier-Poisson *et al.*, 2015).

Mucosal routes for vaccine administration are convenient to stimulate the mucosal immune response, therefore local and systemic immunity. The nasal administration has already been successfully exploited for vaccine administration (publication 2, Nasal nanovaccines) thanks to the non-invasive approach, favouring a good patient compliance and triggering the mucosal immune response.

In this work, we showed that porous maltodextrin nanoparticles retained the antigen (OVA) in the nasal cavity for 6 hours, whereas free OVA resides time in the nose for 1h30 min (Figure 8 publication 3, Bernocchi *et al.*). Other nanoparticles have been evaluated elsewhere to implement the nasal retention time of antigens. Slütter *et al.* showed that also TMC nanoparticles decrease the protein clearance rate in the nose, compared to OVA solution. However, TMC nanoparticles extend the protein nasal residence time of 30min and PLGA nanoparticles do not affect the OVA clearance (Slutter *et al.*, 2010). Similarly, the encapsulation of ¹²⁵I-HBsAg in glycol chitosan-coated PLGA nanoparticles showed a higher nasal retention compared not only

to the plain antigen, but also to the chitosan-PLGA carrier (D. Pawar *et al.*, 2013). Generally, glycol-chitosan particles showed a better mucoadhesiveness than chitosan, increasing also the nasal permanence of bovine serum albumin (BSA) (D. Pawar *et al.*, 2016). Saito *et al.* showed that carboxy-vinyl polymer increased the nasal residence time of whole inactivated influenza vaccine in mice and monkeys up to 6 hours whereas significant differences with the whole inactivated influenza vaccine group are observed after 2h30min (Saito *et al.*, 2016).

Supplementing the particulate formulation with cationic adjuvants potentially produce a synergistic effect with the mucoadhesive polymer, prolonging the antigen persistence in the mucosa. Bento *et al.* showed that the addition of compound 48/80, mast cell activator, to chitosan nanoparticles improved the OVA nasal residence up to 24 hours, contrasting the mucociliary clearance (Bento *et al.*, 2015).

Nanoparticles biodistribution following nasal administration is relevant for safety issues. Similarly to the nanoparticles used in this work Supramolecular Biovectors (SMBV) showed the possibility to improve the delivery of morphine to the brain after nasal administration (Betbeder *et al.*, 2000). This mechanism was not related to the nose-brain transcytosis of the nanoparticle as no morphine loading in SMBV was observed.

The olfactory tissue, resident in the upper portion of the nasal cavity, form a direct pathway to the brain thanks to the olfactory neurons inserted in the cribriform palate. However, in case of vaccine administration, the nose-to-brain delivery of antigen, adjuvant or nanoparticle should be avoided.

A case of toxicity has been reported after the nasal administration of virosomes adjuvanted with heat labile toxin as adjuvant of a split flu vaccine (NasalFlu). This vaccine produced transient facial nerve paralysis (i.e. Bell's palsy) (Wong *et al.*, 2005). For this reason, it is crucial to assess the biodistribution of the formulation.

Ducournau *et al.* indeed investigated the biodistribution of porous maltodextrin carriers (NPL) used hereby, after nasal administration. They reported no brain passage of these carriers (Ducournau *et al.*, 2016). This carrier lasted in the nasal cavity for about 24h. In this time lag the mucociliary clearance progressively removed nanoparticles from the epithelium. Nanoparticles, most likely entrapped in the mucus, were transported to the pharynx and to the esophagus, then to the stomach, the gut and finally excreted *via* the feces.

Conversely to what has been observed for maltodextrin nanoparticles, lactoferrin-conjugated poly(ethyleneglycol)-poly(ϵ -capro-lactone) (Lf-NP) nanoparticles reached the brain after nasal

administration and increased coumarin-6 delivery in the olfactory bulb (Liu *et al.*, 2013). Lf-NP drug delivery system would not be suitable for vaccine administration.

In order to prime the immune response, the formulation has several pathway possibilities. In these studies we analyzed the nanoparticles and the antigen endocytosis and transcytosis.

Dombu *et al.* showed that these NPL were mainly endocytosed by a clathrin-dependent pathway and *in vitro*, they were exocytosed by airway epithelial cells (C. Y. Dombu *et al.*, 2010). We established an *in vitro* model (Transwell® model) to evaluate the transcytosis and the paracellular passage of the formulation. Hence we showed in the Transwell® model that NPL did not cross the airway epithelium and did not influence the TJ opening (Figure 4 publication 3, Bernocchi *et al.*). However, *in vitro* models do not entirely embrace the complexity of *in vivo* kinetics mechanisms.

Our *in vivo* results confirmed the endocytosis of the NPL and the lack of NPL passage across the airway epithelium (Figure 9 publication 3, Bernocchi *et al.*). The lack of antigen transcytosis supports or not the evidence that epithelial cells are involved in the immune response as accessory cells (T. L. Li *et al.*, 2013; Salik *et al.*, 1999). Other cells like macrophages and dendritic cells are specialized antigen presenting cells that can be activated by the antigen. However, *in vivo* we could not detect OVA. Hence we do not know the mechanism implied in the activation of the immune system.

The intracellular antigen delivery is rather enhanced by the NPL formulation (Figure 6 publication 3, Bernocchi *et al.*). Nonetheless the amount of antigen delivered is sufficient to trigger an effective immune response, stronger than the unformulated antigen (Dimier-Poisson *et al.*, 2015).

Universal influenza vaccine

Currently different strategies are explored to develop an effective universal influenza vaccine. Many approaches are based on the activation of broad protective immunity by using viral antigenic proteins. These strategies aim to stimulate humoral response, by means of HA, NA or M2e, or cellular responses, *via* NP or M1. While humoral response is essential in influenza disease to prevent the infection (by means of neutralizing and non-neutralizing antibodies) the cellular response is needed to decrease the severity of the illness and therefore the mortality.

Studies aiming the stimulation of the innate immunity have been performed using the bystander activation of T lymphocytes by vaccination with live viruses (Goodridge *et al.*, 2016). For

instance the vaccination with an attenuated strain of *Bordetella pertussis* protected against lethal challenge with Influenza A (R. Li *et al.*, 2010)

Other approaches are based on antigen delivery platform improvement such as recombinant viral vectors, DNA and RNA vaccines, virus-like-particles/virosomes and adjuvants (Wiersma *et al.*, 2015). It is advantageous to enhance CD8⁺ activation, which is usually poor in case of subunit vaccines.

Firstly we associated the adjuvanted targeted fusion protein CTA1-3M2e-DD with NPL (Part II: *Results*). Different formulations were investigated (notably 1:0.5 and 1:5 antigen:NPL by mass ratio) to address a potential difference in the immune response.

A critical issue of pharmaceutical vaccine formulations is the stability. This should be taken into account especially when considering drugs that easily degrades, such as proteins. An often adopted solution to improve the stability of antigens is freeze-drying. However, the colloidal stability of the nanoparticle formulation may be compromised by the reconstitution of the suspension, since aggregates may irreversibly form and precipitate, thus rendering the preparation useless. Even if lyophilization may be advantageous to avoid stocking liquid formulations, freeze-dried vaccine still requires the cold chain to assure the stability (Kumru *et al.*, 2014).

To achieve a universal influenza vaccine the combination of different approaches is attractive. To obtain broader protection the vaccine can be implemented by the addition of multiple antigens, while avoiding the administration of WIV. Unfortunately, hemagglutinin, a key antigen of influenza vaccines, undergoes seasonal drift. Hence to develop a universal flu vaccine the conserved stalk domain of HA can be administered. Yassine H.M. *et al.* have recently linked HA stabilized-stem region on ferritin nanoparticles and showed that these particles elicited complete and partial protection against heterosubtypic challenge in mice and ferrets respectively (Yassine *et al.*, 2015). The use of HA as vaccine antigen seems to be necessary to develop a universal influenza vaccine. Consequently, in our work we associated HA to the NPL to obtain a broader immunity.

Even if the nasal epithelium has a limited permeability, the accessibility of nose-associated lymphoid tissues and the mimicry of the natural route of influenza infection make the nasal route ideal for vaccination. Moreover, the lack of injection confers to this route rapidity and easiness, which are ideal characteristics for mass vaccination in case of pandemics. However, concerning influenza vaccines, FluMist® and Nasovac™ are the only influenza nasal vaccines

commercialized, in U.S.A. and Asia, respectively. These vaccines are live attenuated and generally considered safe. The possibility of virus reversion has been discussed by the scientific community and should be taken into account as possible risk (Rimmelzwaan *et al.*, 2001). Even if LAIV intranasal administration can cause mild flu symptoms, such as runny nose, these vaccines are generally well tolerated. Nevertheless, patients suffering from asthma condition should not receive these vaccines (J. M. Kelso, 2012). Additionally, current nasal LAIV provide an administration schedule with two vaccinations, 1 month apart: this is a disadvantage compared to a single shot.

CONCLUSION AND PERSPECTIVES

Nanoparticles are promising tools for protein delivery in the mucosa, useful to stabilize proteins and to provide a depot effect, more advantageous than multiple administrations. We studied the mechanisms of interaction of porous cationic polysaccharide nanoparticles with the nasal mucosa. These nanoparticles are ideal vectors for the administration of drugs, especially of proteins. They are able to associate high amounts of proteins, to deliver them effectively into cells and they are totally bio-eliminated.

Concerning vaccine application, an ideal influenza vaccine should be administered in single dose *via* a non-invasive mucosal route and should trigger local and systemic protection. Moreover it should be a recombinant or split vaccine, free from egg-contaminants such as OVA, that can provoke adverse reactions in sensitive subjects. Still, recombinant vaccines need the co-administration of adjuvants to produce an effective immune response. However, the market has a great need for new adjuvants, since only a few (e.g. aluminium salts and AS01) are approved by regulatory affairs.

Great progresses have been achieved in influenza vaccine production. Flucelvax®, a trivalent inactivated influenza vaccine produced in cell culture, and Flublok®, the first recombinant flu vaccine have reached the market in the last years. Unfortunately, these are injectable vaccines and the only mucosal influenza vaccine marketed is the Flumist®, a LAIV one. The only particles that are marketed as influenza vaccine are virosomes (Infexal®). Therefore we trust that these approaches will soon be combined to obtain an ideal single dose recombinant adjuvanted vaccine delivered by nanoparticles and administered through the nasal mucosa.

The development of a safe and efficient delivery system is needed.

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